Monocytes of Individual Human Subjects Display Heterogeneous Bacterial Uptake and Antilisterial Activity

GEROLD ZERLAUTH,^{1*} HEIDI E. CHEHADEH,¹ ELISABETH MAIER,¹ ZSUZSA SCHAFF,²
MARTHA M. EIBL,³ AND JOSEF W. MANNHALTER¹

Immuno AG,¹ and Institute of Immunology, University of Vienna,³ Vienna, Austria, and 1st Institute of Pathology and Experimental Cancer Research, Semmelweis Medical University, Budapest, Hungary²

Received 19 October 1995/Returned for modification 1 December 1995/Accepted 18 April 1996

Peripheral blood monocytes (Mo) of normal human donors simultaneously exhibit two subsets differing in their functional activity towards the facultative intracellular bacterium *Listeria monocytogenes*. One subset (on average, 25% of total Mo) was characteristically able to ingest a large number of *L. monocytogenes* bacteria and permitted intracellular growth of these bacteria. The other Mo subpopulation (on average, 75% of total Mo) was far less active in phagocytosing *L. monocytogenes* and restricted intracellular *L. monocytogenes* growth. Electron microscopy revealed that the *Listeria*-permissive Mo subset allowed the bacteria to escape to the cytosol, a mechanism by which these bacteria evade the lethal attack of phagocytes. The *Listeria*-restrictive Mo subset, on the other hand, confined the bacteria to the phagolysosomes, where they were exposed to the killing mechanisms of the Mo. Permissiveness for *L. monocytogenes* growth was further associated with differences in the capacity of the Mo subsets to synthesize tumor necrosis factor alpha (TNF- α), an important mediator in the defense against intracellular bacteria. Following challenge with *L. monocytogenes*, the *Listeria*-restrictive Mo subset secreted two to six times more TNF- α than did the *Listeria*-permissive Mo subset. Enhanced TNF- α secretion was paralleled by increased accumulation of TNF- α mRNA as assessed by quantitative PCR. Despite these functional differences, the two Mo subsets were indistinguishable with respect to expression of cell surface markers known to be involved in adherence and phagocytosis of microbes. A speculative physiological role of the two Mo subsets may lie in the dual function of Mo as microbicidal effector cells and accessory cells for antigen-specific immune reactions.

The current concept of host response to infection with the facultative intracellular bacterium *Listeria monocytogenes* suggests that both phagocytic cells and T cells are activated by listerial infections and involved in antibacterial defense. Although T cells are the cells that eliminate *L. monocytogenes* from the infected host (19), peripheral blood monocytes (Mo) appear to be able to defend themselves against infection with *L. monocytogenes* on their own if provided with the right cytokines (16, 24). This was successfully shown in studies using T-cell-deficient mice. When cytokines with the capacity to activate mononuclear phagocytes were present, these animals not only survived *L. monocytogenes* infection but were even able to eliminate the invading bacteria (1, 20).

The present study was designed to investigate the innate antibacterial defense system of human mononuclear phagocytes. By using *in vitro* infection of human Mo with *L. monocytogenes* as a model system, bacterial uptake by Mo, bactericidal activities, cytokine secretion, and gene expression in response to bacterial challenge were studied.

MATERIALS AND METHODS

Preparation of *L. monocytogenes* stock. *L. monocytogenes* EGD (ATCC 15313) was grown to the mid-log phase in tryptic soy broth (Difco Biologicals, Detroit, Mich.), washed three to four times in Hank's balanced salt solution (HBSS), and frozen at -70°C . For each experiment, a frozen vial was thawed rapidly, sonicated to dispense the bacteria, and diluted in HBSS to the desired concentration.

Isolation of human Mo and preparation of Mo monolayers. Human peripheral blood mononuclear cells were obtained from the venous blood of healthy volunteers and isolated by buoyant density gradient centrifugation as described by Bøyum (7). The resulting mononuclear cells were suspended in RPMI 1640

medium (Flow Laboratories, Irvine, United Kingdom) supplemented with 2 mM L-glutamine and 15% pooled, heat-inactivated (30 min, 56°C) human AB serum (complete medium), and Mo were prepared by adherence to plastic as described previously (41). To prepare Mo for phagocytosis assays, killing assays, and fluorescence-activated cell sorter (FACS) analysis, 2×10^7 mononuclear cells in 1 ml of complete medium were added to each well of six-well culture plates (Costar, Badhoevedorp, The Netherlands); for electron microscopy, 2×10^7 mononuclear cells were adhered to plastic petri dishes (35 by 10 mm; Flow Laboratories), and for RNA isolation, 10^8 mononuclear cells in 10 ml of complete medium were adhered to a 260-ml tissue culture flask (Nunc, Roskilde, Denmark). After incubation for 90 min at 37°C in a CO_2 incubator (5% CO_2 in humidified air), the nonadherent cells were aspirated and the adherent cells were washed three times with warm (37°C) RPMI 1640 medium without supplements and further incubated in complete medium overnight. Adherent cells prepared in this way contained $>91\%$ monocytes as judged by nonspecific esterase staining (25). Viability, assessed by trypan blue exclusion, was consistently $>95\%$. In all experimental setups, antibiotics were omitted from the culture media to prevent any possible influence on growth of bacteria in the later evaluation of bactericidal activity (11).

***In vitro* infection and assessment of intracellular *L. monocytogenes*.** A fluorescence-based method was used to determine the percentage of *Listeria*-infected Mo and to differentiate between intracellular bacteria and bacteria attached to the Mo surface (13). First, the bacteria were labeled with a fluorochrome by incubating 1×10^8 to 2×10^8 *L. monocytogenes* bacteria (suspended in 1 ml of phosphate-buffered saline [PBS; pH 7.2]) in the presence of 0.1 mg of fluorescein isothiocyanate (FITC) per ml (Sigma, Deisenhofen, Germany) at 37°C for 2 h. The FITC-labeled bacteria were then pelleted at $10,000 \times g$ for 5 min, washed free of unbound fluorochrome by four washes with GHBSS (HBSS supplemented with 0.02% gelatin), and used for infection of Mo.

For infection, Mo monolayers prepared in six-well plates were washed three times with warm (37°C) GHBSS and overlaid with 1 ml of GHBSS-FCS (GHBSS supplemented with 5% heat-inactivated fetal calf serum [Flow Laboratories]), to which FITC-labeled *L. monocytogenes* had been added to yield an infection ratio of 10 or 50 bacteria per Mo. After 30 min, detachment of some Mo was observed. These detached Mo were aspirated and washed free of noningested bacteria by differential centrifugation (three consecutive washes with HBSS at $300 \times g$ for 5 min). Mo remaining adherent in the six wells were also washed three times with HBSS. Adherent and detached Mo (a minimum of 100 to 200 cells) were assayed for Mo-associated bacteria with a fluorescence microscope (Aristoplan; Leitz, Wetzlar, Germany) equipped with a mercury arc lamp with a 520-nm FITC filter and oil immersion. To differentiate between cell-associated and ingested bacte-

* Corresponding author. Mailing address: Immuno AG, Industriestrasse 20, A-1221 Vienna, Austria. Phone: 43 1 20100 2611. Fax: 43 1 20100 590.

ria, counterstaining with ethidium bromide was performed with the effect that extracellular bacteria fluoresced red-orange while intracellular bacteria fluoresced green. Uptake of bacteria is expressed as an infection index, calculated as (percent monocytes containing 1 or more bacteria) $\times$ (mean number of bacteria per positive cell) (14). To determine the total number of Mo, each field was evaluated with visible light.

Determination of the Mo antilisterial activity. Mo monolayers in six-well plates were washed, overlaid with 1 ml of GHBSS-FCS containing *Listeria* bacteria (infection ratio of *L. monocytogenes*/Mo, 10:1), and incubated for 90 min at 37°C in a CO₂ incubator (5% CO₂ in humidified air). During this incubation period, the two Mo subsets separated. The Mo subset with high phagocytic activity detached from the plastic surface, while the low-phagocytic Mo subset remained adherent. After the 90 min of phagocytosis, noningested *L. monocytogenes* bacteria were removed from the adherent Mo subpopulation by washing the plates three times with warm (37°C) RPMI 1640 medium without supplements and from the detached Mo subset by differential centrifugation as described above. Then, the number of viable *L. monocytogenes* bacteria ingested by each Mo subset was determined by assessment of CFU. To do this, Mo were lysed in ice-cold distilled water containing 0.2% saponin. Serial dilutions of the lysates were then plated on tryptic soy agar plates. The number of bacterial colonies was counted 24 h after plating with a colony counter (BioFoss, The Chantry, United Kingdom), and the results are expressed as CFU at time zero (T0).

For determination of antilisterial activity, Mo remaining adherent after the phagocytosis period were overlaid with 1 ml of fresh GHBSS-FCS and incubated for an additional 90 min at 37°C in a CO₂ incubator (5% CO₂ in humidified air). The detached Mo were suspended in 1 ml of fresh GHBSS-FCS, seeded into a new six-well plate, and also incubated for 90 min. The numbers of viable bacteria at this time (CFU at T90) were determined in both Mo subsets as described above. The antilisterial activity was calculated as the percent change in number of bacteria, i.e., (CFU per milliliter at T90/CFU per milliliter at T0) $\times$ 100.

Transmission electron microscopy of *Listeria*-infected Mo. Mo monolayers adherent to petri dishes (35 by 10 mm) were washed and infected with *L. monocytogenes* at a ratio of 10 *L. monocytogenes* bacteria per Mo. Mo remaining attached during *L. monocytogenes* challenge were washed free of noningested bacteria, fixed in situ with 2.5% glutaraldehyde in PBS (pH 7.4) at 4°C for 45 min, washed in PBS, and postfixed in 2% OsO₄ at 4°C for 60 min. Cells that had detached during the phagocytosis period were collected, separated from non-ingested bacteria by differential centrifugation, and fixed separately as described above. After dehydration and embedding in Polybed (Polysciences Inc., Warrington, Pa.), ultrathin sections prepared from the specimens were double-contrasted with uranyl acetate and lead citrate and examined with a JEOL 100 B electron microscope.

Phenotypic characterization of Mo. Expression of Mo surface antigens was analyzed with the following FITC- or phycoerythrin-labeled monoclonal antibodies (MAb): an anti-major histocompatibility complex class II MAb reacting with a monomorphic region of HLA-DR, Leu15 (an anti-CD11b antibody specific for CR3), and LeuM3 (anti-CD14; all purchased from Becton Dickinson, San Jose, Calif.); anti-human FcRIII (anti-CD16, 3G8), anti-human FcRII (anti-CD32, IV.3), and anti-human FcRI (anti-CD64, 32.2; all obtained from Medarex, West Lebanon, N.H.); and anti-human lymphocyte function-associated antigen 1 (anti-CD11a, IOT16) and anti-human intracellular adhesion molecule 1 (anti-CD54, IOL54; both from Immunotech, Marseilles, France). FITC-labeled F(ab')₂ anti-mouse immunoglobulin G (IgG) and IgM from An der Grub (An der Grub, Austria) was used as the second antibody for analysis of cells incubated with unlabeled lymphocyte function-associated antigen 1 antibody. Simultest controls (IgG1 FITC and IgG2a phycoerythrin) from Becton Dickinson were used as isotype controls.

Preparation and *L. monocytogenes* infection of Mo as well as removal of noningested bacteria were performed as described above. The detached Mo were processed directly for staining. Cells remaining adherent following infection with *L. monocytogenes* were first detached from the plastic surface by gentle scraping with a rubber policeman. Staining was carried out as reported previously (39). In brief, 3×10^5 to 5×10^5 cells of each Mo subset were incubated (30 min, 4°C) with saturating amounts of the respective MAb in 70 μ l of PBS containing 0.1% NaN₃ and 2% human serum IgG (Endobulin; Immuno AG, Vienna, Austria). Endobulin was included in our staining buffers to inhibit FcR-mediated binding of MAb to the monocyte surface. If a second-step antibody was required, the cells were washed twice and again incubated for 30 min at 4°C. Fluorescence was evaluated with a cytofluorograph (FacStar Plus; Becton Dickinson, Mountain View, Calif.).

Quantitation of lymphokine mRNA accumulation by reverse transcription-PCR. For quantitative determination of lymphokine gene expression, total RNA was first isolated from both Mo subpopulations by a single-step method as described previously (10). mRNA from the RNA isolates was then reverse-transcribed into double-stranded cDNA with a cDNA synthesis kit (Boehringer GmbH, Mannheim, Germany) with oligo(dT)₁₅ primer. The resulting cDNA was dissolved in 10 μ l of double-distilled water per microgram of RNA.

To perform quantitative PCR, serial dilutions of known quantities of competitor DNA, i.e., a DNA fragment containing the primer-annealing regions of the corresponding reverse transcription-PCR amplicon set (MIMIC; Clontech, Palo Alto, Calif.) were coamplified with constant amounts of target cDNA (34). The

conditions for PCR were as follows: denaturation, 1 min at 94°C; annealing, 2 min at 60°C; extension, 3 min at 72°C. Thirty-five cycles were performed in a thermal cycler (60/2; Biomed, Theres, Germany). An aliquot of each reaction mixture was run on a 2% NuSieve 3:1 agarose gel (FMC BioProducts, Rockland, Maine) in TBE buffer (0.5 M Tris, 0.05 M boric acid, 1 mM EDTA), and bands were quantified directly with a video image analyzer system (ATS 800; Hirschmann, Taufkirchen, Germany) after staining with ethidium bromide (9). The data from the image analyzer were plotted as the log₁₀ of the target-to-competitor ratio versus the log₁₀ of the number of competitor molecules. The amount of target cDNA in the sample could be determined from the dilution in which the quantities of competitor and target PCR products were equal. This value was calculated by linear regression analysis of the data from four to five target-competitor pairs (30).

To control for variations in extraction of RNA and reverse transcription procedures, the copy numbers of the cytokine mRNAs were normalized relative to the level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA in the respective sample.

The following primers and MIMICS (purchased from Clontech) were used: (i) hTNF- α (human tumor necrosis factor α) sense primer (5' GAGTGACAA GCCTGTAGCCCATGTTGTAGCA 3') and antisense primer (5' GCAATGAT CCAAAGTAGACCTGCCAGACT 3'; amplification product size, 444 bp) and hTNF- α MIMIC (617 bp); (ii) hIL-6 (human interleukin-6) sense primer (5' ATGAACCTCTTCTCCACAAGCGC 3') and antisense primer (5' GAAGAG CCCTCAGGCTGGACTG 3'; amplification product size, 627 bp) and hIL-6 MIMIC (500 bp); (iii) hIL-1 β sense primer (5' ATGGCAGAAGTACCTAAGC TCGC 3') and antisense primer (5' ACACAATTGCATGGTGAAGTCAGTT 3'; amplification product size, 801 bp) and hIL-1 β MIMIC (630 bp); and (iv) hGAPDH sense primer (5' TGAAGGTCCGAGTCAACGGATTGGT 3') and antisense primer (5' CATGTGGCCATGAGGTCCACCAC 3'; amplification product size, 983 bp) and hGAPDH MIMIC (630 bp).

Assay for TNF- α . TNF- α in culture supernatants was assayed with a commercial enzyme-linked immunosorbent assay (ELISA) kit (TNF- α Quantikine; British Biotechnology, Abingdon, United Kingdom) as described in the manufacturer's assay protocol. Results are expressed as picograms of TNF- α produced by 10⁶ cells during the indicated period of time.

Anti-human TNF- α antibody. Anti-human TNF- α MAb (Sanbio, AM Uden, The Netherlands) was used to inhibit the function of TNF- α secreted by activated Mo (41). An amount capable of neutralizing three times the physiological levels of TNF- α was added to the Mo of the low-phagocytic subset after the 90-min phagocytosis period, and the antibody remained present during the 90-min assay for antilisterial activity. The mouse myeloma immunoglobulin MOPC 21 (Sigma), which does not bind to human Mo, was used as an isotype control.

RESULTS

Two subsets which differ in their capacity to ingest *L. monocytogenes* are simultaneously present in the Mo of normal human donors. Adherent Mo prepared from the peripheral blood of individual human donors were infected with the facultative intracellular bacterium *L. monocytogenes*. This infection revealed the existence of two subpopulations within the same Mo preparation. One Mo subset ingested *L. monocytogenes* with a high capacity (up to 20 bacteria per infected Mo) and was further characterized by rapid detachment from the plastic surface following bacterial uptake. The second Mo subset ingested only a few bacteria (two to three per infected cell) and remained adherent (these differences in adherence made it easy to separate the two Mo subsets).

In general, cells with a high capacity for *L. monocytogenes* ingestion showed an infection index of 168 after a 30-min incubation period at an infection ratio of 10 bacteria per Mo. Extending the time span to 90 min yielded an infection index of 184. When Mo were challenged for 30 min with a 50:1 bacterium/Mo ratio, an infection index of 210 was found. In contrast, the cells remaining adherent during *L. monocytogenes* infection displayed fewer internalized bacteria with infection indices of 10 (30 min, bacterium/Mo ratio of 10:1), 36 (90 min, bacterium/Mo ratio of 10:1), and 58 (90 min, bacterium/Mo ratio of 50:1).

For discrimination of extracellular and intracellular bacteria, we utilized FITC-labeled bacteria and ethidium bromide as a counterstain with the effect that extracellular bacteria fluoresced red-orange, while bacteria residing in viable Mo and therefore not accessible to ethidium bromide fluoresced green

FIG. 1. Comparable expression of surface markers on Mo with low and high phagocytic capacities for *L. monocytogenes*. Mo subsets were stained with MAb and subjected to FACS analysis. Shaded peaks show the log of fluorescence intensity of cells exposed to the MAb indicated; open areas depict log fluorescence intensity of cells exposed to isotype controls. The percentages of cells staining positive for the indicated marker in this representative experiment are shown in the top right-hand corner of each histogram. Similar results were obtained in three to six additional experiments.

(13). The results of these experiments revealed that in the highly infectible Mo subset, 65% of the cell-associated bacteria were intracellular. The extracellular bacteria, however, were firmly bound to the Mo and could not be removed by repeated washing steps. In the Mo subset containing only a few bacteria following infection, all *L. monocytogenes* bacteria were intracellular. This suggests that the low number of intracellular bacteria in this Mo subset is not due to a failure in the capacity of the cells to internalize the bacteria but is possibly the result of decreased binding of *L. monocytogenes*. Once bound to the cell surface, the bacteria are efficiently internalized.

The percentage of cells capable of high *L. monocytogenes* uptake and detaching upon infection with *L. monocytogenes* varied considerably between individual donors. In a series of 30 experiments, the infection strategy generally employed (i.e., incubation of Mo monolayers with *L. monocytogenes* for 90 min) resulted in an average of 25% (range, 8 to 35%) detaching Mo. Interestingly, the number of detaching Mo was not affected when the bacterium/Mo ratio was increased from 10:1 to 50:1 (data not shown).

Both Mo subsets were >91% pure as assessed by nonspecific esterase staining and FACS analysis. The viability of these cells as determined by trypan blue exclusion exceeded 95% at all stages of the experiments. The viability of the detached cells was further corroborated by their ability to readhere after detachment in response to heat-killed listeriae. For these experiments, heat-killed listeriae were used for the detachment to allow cell cultivation over extended periods of time. Eighty percent of the cells detaching were found to readhere again, indicating a viable population of cells. A second round of

challenge with heat-killed listeriae led to detachment of virtually all cells. In contrast, the Mo subpopulation remaining adherent after the first *L. monocytogenes* challenge did not detach in measurable numbers upon a second challenge with *L. monocytogenes* (data not shown).

The two Mo subsets cannot be distinguished phenotypically. Expression of receptors of both the integrin and the immunoglobulin superfamilies, cell surface receptors known to be associated with adherence of bacteria to, as well as bacterial entry into, phagocytic cells, was analyzed with a cytofluorometer (23). The representative experiment depicted in Fig. 1 demonstrates that expression of the cell surface markers tested did not differ in the two Mo subsets; comparable expression levels of CD14, HLA-DR, LFA-1, CR3, ICAM-1, and Fc receptors I, II, and III were observed. From Fig. 1, it can also be deduced that the surface density of the individual markers is comparable in the Mo subsets with both high and low *L. monocytogenes* infection capacities. Statistical analysis of three to six experiments performed with cells from different donors did not reveal a significant difference in any of the markers studied (P was always >0.1; data not shown).

The two Mo subsets exhibit different antilisterial activity. To examine the permissiveness of the two Mo subsets for intracellular growth of ingested *L. monocytogenes*, Mo monolayers were exposed to a 10-fold excess of *L. monocytogenes* for 90 min and separated into the two subsets as described above. Subsequently, free bacteria were removed, the CFU at this time (T0) was determined, and the antilisterial activities of the two Mo subsets were assessed after a second 90-min incubation period (CFU at T90). Figure 2 shows that the Mo subset which

FIG. 2. Mo from individual human donors contain subsets with different antilisterial activities. The number of surviving *L. monocytogenes* bacteria in highly infectible Mo subsets as well as in Mo subsets with low infectivity was determined by assessing CFU at the beginning of the killing assay (T0) and 90 min later (T90). The bars indicate percent changes in number of *L. monocytogenes* bacteria after a 90-min killing assay. Negative values reflect killing of bacteria; positive values indicate bacterial growth. The data are given as means $\pm$ standard deviation.

was highly infectible with *L. monocytogenes* supported intracellular listerial growth. Bacteria engulfed by this subset yielded on the average a (2.43 ± 1.13) -fold increase ($n = 11$) in bacterial number within the second 90-min incubation period. In contrast, the Mo subset containing only a few intracellular bacteria was found to be restrictive for listeriae (on average, a reduction in listerial growth was observed).

Since attachment of Mo to surfaces is known to provide activation signals for these cells (18), it seemed appropriate to test whether it is the attached state which enables Mo to express antilisterial activity. To address this question, Mo remaining adherent following infection with *L. monocytogenes* were detached from the plastic surface by the use of EDTA. Subsequently, the EDTA-detached cells were washed, suspended in fresh medium, and tested for their antilisterial effect. In parallel, the killing capacity of *Listeria*-infected monolayers of Mo which were not detached with EDTA was assessed. In a series of three experiments, EDTA-detached Mo in suspension were found to be as restrictive for the growth of *L. monocytogenes* as the adherent Mo were, indicating that the adherent state per se does not determine the antilisterial activity of Mo (data not shown).

Different antilisterial activities of the two Mo subsets are reflected by the intracellular location of the bacteria. *L. monocytogenes* belongs to the group of facultative intracellular bacteria which evade killing by an active mechanism which results in escape from the phagolysosome to the cytosol of the cell, thereby evading the lethal attack of the phagocyte (38). Since the two Mo subsets differed in their antilisterial activity, we hypothesized that this might be reflected by the intracellular location of the ingested bacteria. Upon examination by electron microscopy, a difference in the intracellular location of ingested *L. monocytogenes* in the two subsets of Mo was in fact detected. *Listeria*-permissive (highly infectible) Mo contain large numbers of bacteria (Fig. 3a), some of which migrate to the cytosol after having dissolved the phagolysosomal membrane (Fig. 3a and b). The bacteria in this Mo subset look healthy, as indicated by an intact bacterial cell wall and electron-dense appearance (Fig. 3b). In contrast, the *Listeria*-re-

strictive Mo subset (Fig. 3c) confined the few engulfed bacteria to the phagolysosome, whose lining remained undisturbed. Moreover, the bacteria within this compartment appear to be destroyed, which is indicated by the electron-sheer and fuzzy appearance of the microorganisms (Fig. 3d).

Antilisterial activity of the Mo subsets correlates with TNF- α production. TNF- α has been shown to play a crucial role in the defense against bacteria, including listeriae (2, 12, 21, 41). We therefore studied TNF- α synthesis of *Listeria*-restrictive and -permissive Mo in response to infection with *L. monocytogenes*. As depicted in Table 1, infection of Mo with *L. monocytogenes* induced rapid TNF- α synthesis in both subsets. The *Listeria*-restrictive subset, however, secreted up to 5.9 times more TNF- α on a per cell basis than the *Listeria*-permissive subset.

This was also reflected by a comparable difference in gene expression as assessed by quantitative competitive PCR. Experiments performed with cells from three different donors demonstrated a reduction of mRNA accumulation for TNF- α in the *Listeria*-permissive subset (Table 2).

This, however, was not due to a general blockade of cytokine synthesis in the *Listeria*-permissive Mo subset, since expression of IL-1 β and IL-6, cytokines known to be coinduced with TNF- α in response to various triggers (33, 42, 43), including listeriae (6), were not impaired. As shown in Table 2, transcript levels of IL-1 β were comparable in the *Listeria*-permissive and the *Listeria*-restrictive Mo subsets, while expression of IL-6 mRNA was even higher in the *Listeria*-permissive subset.

To test further for the importance of TNF- α for the Mo antilisterial activity, MAb anti-TNF- α or control antibody of irrelevant specificity (mouse myeloma immunoglobulin MOPC 21) was added to the infection mixture. Antibody was added at a concentration sufficient to bind 25 ng of TNF- α . This binding capacity exceeds the highest amount of TNF secreted in our system by a factor of 3. Data obtained with Mo from six different donors (Table 3) revealed that the presence of anti-TNF- α antibody (but not of control antibody) abrogated the antilisterial activity in the *Listeria*-restrictive Mo subset.

To test whether the *Listeria*-permissive Mo subset could be induced to produce larger amounts of TNF- α , lipopolysaccharide (LPS), a potent trigger of TNF- α synthesis, was added to these cells. The results indicated that addition of 1 μ g of LPS (*Salmonella minnesota*; Sigma) per ml augmented TNF- α secretion (4.04 ± 0.46) -fold ($n = 4$). Interestingly, the antilisterial activity was not affected by the increased TNF- α production of these cells: the Mo remained permissive for *L. monocytogenes* (Table 3). A comparable increase in TNF- α release $[(3.53 \pm 1.53)$ -fold; $n = 4$] was also observed after addition of LPS to the *Listeria*-restrictive subset.

An additional set of experiments was performed to test whether the *Listeria*-permissive subset could make use of externally added TNF- α . The presence of 50 ng of recombinant TNF- α per ml in the incubation mixture, however, did not evoke antilisterial activity in the *Listeria*-permissive subset $[(2.43 \pm 1.13)$ -fold increase in bacterial numbers in the absence of exogenous TNF- α ; (2.36 ± 0.62) -fold increase in the presence of exogenous TNF- α ; by way of comparison, the *Listeria*-restrictive subset was slightly bactericidal] (Table 3). Therefore, exogenous TNF- α failed to activate listericidal activity of the *Listeria*-permissive subset of Mo, despite the fact that the concentration of recombinant TNF- α used in these experiments was higher than the maximal amount of TNF- α secreted in response to *L. monocytogenes* infection (Table 1).

FIG. 3. Electron microscopic examination of the *Listeria*-permissive (a and b) and -restrictive (c and d) Mo subsets infected with listeriae for 90 min. (a) Mo packed with a large number of *L. monocytogenes* bacteria, located in phagolysosomes. (b) Extracellular *L. monocytogenes* entering the cell (labeled 1) and intracellular *L. monocytogenes* bacteria in the phagosome (labeled 2) and partly (labeled 3) and completely (labeled 4) free in the cytoplasm. Contrast, uranyl acetate and lead citrate. Magnifications, $\times 10,000$ (a) and $\times 20,000$ (b). (c) One decaying *L. monocytogenes* bacterium near the nucleus (arrow). (d) One intact and two destroyed *L. monocytogenes* bacteria in a phagosome (arrows). Contrast, uranyl acetate and lead citrate. Magnifications, $\times 10,000$ (c) and $\times 20,000$ (d).

DISCUSSION

In the present study, we focused on the very early response of human Mo to infection with *L. monocytogenes* and found evidence for cell subsets with different antilisterial effector functions in a Mo population derived from individual human subjects. Differences in phagocytosis of *L. monocytogenes*, control of intracellular *L. monocytogenes* growth, and production of TNF- α were observed. High antilisterial activity was associated with a low capacity to ingest *L. monocytogenes* and the secretion of high amounts of TNF- α , whereas the Mo subset which could not restrict intracellular growth of *L. monocytogenes* phagocytosed many of these bacteria; however, this was not followed by substantial TNF- α production. Despite these functional differences, the two Mo subsets were phenotypically indistinguishable. The membranes of both Mo subsets displayed comparable amounts of receptors known to be involved in the adherence of bacteria to cells in bacterial uptake. This is in contrast to previous studies in which functional differences were accompanied by differences in expression of cell surface structures such as Fc receptors or CR3 (29, 31, 35, 36).

Our finding, however, does not rule out the possibility that

the same receptor in one, but not the other, subset of Mo may be utilized for enhanced bacterial uptake. Such a function could be envisaged in CR3/CD11b, a member of the leukocyte integrin family. This structure is known to mediate enhanced

TABLE 1. Release of TNF- α by Mo subsets permissive and restrictive for listerial growth^a

Expt	TNF- α released (pg/ 10^6 Mo at 90 min)	
	<i>Listeria</i> -restrictive Mo	<i>Listeria</i> -permissive Mo
1	4,066	694
2	8,616	2,968
3	3,000	588
4	793	550
5	483	191

^a Mo subsets were obtained by infection of adherent Mo with *L. monocytogenes* for 90 min as outlined in Materials and Methods. After the 90-min infection period, the cells were washed free of noningested bacteria and incubated for an additional 90 min in fresh GHBSS-FCS. The amount of TNF- α released by the two Mo subsets during this incubation period was measured by ELISA and found to differ significantly ($P < 0.05$; paired Student *t* test).

TABLE 2. Comparison of cytokine mRNA levels in the two Mo subsets as determined by quantitative competitive PCR^a

Mo subset	% Cytokine mRNA level ^b		
	TNF- α	IL-1 β	IL-6
Uninfected Mo	3.3 $\pm$ 0.14	1.45 $\pm$ 0.35	0
<i>Listeria</i> -permissive Mo	35.3 $\pm$ 14	77.7 $\pm$ 16.2	204.8 $\pm$ 8.5
<i>Listeria</i> -restrictive Mo	100	100	100

^a The ethidium bromide-stained PCR products were directly quantified by video image analysis.

^b The data are expressed as the percentage of mRNA molecules (mean $\pm$ standard deviation [$n = 3$]) for TNF- α , IL-1 β , and IL-6 in the *Listeria*-permissive Mo subset in comparison with the *Listeria*-restrictive Mo subset (100%). Uninfected Mo served as controls. The number of mRNA molecules for each sample was normalized to 10⁶ mRNA molecules of GAPDH.

phagocytosis after activation of the cells (40) (possibly by involvement in the organization of cytoskeletal events required for ingestion [reviewed in reference 8]) and functions as a principal mediator of uptake of intracellular parasites (32), including listeriae (14). Likewise, the functions of Fc receptors, the mannose-fucose receptor, and the fibronectin receptor are known to be regulated in a complex manner by inflammatory and infectious stimuli (4).

Alternatively, it may be assumed that bacterial uptake of the two Mo subsets involves different receptors. This was recently demonstrated by Drevets et al. for phagocytosis of *L. monocytogenes* by murine macrophages (15). These authors found that in *Listeria*-restrictive macrophages, two-thirds of the bacteria were ingested via CR3, while in *Listeria*-permissive macrophages, only one-third of the bacteria utilized this pathway and the majority of the bacteria were taken up by other means. This mode of uptake, which has yet to be determined, also allowed escape of *L. monocytogenes* from the phagolysosome into the cytosol. In this context, it is interesting to note that, in the human system described here, the *Listeria*-permissive Mo subset also contained intracytosolic *L. monocytogenes*, while in the *Listeria*-restrictive Mo, the bacteria were confined to the phagolysosomes. This finding suggests that different modes of listerial uptake may be operational in our system as well. Studies to address this question are currently in progress in our laboratory.

In vivo studies in the mouse system clearly showed that the presence of TNF- α has a beneficial effect on the outcome of a *Listeria* infection (20, 21, 28). Bactericidal functions of mononuclear phagocytes are known to be under cytokine control (22), with TNF- α playing a pivotal role in the activation of Mo for listericidal and mycobactericidal activities (5, 41). In vitro experiments with human Mo revealed that TNF- α was able to augment the Mo antibacterial activity (5, 12, 41). In the present study, *Listeria*-restrictive Mo were found to synthesize up to six times more TNF- α than that synthesized by *Listeria*-permissive Mo. The increased amount of synthesized TNF- α was paralleled by a higher level of accumulated TNF- α mRNA in the *Listeria*-restrictive Mo subset.

The reduced level of TNF- α mRNA in the *Listeria*-permissive subset, however, is not the consequence of a general blockade of cytokine production in these cells. Gene expression of IL-1 β and IL-6, both known to be coinduced along with TNF- α (6, 33, 42, 43), did not follow the same pattern. In both Mo subsets, *L. monocytogenes* infection was followed by comparable expression of IL-1 β mRNA, while IL-6 mRNA was expressed to a higher extent in the *Listeria*-permissive subset. This is in line with several reports which have shown that

inflammatory cytokines are in fact regulated differently (17, 26, 37).

A correlation between impaired TNF- α production and the loss of antilisterial activity does not necessarily imply a causal relationship. The finding that the permissive Mo subset could be rendered restrictive neither by addition of exogenous recombinant TNF- α nor by triggering of endogenous TNF- α production with LPS may argue against the role of TNF- α in the activation of enhanced antibacterial activity. This apparent paradox, however, might be explained by the possibility that the *Listeria*-permissive Mo subset does not express receptors for TNF- α or expresses functionally inactive TNF- α receptors which would impair the capacity of these cells to respond to this cytokine. Alternatively, TNF- α may not be the only cytokine required to induce antibacterial activity. There is evidence that activation of Mo for enhanced bactericidal activity is a multifactor process (27, 41). A publication by Bermudez et al. (3) showed that IL-6 decreased the ability of macrophages to inhibit growth of *Mycobacterium avium*, another intracellular bacterium. This effect was caused by the down-regulation of membrane TNF receptors, which were decreased by 78% in macrophages exposed to IL-6. In light of these findings, the high level of IL-6 mRNA observed in the *Listeria*-permissive Mo subset described in the present study may help to explain the impaired capacity of these cells to restrict the growth of *L. monocytogenes*.

Our results suggest that two subsets of mononuclear phagocytes which may fulfill different tasks exist. The *Listeria*-restrictive Mo subset could be envisaged as the cells that kill *L. monocytogenes* bacteria and direct the killed bacteria to an exogenous antigen-processing pathway. In addition, these cells produce autocrine activation signals such as TNF- α . The *Listeria*-permissive Mo subset, on the other hand, which harbors growing listeriae in its cytoplasm, could present their intracytoplasmically processed bacterial antigens via major histocompatibility complex class I, which then would lead to the induction of cytotoxic CD8⁺ T cells (22). Whether the two Mo subsets are specialized mononuclear phagocytes or just cells in different stages of differentiation remains to be shown.

TABLE 3. Effects of anti-TNF- α antibody, exogenously added LPS, or recombinant TNF- α on the listericidal activity of the two Mo subsets^a

Additive ^b	Fold change in no. of listeriae ^c	
	<i>Listeria</i> -restrictive Mo subset	<i>Listeria</i> -permissive Mo subset
None	0.88 $\pm$ 0.3 ($n = 11$)	2.43 $\pm$ 1.13 ($n = 11$)
Anti-TNF- α Ab	2.42 $\pm$ 0.5 ($n = 6$)	ND ^d
Irrelevant Ab	0.9 $\pm$ 0.1 ($n = 3$)	ND
LPS (1 μ g/ml)	1.25 $\pm$ 0.17 ($n = 3$)	2.2 $\pm$ 0.9 ($n = 3$)
rTNF- α (50 ng/ml)	0.9 $\pm$ 0.3 ($n = 4$)	2.36 $\pm$ 0.62 ($n = 4$)

^a Mo subsets were obtained by infection of adherent Mo with *L. monocytogenes* for 90 min. After the 90-min infection period, cells of both Mo subsets were washed free of noningested bacteria and their antilisterial activities were assessed after incubation for an additional 90 min in fresh GHBSS-FCS with the indicated additives.

^b Ab, antibody; rTNF- α , recombinant TNF- α .

^c Data are given as the fold change in number of *L. monocytogenes* bacteria during the 90-min killing assay (mean $\pm$ standard deviation) (values of >1 indicate bacterial growth, whereas values of <1 reflect killing of listeriae). The difference in the antilisterial activity between the *Listeria*-permissive and the *Listeria*-restrictive Mo subsets was significant ($P < 0.001$). Also, a statistically significant difference in antilisterial activities of *Listeria*-restrictive Mo in the presence and absence of anti-TNF- α antibody was found ($P < 0.001$).

^d ND, not determined.

ACKNOWLEDGMENTS

We thank Elisabeth Hopfner and Peter Zauner (flow cytometry) for excellent technical assistance and Myrna Pfundner for help in preparing and typing the manuscript.

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