Phagocytosis-Associated Functions in Neonatal Monocyte-Derived Macrophages

CHRISTIAN P. SPEER, MANFRED GAHR, MARION WIELAND, AND STEFAN EBER

Department of Pediatrics, University of Göttingen, Federal Republic of Germany

ABSTRACT. Using a Teflon culture system we analyzed different aspects of cell maturation and phagocytic activities in neonatal monocyte-derived macrophages. Morphological and cytochemical characteristics as well as the protein composition of neonatal macrophages were identical with those of adult controls; actin binding protein (265,000 Da), myosin (210,000), α -actinin (102,000) and actin (42,000) could be identified in cells from either source. All phagocytic functions were shown to be perfectly normal in neonatal macrophages when compared with adult cells: random migration, chemotactic response to zymosan-activated serum and formyl-methionyl-leucyl-phenylalanine, ingestion, and killing of *Staphylococcus aureus*, phagocytosis-associated chemiluminescence, production of oxygen intermediates (superoxide anion, O_2^- ; hydrogen-peroxide, H_2O_2). Phorbol myristate acetate-stimulated O_2^- -generation by 1×10^5 macrophages was 11.8 ± 4.7 nmol/h for neonates, and 10.2 ± 3.9 for controls; production of H_2O_2 was 7.6 ± 3.5 nmol/h in neonatal macrophages and 6.4 ± 2.8 in adult controls. It is unlikely, then, that the increased susceptibility of human neonates to systemic bacterial infections can be related to an abnormality in the essential phagocyte functions of macrophages. (*Pediatr Res* 24: 213–216, 1988)

Abbreviations

FMLP, formyl-methionyl-leucyl-phenylalanine
PMA, phorbol myristate acetate
 O_2^- superoxide anion
 H_2O_2 , hydrogen peroxide
HBSS, Hanks' balanced salt solution
SDS, sodium dodecyl sulfate
 $\cdot OH$, hydroxyl radical
PBS, phosphate-buffered saline

In newborns, who suffer an increased susceptibility to serious systemic infections, subnormal phagocyte functions have been described. Neutrophils from neonates showed a decreased deformability (1) and a marked deficiency in movement toward different chemotactic stimuli (2–6). In addition generation of $\cdot OH$ by neonatal neutrophils was shown to be decreased (7). However, neonatal monocytes exhibited basically normal phagocytic functions when compared with adult cells (8). The functions of neonatal macrophages have not been fully explored. Macrophages from newborns synthesize humoral host defense factors such as complement components C2, C4 to a similar extent as phagocytes from adults (9, 10); fibronectin production

was shown to be decreased in neonatal macrophages (9). Macrophages from newborns who had been activated by interferon- γ effectively killed or inhibited replication of toxoplasma gondii (11), presentation of bacterial Ag, and production of interleukin 1 was normal in cord blood monocytes/macrophages (12, 13). However, it has been suggested, that the decreased production of interferon- γ by human neonatal cells (13) could be due to a functionally immature macrophage (14).

Our study was initiated to examine the principal phagocyte activities of monocyte-derived macrophages in response to inflammation, i.e. chemotaxis, phagocytosis, killing, and generation of oxygen metabolites. Morphological studies included a detailed analysis of the protein composition of macrophages.

MATERIALS AND METHODS

Cell separation. Umbilical venous blood was withdrawn from the placenta into heparin (1–20 U/ml) immediately after delivery of healthy term newborns who were products of an uncomplicated pregnancy, normal labor, and vaginal delivery ($n = 78$); general anesthesia was not used in any of the deliveries. Mothers were not receiving a special diet or medications, including antibiotics, within 4 wk before delivery. Blood from healthy adult donors similarly anticoagulated was drawn simultaneously with the procurement of cord blood. Mononuclear cells were isolated by dextran sedimentation and separation by Ficoll-Hypaque density gradient as previously described (15).

Mononuclear cells were resuspended in RPMI 1640 (with Pen-Strep, L-glutamine and HBSS, Flow Laboratories, Meckenheim, FRG) at a final concentration of 1×10^6 cells/ml. A total of 5 ml samples was placed into 100-mm diameter culture dishes (Falcon, Oxnard, MA). After a 2 h incubation at $37^\circ C$ in 5% $CO_2/95\%$ air, nonadherent cells were removed by gently aspirating the supernatant fluid and repeated washings with media (three times). Monocyte suspensions were then prepared by recovering adherent cells after incubation in PBS, pH 7.4, containing 2 mmol EDTA at $4^\circ C$ for 15 min and gently resuspending with a rubber policeman. These suspensions contained about 90% monocytes, identified by nonspecific esterase stain.

Cells were pooled, centrifuged, and resuspended at a concentration of 1×10^5 /ml in RPMI 1640 without serum. In a volume of 50 ml the cell suspension was added to Teflon culture bags and the cells were cultured for 10 days at $37^\circ C$ in 5% $CO_2/95\%$ air. After 10 days in culture the macrophages were washed and again examined for nonspecific esterase activity and in some experiments for peroxidase activity (16). More than 95% of the macrophages were viable by a trypan blue exclusion test. In air-dried cell smears macrophages were identified by monoclonal mouse anti-human macrophage antibodies (M 780) detected by use of alkaline phosphatase labeled antibodies (Dakopatts, Giostrup, Denmark).

SDS-polyacrylamide gel electrophoresis of macrophages. Approximately 5×10^6 macrophages were lysed in 0.025 M Tris-(hydroxymethyl) aminomethane-HCl buffer, pH 8.2, containing

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Correspondence Christian P. Speer, M.D., Universitäts-Kinderklinik, Robert-Koch-Str. 40, D-3400 Göttingen, Federal Republic of Germany.
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0.19 M glycerine, 5% w/v SDS, and 5% w/v mercaptoethanol. The samples were incubated at 60° C for 15 min, diluted with aqua dest. (1:5), and kept at 100° C for 7 min. After centrifugation (10 min at 48,000 × *g*) the supernatants were diluted 1:2 with sample buffer according to Laemmli (17). SDS-polyacrylamide gel electrophoresis was performed using the discontinuous buffer system (17) with a gradient of acrylamide concentration ranging from 5–17%. Proteins were stained with Coomassie blue G 250. Molecular weight markers (Bio-Rad Laboratories, Richmond, CA) were applied to the same gel. The standard proteins were myosin (apparent mol. wt. 200,000), β -galactosidase (116,250), phosphorylase B (92,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400). In addition erythrocyte membrane proteins extracted according to Dodge *et al.* (18) were used as reference samples. The mol. wt. of the prominent macrophage proteins were determined by applying a calibration curve from known standards according to Weber and Osborn (19).

Chemotaxis. Chemotaxis of macrophages was evaluated using a modification of the Boyden chamber technique (Celloplex, Basel, Switzerland) as recently described (20). Pooled serum activated by zymosan (50 mg/ml Sigma, Munich, FRG) and FMLP 10^{-5} M (Sigma) were used as chemoattractants.

Phagocytosis and bactericidal assay. A modification of the method of Quie *et al.* was used (21). Phagocytosis by macrophages was measured as the decrease in the number of viable extracellular bacteria during incubation of bacteria and macrophages in the presence of serum. The 1 ml reaction mixture consisted of 0.3 ml HBSS, 0.1 ml pooled human serum, approximately 5×10^6 bacteria (*Staphylococcus aureus* 502 A) in 0.3 ml HBSS and 2.5×10^6 macrophages in 0.3 ml HBSS. The tubes were chilled in an ice bath, and then serum, bacteria, and phagocytes were added in that order. Samples were incubated at 37° C and aliquots (50 μ l) were removed at 0, 30, 60, and 90 min. The aliquots were added to ice cold HBSS (450 μ l) and the cells immediately centrifuged at 200 × *g* for 2 min at 40° C and the supernatant was diluted to make agar pour plates for colony counting. Intracellular killing of *S. aureus* was measured as recently described (20).

Chemiluminescence. Luminol-dependent chemiluminescence was measured using a luminometer (LKB-Wallac 1250); details have been recently published (20).

Measurement of O_2^- and H_2O_2 . Release of O_2^- by 1×10^5 macrophages/ml in suspension was determined by superoxide dismutase inhibitable reduction of cytochrome C using PMA (Consolidated Midland Corporation, Brewster, NY) or opsonized zymosan as stimulus (22). The formation of H_2O_2 by macrophages (1×10^5 /ml reaction mixture) was assayed by measuring the amount of fluorescence due to the oxidation of homovanillic acid by H_2O_2 catalyzed by horseradish peroxidase as recently described (20). Assays were performed in duplicate.

RESULTS

Morphology and cytochemical characteristics of cord blood macrophages. After 10 days in culture monocyte-derived macrophages from neonates and adults demonstrated similar heterogeneous morphological changes: increase in cell size, ovaloid shape, and development of a few dendritic processes. A total of $96.6 \pm 4.1\%$ of the cord blood macrophages stained for esterase and the corresponding percentage of control macrophages was 98.2 ± 3.4 (mean $\pm$ 1 SD, *n* = 12). Peroxidase staining was negative in cells from either source. A total of $93.3 \pm 2.5\%$ of the neonatal macrophages was detected by monoclonal mouse anti-human macrophage antibodies (Dako-macrophages, EBM 11, Dako Corp., Santa Barbara, CA) and 98.8 ± 0.8 of controls.

SDS-polyacrylamide gel electrophoresis. In macrophages from neonates and adults several prominent protein bands could be

Fig. 1. SDS-polyacrylamide gel electrophoresis of macrophage proteins, erythrocyte membrane proteins and molecular weight markers. Right, apparent m.w. of the marker proteins. Left, m.w. of prominent macrophage proteins. a, macrophage extract from adult donor; b, macrophage extract from a newborn infant; c, erythrocyte membrane proteins which were used as standards: spectrin (220,000–240,000), band 3 (90,000–120,000), actin (42,000), glyceraldehyde-3-phosphate dehydrogenase (35,000), band 7 (27,000–29,000), and Hb (17,000) (28); d, low m.w. markers; e, high m.w. markers.

found (Fig. 1). On the top of the gel there is a triplet of proteins containing actin-binding protein (265,000), an unidentified second band (240,000), and myosin (210,000). A distinct band at 102,000 Da probably represents α -actinin. The next protein (55,000) is most likely vimentin. Actin (42,000) can be identified by comparison with erythrocyte actin. A triplet of bands with a mol. wt. ranging from 32,500 to 35,500 is found below actin. The first of the three bands comigrates with erythrocyte glyceraldehyde-3-phosphate dehydrogenase. The protein with a mol. wt. of 28,000 Da is similar to tropomyosin. Some bands with an apparent mol. wt. of about 18,000 Da should contain profilin and myosin light chain. Two prominent bands with a mol. wt. of 47,500 and 22,800 could not be identified. All proteins are present to an identical amount in macrophages from newborns and adult controls.

Random migration and chemotaxis. As demonstrated in Table 1 during a 90-min assay random migration of cord blood macrophages was similar to that of adult controls. In addition the chemotactic response to zymosan-activated serum was identical in macrophages from neonates and adults. A mildly decreased chemotaxis of cord blood macrophages was observed in response to the chemotactic peptide FMLP.

Phagocytosis and bactericidal capacity of macrophages. As shown in Figure 2 neonatal macrophages exhibited normal phagocytosis of opsonized *S. aureus*. Similarly neonatal macrophages killed *S. aureus* as efficiently as macrophages from adults.

Chemiluminescence. After stimulation with PMA macrophages from neonates and adult controls generated small amounts of chemiluminescence (Fig. 3); both peak values and kinetics of the reaction were identical in cells of either source. Chemiluminescence induced by opsonized zymosan gradually increased to reach a peak at about 15 min; the peak value was about two-third of that achieved with PMA.

Production of O_2^- and H_2O_2 . Generation of oxygen metabolites by neonatal macrophages and the extent of O_2^- and H_2O_2 released by macrophages from neonates and adult controls are summarized in Table 2.

Production of superoxide anion in unstimulated macrophages was slightly elevated in cells from neonates. However, after stimulation with PMA and during phagocytosis of opsonized zymosan macrophages from neonates and adults similarly generated O_2^- and H_2O_2 .

DISCUSSION

Macrophages that are present in various anatomical sites, play a central role in cellular host defense; their main functions include response to inflammation, immunoregulatory activities, microbicidal as well as tumoricidal capacities (23). Macrophages are motile cells that are capable of migrating directionally along chemotactic gradients (24). Branching network of actin filaments are the principal structures observed in the moving regions of cytoplasm (25).

The antimicrobial and anticellular activities of macrophages depend to a large extent on their ability to ingest various particles and to reduce oxygen molecules to reactive intermediates (O_2^- , H_2O_2 , $\cdot OH$). These phagocytic functions of macrophages have not been evaluated in the human neonate. Using a Teflon culture system we analyzed different aspects of cellular activities in nonadherent monocyte-derived macrophages.

Morphological and cytochemical characteristics of neonatal macrophages were identical with those of adult controls (9). In a detailed analysis of the protein composition of macrophages no quantitative and qualitative differences were found between cells from either source.

Actin binding protein, α -actinin, myosin, and actin could be identified in both macrophages. Random migration and a chemotactic response to zymosan-activated serum was identical in cells from newborns and adults. Additionally ingestion of opson-

ized *S. aureus* was normal in neonatal macrophages as well as in monocytes from newborns (8).

During phagocytosis of opsonized zymosan and perturbation with PMA macrophages from neonates and adults generated considerable amounts of O_2^- and H_2O_2 ; no differences in the production of either oxygen metabolites were found. Hydroxyl radical production in neonatal monocytes was also shown to be normal (27).

Generation of oxygen radicals in monocytes and monocyte-derived macrophages activated by lipopolysaccharide was identical in cells from newborns and adults (10, 27). However, generation of chemiluminescence was similarly depressed in macrophages of either source, when compared with monocytes (20); this disparity between a normal generation of oxygen metabolites and a decreased chemiluminescence might be explained at least in part by a reduction in the content of excitable substrates

Table 1. Random migration and chemotaxis of macrophages from neonates and adult controls in response to zymosan-activated serum or FMLP (10^{-5} M)

Chemoattractant	Macrophage migration (μm)*	
	Neonate	Adult
None	47.7 $\pm$ 7.3†	53.2 $\pm$ 6.4
Zymosan-activated serum	128 $\pm$ 25.3	141 $\pm$ 29.6
FMLP	73.9 $\pm$ 16	89.8 $\pm$ 12.3

* The migratory distance was expressed in μm .

† Mean $\pm$ SD of averages of duplicate samples; 12 paired experiments were performed with macrophages from either source.

Fig. 3. Generation of chemiluminescence by 1×10^5 macrophages from neonate and adult controls after stimulation with PMA. Mean values ($\pm$ SD) are plotted for six experiments with either cell.

Fig. 2. Phagocytosis of *S. aureus* by macrophages from neonates and adults (a). Bactericidal activity of macrophages from neonates and adults. Values represent mean $\pm$ SD for six experiments performed in duplicate (b).

Table 2. Generation of O_2^- and H_2O_2 in macrophages from neonates and adults

Stimulus	Macrophages	O_2^- (nmol/ 10^5 cells/h)	H_2O_2 † (nmol/ 10^5 cells/h)
None	Neonate	$4.1 \pm 1.9\ddagger$	1.2 ± 0.6
	Adult	2.5 ± 0.9	0.7 ± 0.5
PMA	Neonate	11.8 ± 4.7	7.6 ± 3.5
	Adult	10.2 ± 3.9	6.4 ± 2.8
Zymosan	Neonate	9.1 ± 2.3	6.1 ± 3.2
	Adult	8.4 ± 2.7	5.9 ± 3.7

* O_2^- was quantitated by its capacity to reduce ferricytochrome c.

† H_2O_2 was measured by following the increase of fluorescence due to the oxidation of homovanillic acid catalyzed by horseradish peroxidase.

‡ Mean $\pm$ SD of averages of duplicate samples ($n = 12$).

during long term culture of macrophages. The bactericidal capacity of neonatal macrophages against *S. aureus* was as effective as that from control cells. Studies of microbicidal activity by neonatal monocytes similarly indicate that phagocytic killing of various microorganisms is approximately normal (8). However, a depression of microbicidal capacity could be demonstrated when neonatal monocytes were challenged by group B Streptococcus (26); the mechanisms of this impairment is unknown.

The results of our study demonstrate that monocytes from newborns and adults similarly mature into macrophages during long-term culture. In addition neonatal macrophages exhibit perfectly normal phagocytic activities when compared with adult cells. It is unlikely, then, that the increased susceptibility of neonates to systemic bacterial infections can be related to an abnormality in the essential phagocyte functions of macrophages.

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