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Mononuclear phagocytes from human bone marrow progenitor cells; morphology, surface phenotype, and functional properties of resting and activated cells

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SUMMARY

After 3–4 weeks culture of human bone marrow cells in medium supplemented with IL-3, macrophage- (M-CSF), and granulocyte-macrophage colony-stimulating factor (GM-CSF), the firmly adherent cells exhibited the morphologic features of mononuclear phagocytes and were strongly esterase-positive. Flow cytometric analysis revealed a rather homogeneous cell population with marked autofluorescence; the large majority of the cells expressed CD14, CD11a,b, and c, Fc receptors for IgG, FcγRI, II, and III, and HLA class II molecules. Interferon-gamma (IFN-γ), bacteria, and bacterial products modulated expression of some of the surface markers, induced and/or enhanced respiratory burst, phagocytic activity, secretion of tumour necrosis factor, and tumouricidal activity; in contrast, these cells were not able to generate reactive nitrogen intermediates.

Keywords macrophage differentiation and activation interferon-gamma bacteria tumour necrosis factor nitric oxide

INTRODUCTION

Utilizing similar techniques as in previous work with rodent bone marrow cells [1–4], it was the aim of the present study to obtain a pure population of human bone marrow-derived mononuclear (BMM) phagocytes. Human bone marrow samples from the iliac crest were propagated in long-term cell culture, the cells becoming adherent after an appropriate time interval were collected, and their phenotypic and functional characteristics determined. The results of this study provide evidence that, by the methods used, a homogeneous cell population exhibiting the characteristics of human mononuclear phagocytes is obtained.

MATERIALS AND METHODS

Materials

Growth factors. Human IL-3 ($3{\cdot}0 \times 10^6$ U/mg) and human GM-CSF ($5{\cdot}9 \times 10^6$ U/mg) were kindly provided by Dr M. Schreier (Sandoz Ltd, Basel, Switzerland); human M-CSF was a gift from Genetics Institute (Cambridge, MA).

Bacteria and bacterial products. The bacteria were selected, grown, harvested and heat-inactivated as previously described [5]; wet weight of washed bacteria was taken as a measure of their amount. Peptidoglycan was isolated from *Moraxella catarrhalis* organisms [6].

Other agents. Recombinant human interferon-gamma (IFN-γ) (biologic activity 10^5 U/ml) was from Professor C. Weissmann (University of Zürich, Switzerland). Human holotransferrin, bovine serum albumin (BSA), lipopolysaccharide (LPS) from *Escherichia coli* 0128:B12, lipoteichoic acid (LTA) from *E. faecalis*, and T-2 toxin from *Fusarium* sp. were from Sigma, St Louis, MO. N^G-monomethyl-L-arginine (NMMA) was purchased from Bachem (Bubendorf, Switzerland), soy bean lecithin (PH 75), was from Nattermann (Köln, Germany). Vitamin D_3 and retinoic acid were kindly provided by F. Hoffmann-La Roche (Basel, Switzerland). 12-*o*-tetradecanoylphorbol-13-acetate (TPA) was purchased from Consolidated Midland Corp. (Brewster, NY). Endotoxin was measured with the Endospecy reagent set from Seikagaku Kogyo Co. (Tokyo, Japan).

Monoclonal antibodies. The MoAbs used in this study are listed in Table 1. MoAb against human tumour necrosis factor-alpha (TNF-α) was kindly provided by Knoll AG (Ludwigshafen, Germany).

Methods

Culture of bone marrow cells. After informed consent, human bone marrow samples obtained by aspiration from the

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Table 1. Reactivity of MoAbs with non-stimulated, adherent bone marrow-derived mononuclear cells

CD	MoAb clone	(Source)	Specificity	Per cent non-stimulated	Per cent positive cells after incubation with
3	UCHT1	(1)	CD3 T cell receptor complex, p20,26	<15	
6	LO-CD6a	(1)	Pan T differentiation antigen	<10	
11a	38	(2)	LFA-1α; leucocytes	90–100	
	YTH-81.5	(1)	LFA-1; leucocytes	90–100	60–70 (LPS)
11b	44	(2)	C3Rα; monocytes,	75–85	ND
	BRC-GM1	(1)	CR3, Mac-1; granulocytes	75–85	ND
11c	3.9	(1)+(2)	p150.95, pan monocyte/macrophage molecule	80–90	ND
14	UCHMI	(2)	p55; monocytes	80–90	ND
	SP-21	(1)	Monocytes	80–90	ND
16	YFC 120.5	(1)	FcγRIII neutrophils,	90–95	ND
	80H3	(1)	FcγRIII monocytes,	5–15	0
	3G8	(2)	FcγRIII NK cells	45–55	60–70 (IFN-γ, LPS MC)
18	YFC 118.3	(1)	LFA-1β	90–100	0
23	BSL/23	(1)	Low affinity FcγR; B cells	70	ND
30	YTH 66-9HL	(1)	Monocytes, T and B cells Reed-Sternberg cells	90–100	0
	W31	(1)	FcγRII; B cells, monocytes, granulocytes	85–95	0
33	WM-54	(1)	gp67; myeloid lineage	70	ND
34	QBEND/10	(1)	Myeloid precursors, endothelial cell marker	10	15–20 (IFN-γ)
35	E 11	(1)	CR1; B cells monocytes, granulocytes	25–50	0
45	F10-89-4	(1)	T200; leucocyte common antigen	75–85	ND
54	15.2	(2)	ICAM-1; leucocytes	80–95	95–100 (IFN-γ)
	84H10	(1)	ICAM	80–95	95–100 (IFN-γ)
64	10.1	(2)	FcγRI; monocytes	65 90	85–100 (IFN-γ)
68	EBM11	(3)	Macrophage cytoplasmic protein	50	0
—	YE2/36 HLK	(1)	HLA-DR	>90	ND
—	YD1/63	(1)	HLA-DR	>90	ND
—	52	(2)	MHC-II (HLA D/DR,DQ)	65–75	80–90 (IFN-γ) 55 (LPS)

Source of MoAb: 1, Serotec; 2, N. Hogg [14,15]; 3, Dako A/S. IFN-γ, interferon-gamma (0·5–25 U/ml); LPS, lipopolysaccharide *E. coli* (1–10 ng/ml); MC, *Moraxella catarrhalis* (1–2·5 μg/ml); 0, not affected by stimulants, results as with non-stimulated cells; NK, natural killer; ND, not done.

iliac crest of healthy adult volunteers or of tumour patients in remission were collected in RPMI 1640 medium supplemented with 50 U/ml heparin (Liquemin, Roche) and depleted of erythrocytes by separation on Histopaque-1083 (Sigma) for 120 min at room temperature. The leucocytes were washed four times, suspended in IMDM supplemented with 15% heat-inactivated (60 min, 56°C) fetal calf serum (FCS; GIBCO, Grand Island, NY) plus antibiotics (50 U/ml penicillin, 50 μg/ml streptomycin), and adjusted to $2–4 \times 10^5$ cells/ml. Recombinant growth factors, vitamin D_3, retinoic acid and TPA were then added in different concentrations and various combinations. Cell suspension (10 ml) was seeded into bacteriologic Petri dishes (diameter 96 mm; Greiner, Nürtingen, Germany) and the cultures incubated for 7 days at 37 C/5% CO_2. Afterwards, 5 ml fresh IMDM (supplemented with 10% FCS plus antibiotics with or without the other admixtures) were added to each of the dishes and incubation proceeded for an additional week. This procedure was continued until a considerable portion of the cells had become adherent, usually on day 18–30 after initiation of the cultures. These cells were used in the experiments described below.

Morphological studies. To characterize the cells, their light microscopic appearance, the cytochemical staining for esterase [3], and their ability to ingest particles such as latex or heat-killed *Listeria monocytogenes* or *Corynebacterium parvum* organisms [3] were assessed. For scanning electron microscopy (SEM), bone marrow cells were cultivated on glass coverslips and processed according to Schroeter *et al.* [7]. The specimens were examined in a SEM 505 (Philips, Eindhoven, The Netherlands). For transmission electron microscopy (TEM), the cells grown

on plastic coverslips (Thermanox) were fixed as previously described [3]. Ultra-thin sections (*ca* 50 nm) contrasted with uranyl acetate and lead citrate were studied in a Philips TEM 420.

Surface phenotype of the cells. Utilizing a panel of currently available MoAbs, the expression of surface markers by the human bone marrow-derived cells was analysed by flow cytometry as previously described [4,8]. For detection of intracellular antigens, the cells were first incubated for 60 min at 0°C in a periodate-lysine-paraformaldehyde solution [9] and washed four times. The MoAbs used were kindly provided by Dr Nancy Hogg or were purchased from Serotec (Blackthorn, Bicester, UK) or from Dako A/S (Glostrup, Denmark).

Functional parameters. Most experiments were performed directly, i.e. without further treatment of the cells (resting cells). In some experiments, bone marrow cells were first incubated for various time intervals with IFN-γ, bacteria and/or bacterial products before their functional activity was determined.

Respiratory burst was measured by incubation for 10 min at 37°C with dihydrorhodamine 123; its oxidation results in the quantitative formation of a bright green fluorescence in the mitochondria (Bursttest, Orpegen, Heidelberg, Germany). Ten thousand cells were analysed each time in the FACS analyser and the results processed with the Consort 30 software (Becton Dickinson). In parallel, reductive capacity (mitochondrial respiration) of control and activated cells (10^5/well, in 96-well microplates) was determined in a MTT tetrazolium assay [10] by measuring absorbance at 570 nm in a Dynatech MR 700 microplate reader.

Phagocytosis. Phagocytosis was measured by the uptake within 60 and/or 120 min at 37°C of standardized fluoresceinated *E. coli* organisms (Phagotest, Orpegen).

TNF-α activity. TNF-α activity was determined in supernatants harvested from 5×10^5 bone marrow cells/ml by measuring their ability to affect the reductive capacity of the TNF-α-sensitive WEHI-164/13 cells in a 3-h MTT tetrazolium cytotoxicity assay [6,11]. The assay was standardized with human rTNF-α (specific activity $8{\cdot}1 \times 10^6$ U/mg protein; Knoll AG, Ludwigshafen, Germany) and its specificity checked with anti-TNF-α MoAb 195 (Knoll; 200 ng/ml MoAb neutralized 1 ng/ml TNF-α).

Nitrite concentrations. Nitrite concentrations of cell-free sample aliquots (100 μl) harvested from bone marrow cells (10^6 cells/ml per 16-mm well) were determined by measuring absorbance at 550 nm in a microplate reader (Dynatech MR 700 [11]).

Tumouricidal activity. Tumouricidal activity of bone marrow cells was determined in a ^{14}C-thymidine release assay [3]. Prelabelled tumour cells [11] were allowed to interact with bone marrow cells at an initial effector/target cell ratio of 1:1, 2·5:1 and 5:1 for the time interval indicated before radioactivity in cell-free supernatants was measured in order to calculate the percentage of specific isotope release.

RESULTS

In early experiments, bone marrow cells from tumour patients in remission and from healthy volunteers were cultured in medium supplemented with IL-3 (1, 2·5, 5 ng/ml), GM-CSF (10, 25, 50, 100 U/ml), M-CSF (5, 10, 25, 50 U/ml), IFN-γ (1, 2·5, 5, 10 U/ml), vitamin D_3 (10^{-8}–10^{-10} M), TPA (10^{-8}–10^{-10} M), and/or retinoic acid (10^{-7}–10^{-10} M), alone or in combination. Only limited numbers of adherent, esterase-positive cells were obtained after culture in medium supplemented with vitamin D_3, retinoic acid, and/or TPA, alone or in combination. Yield and quality of adherent, esterase-positive cells were highest when the medium was supplemented with a combination of IL-3 (1·0 ng/ml), M-CSF and GM-CSF (5 U/ml each). The outcome was similar irrespective of whether the growth factors were permanently present in these concentrations or were diluted out. Therefore, in subsequent experiments, the growth factors were present only during the first 7-day period in the concentrations mentioned but were no longer replaced with the later medium supplements. This procedure yielded adherent, esterase-positive cells in considerable numbers. Endotoxin determinations showed that the medium alone was always free of endotoxin; medium supplemented with BSA and/or FCS and growth factors revealed a comparable, low endotoxin level.

The experiments reported here, obtained with bone marrow cells from nine donors, led to similar results. Phase-contrast microscopic analysis of adherent, freshly removed cells revealed a morphologically rather uniform population of cells with macrophage-like structure and nuclei. These cells were all strongly esterase-positive (not shown). By SEM, the round-shaped cells exhibited the characteristic macrophage surface morphology (Fig. 1). By TEM, the cells displayed a polymorphous nucleus rich in euchromatin, with one or two excentrically located nucleoli. The cytoplasm contained groups of oval-shaped mitochondria of crista type, short profiles of rough endoplasmic reticulum and a few polyribosomes. The large Golgi complex and abundant primary lysosomes scattered throughout the cytoplasm were the most striking features of these cells (Fig. 1). In none of the experiments could other cell types, in particular fibroblasts, lymphocytes or neutrophils, be identified with these morphological methods.

Flow cytometric analysis by the characteristic 90° light angle scatter and volume distribution showed that the cells were rather homogeneous (Fig. 2). These cells exhibited intensive autofluorescence. A high proportion of the cells expressed the gp55 protein recognized by CD14 MoAbs (Fig. 3), the leucocyte adhesion molecules of the LFA-1/CR3/p150.95 (CD11a, b, c, and CD18), and ICAM-1 (CD54) families (Fig. 3), and HLA class II antigens (Table 1). In contrast, these cells did not express surface markers considered to be selectively expressed by T or B lymphocytes [4]. Fc receptors for IgG, FcγRI, FcγRII and FcγRIII were expressed by a high percentage if not all cells already in their resting state (Table 1, Fig. 3). CD68 MoAb, known to recognize intracellular proteins of macrophages, labelled an intermediate portion of the cells (Table 1). Expression of adhesion molecules, FcγR, and MHC class II molecules could still be somewhat enhanced by IFN-γ (1 U/ml). Interestingly, incubation with LPS clearly diminished expression of HLA class II antigen (Table 1).

The reductive capacity and the respiratory burst of the cells were differently affected, depending on the type and concentration of the agent. IFN-γ (0·1–25 U/ml) was without effect, whereas interaction with Gram-negative organisms (e.g. *Clostridium perfringens*, *M. catarrhalis*; 1–100 μg/ml) and LPS (1–100 ng/ml) resulted in a significant enhancement (20–50% increase over control; data not shown). Unstimulated human bone marrow-derived mononuclear cells expressed considerable long-term phagocytic activity. Such activity could be markedly enhanced by bacteria, bacterial products, and IFN-γ. A typical

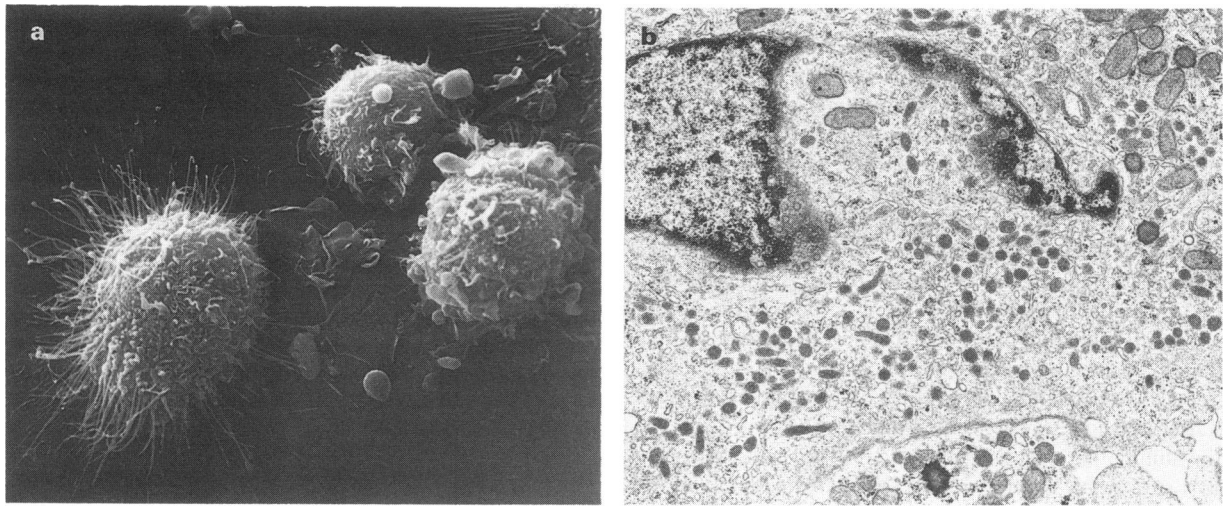

Fig. 1. (a) Surface morphology (× 1940) and (b) section of transmission electron microscopy (TEM) morphology of human bone marrow-derived adherent cells (× 10 600).

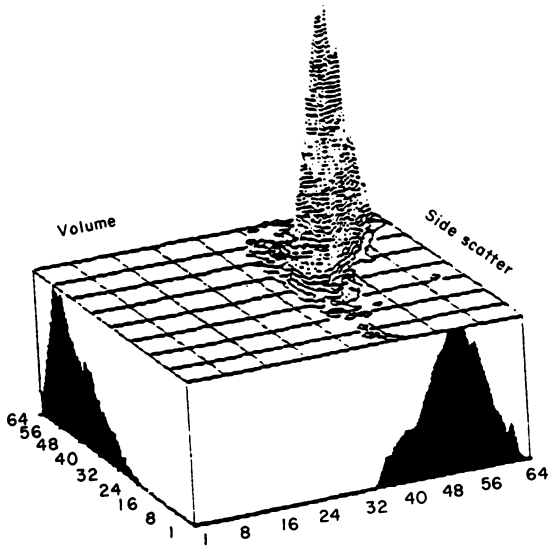

Fig. 2. Volume/light scatter distribution of human bone marrow-derived mononuclear cells.

short-term experiment regarding the modulation of phagocytic activity is given in Table 2.

Resting bone marrow-derived mononuclear cells constitutively secreted minor amounts of TNF-α (Fig. 4). Incubation of the cells with Gram-negative and Gram-positive organisms and/or their products or with IFN-γ markedly enhanced its secretion. TNF-α activity was consistently neutralized by MoAb against human TNF-α (not shown) but was not affected by the L-arginine analogue, NMMA (10^{-4} M; not shown).

Resting bone marrow-derived mononuclear cells did not secrete NO/NO_2^- in detectable amounts ($< 0{\cdot}25$ μM/10^6 cells per 24 h) and stimulation with a wide range of bacteria (including *Clostridium perfringens* and Bacille Calmette–Guérin (BCG)), bacterial products, and T-2 toxin from *Fusarium* sp. for various time intervals did not affect this capacity (data not shown).

Resting BMM phagocytes affected the viability of the tumour cell lines differently; while some (YAC-1 lymphoma)

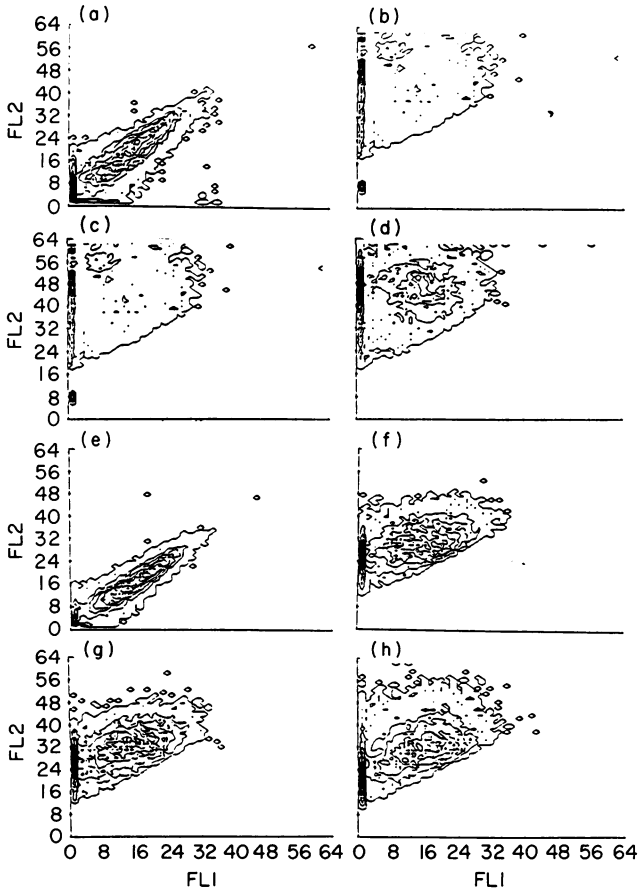

Fig. 3. Flow cytometric analysis of unstimulated human bone marrow-derived mononuclear phagocytes. (a) Background staining (control). (b) Staining with anti-CD14. (c) Anti-C3bi (CD11b). (d) Anti-ICAM-1 (CD54). (e) Background staining (control). (f) Staining with anti-FcγRI (CD64). (g) Anti-LFA-1 (CD11a). (h) Anti-monocyte (CD30).

were efficiently killed, others were only moderately affected, and some were resistant (Table 3). Incubation of BMM phagocytes for 24 h with a potentially stimulatory agent and subsequent 36-h interaction with targets in the continuing presence of the stimulator enhanced the lytic activity only little (not shown). Clearly higher lytic activity was achieved when the potential effector cells were first cultured for 24 h in medium alone, then incubated for 48 h with the stimulating agent and subsequently interacted for a further 48 h in the continuing presence of the stimulator with target cells (Table 3). IFN-γ, Gram-negative and Gram-positive bacteria and bacterial products, in concentrations readily achieved in the course of infection, were all able to induce in these cells lytic activity which was expressed against all target cell lines examined. However, the extent of target cell lysis varied considerably depending on both the target cell type and the stimulating agent. While L-1210 leukaemia cells were similarly susceptible to effector cells stimulated with any of the agents, P-815 murine mastocytoma cells were selectively killed by IFN-γ-treated effector cells. IFN-γ, *M. catarrhalis*, and *C. parvum* organisms enhanced the spontaneous lytic activity expressed against WEHI-164/13 fibrosarcoma, while the human MIA pancreas carcinoma was particularly susceptible to mononuclear cells stimulated by LPS. To assess the possible role of TNF-α and reactive nitrogen intermediates as mediators of the

Table 2. Basal short-term phagocytic activity of human bone marrow-derived mononuclear cells is enhanced by pretreatment with *Moraxella catarrhalis* organisms or by IFN-γ

Pretreatment of the cells	Mean fluorescence after 120 min at 37°C*
None, unstimulated controls, 24 h 37°C	49·8
M. catarrhalis 5 μg/ml, 24 h 37°C	73·1
IFN-γ 2·5 U/ml, 24 h 37°C	86·6

* Phagocytosis of fluoresceinated *Escherichia coli* organisms.

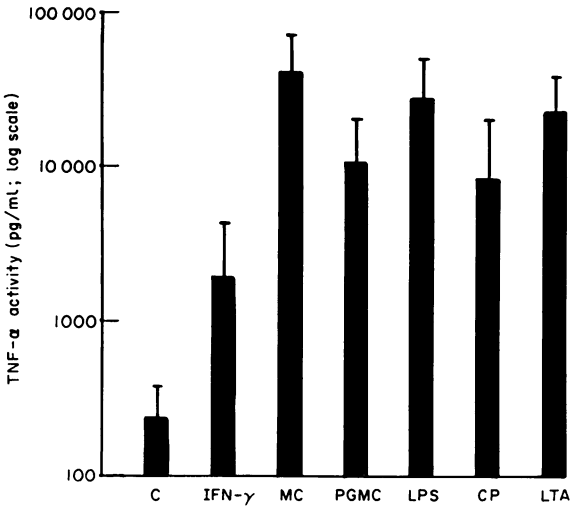

Fig. 4. Ability of bacteria and bacterial products to induce in bone marrow mononuclear cells the secretion of tumour necrosis factor-alpha (TNF-α). Values (±s.d.) represent TNF-α activity against WEHI-164/13 cells in supernatants harvested after 24 h interaction and are means from 8 to 12 experiments, each performed in triplicate. C, Controls (resting cells); IFN-γ, 2·5 U/ml; MC, *Moraxella catarrhalis* organisms, 5 μg/ml; PGMC, peptidoglycan from *M. catarrhalis*, 5 μg/ml; LPS, lipopolysaccharide *E. coli* 0128:B12, 1 μg/ml; CP, *Corynebacterium parvum* organisms, 50 μg/ml; LTA, lipoteichoic acid from *E. faecalis*, 5 μg/ml.

Table 3. Cytolytic activity of resting and stimulated human mononuclear cells expressed against various target cell lines

Target cell type	Interaction of target cells with mononuclear cells: Unstimulated control	Stimulated with: IFN-γ	MC	PGMC	LPS	CP	LTA	General characteristics of target cells
P-815, murine mastocytoma	4±4	85±8*	7±5	ND	15±6*	10±3*	ND	Susceptible to killing by mononuclear phagocytyes and T cells, resistant to TNF-α
WEHI-164/13, murine fibrosarcoma	31±8	52±10*	58±12*	36±8	33±7	48±9*	39±7	Highly sensitive to TNF-α
L-1210, murine lymphocytic leukaemia	27±8	86±12*	77±11*	76±10*	83±8*	75±9*	80±8*	Rather resistant to rodent activated BMM phagocytes
YAC-1, murine lymphoma	73±12	84±6	56±9	86±9	88±7	78±8	75±9	Sensitive to NK cells, intermediate sensitivity to rodent BMM phagocytes
MIA, human pancreas carcinoma	0±0	14±2*	38±5*	11±3*	52±8*	12±3*	38±3*	Resistant to rodent activated BMM phagocytes

Bone marrow-derived effector cells were first cultured for 24 h in medium alone, then incubated for 48 h with one of stimulating agents, and finally interacted for 48 h with prelabelled targets (initial effector/target cell ratio 2·5:1) in the continuing presence of the stimulating agent. Values represent per cent net isotope release (±s.d.) and are means from four to six experiments, each performed in triplicate. Concentrations of stimulating agents: IFN-γ 2·5 U/ml; *Moraxella catarrhalis* (MC) 2·5 μg/ml; peptidoglycan MC (PGMC) 5 μg/ml; lipopolysaccharide (LPS) *E. coli* 1 μg/ml; *Corynebacterium parvum* (CP) 50 μg/ml; lipoteichoic acid *E. faecalis* (LTA) 5 μg/ml.

* These values were statistically significantly different ($P < 0.001$; Mann-Whitney *U*-test) from unstimulated controls.

BMM, bone marrow-derived mononuclear; ND, not done.

cytolytic activity, the effects of anti-human TNF-α and of the L-arginine analogue, NMMA, on the interaction were investigated. Macrophage-mediated killing of WEHI-164/13 targets was abolished by anti-TNF-α, while the cytolytic activity expressed against the other target cell lines was not affected by the presence of the anti-serum. Moreover, the outcome of the interaction of effectors and targets was the same quite irrespective of the presence of NMMA (10^{-4} M; not shown).

DISCUSSION

It was the aim of the present study to induce human mononuclear phagocytes from bone marrow precursors, utilizing the extensive experience gained previously in rodents [2–5,8,11–13]. Adherent cells harvested after 3–4 weeks culture in IMDM medium supplemented with IL-3, GM-CSF, and M-CSF, proved to be rather homogeneous, esterase-positive, exhibited marked autofluorescence and the morphologic and flow cytometric features (volume/scatter distribution and expression of surface markers) of mononuclear phagocytes (Figs 1 and 2). By the same methods, no evidence for the presence of other cells, in particular fibroblasts, T or B cells, or neutrophils, could be found. Without further stimulation, these cells were able to secrete small amounts of TNF-α (Fig. 4), to trigger a respiratory burst, to engulf particles (Table 2), and to kill some tumour cell types (Table 3). Interaction with IFN-γ, bacteria and/or bacterial products enhanced and/or expanded the functional capacities of these cells (Tables 2 and 3, Fig. 4). These findings were in keeping with earlier work on monocytes and macrophages [14–16], suggesting that the present adherent bone marrow-derived cells belong to mononuclear phagocytes.

Although experience in the human system is still limited, it appears likely that cell proliferation and differentiation along the mononuclear phagocyte lineage are more efficiently enhanced in the presence of a combination of growth factors, i.e. IL-3, GM-CSF, and M-CSF, than with M-CSF alone. Their combined presence seemed to be particularly critical in the early, inductive phase; however, further work is required to define the optimal culture conditions.

On the other hand, the percentage of adherent unstimulated cells expressing leucocyte adhesion molecules, FcγR, or HLA class II molecules, was much higher in humans (Table 1) than in rodents [4,8]. This difference may be a consequence of their prolonged *in vitro* culture and/or the presence of limited endotoxin activity in the media supplemented with FCS and growth factors. Another striking difference between phagocytes of rodent [5,11,13] and human origin concerns their ability to respond to certain stimuli with the production of reactive nitrogen intermediates. Despite the examination of a large array of stimuli, we were in no case able to induce in human BMM phagocytes the production of even low amounts of nitric oxide and/or nitrite, which is in keeping with unpublished observations made in various other laboratories. In contrast, secretion as well as mediation of tumour cell killing by TNF-α appears to be similarly regulated in human and rodent mononuclear phagocytes. The present findings showing that some tumour cell lines were susceptible to unstimulated mononuclear cells while others are selectively killed by effector cells that had been activated with a particular stimulus, are in keeping with earlier work with human monocytes and rodent mononuclear phagocytes [11,17,18]. These data suggest that (i) the various stimulatory agents are able to trigger different effector mechanisms; and (ii) different target cell types are susceptible to different killer mechanisms.

Long-term bone marrow culture *in vitro* constitutes a useful model for the study of autorenewal, proliferation, and differentiation of haematopoietic cells [19,20]. Depending on the culture conditions and possibly other variables, previous studies demonstrated the heterogeneity of human haematopoietic cells, resulting in a majority of macrophages [19,20], fibroblastoid cells [21,22], or lymphocytes [21,23]. To our knowledge, the present study is the first successful approach to arriving at a more or less homogeneous population of human BMM phagocytes. It may provide a new starting point for the analysis of the complex pathways involved in the development and regulation of the functional activities of these versatile cells [24].

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