Characteristics of human monocytes cultured in the Teflon culture bag

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Summary. Blood monocytes from healthy volunteers were isolated by Ficoll-Isopaque centrifugation and cultured (together with lymphocytes) in medium 199 with 20% heat-inactivated newborn calf serum in a Teflon culture bag. Quantifiable data on survival showed that up to 21 days of culture, approximately 40% of the initial number of monocytes were still viable. Such cultures could be maintained for more than 8 weeks without refeeding.

The monocytes exhibited the morphology of macrophages after 5–7 days of culture, and increased in size during culture. Less than 1% of the cells became giant cells even after long culture periods. Almost all cultured monocytes were positive for α-naphthyl butyrate esterase, whereas the peroxidase-positive granules disappeared during the first week of culture. After longer culture times increasing amounts of lysozyme and angiotensin-converting enzyme were detected in the culture supernatants. Phagocytosis of staphylococci did not decrease appreciably during culture, and the same holds for intracellular killing of these bacteria. Chemotactic activity decreased during culture, whereas the chemokinetic response of the monocytes persisted.

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INTRODUCTION

Mononuclear phagocytes originate in the bone marrow and are transported via the blood stream to the tissues, where they exert their functions (van Furth, Cohn, Hirsch, Humphrey, Spector & Langevoort, 1972). The origin and kinetics of the mononuclear phagocytes have been studied most extensively in the mouse, both under steady-state and inflammatory conditions (van Furth & Cohn, 1968; Crofton, Diesselhoff-den Dulk & van Furth, 1978; Diesselhoff-den Dulk, Crofton & van Furth, 1979; Blussé van Oud Alblas & van Furth, 1979; Blussé van Oud Alblas, van der Linden-Schrever & van Furth, 1981). The most primitive mononuclear phagocyte in the bone marrow, the monoblast, which has been recognized in both mouse (Goud, Schotte & van Furth, 1975) and man (van der Meer, van de Gevel, Beelen, Fluitsma & van Furth, unpublished), divides to form promonocytes, which in turn divide to produce monocytes (van Furth & Diesselhoff-den Dulk, 1970). The latter are released into the circulation, which they leave after a short time (van Furth & Cohn, 1968; Crofton *et al.*, 1978; Diesselhoff-den Dulk *et al.*, 1979) to enter the tissues where, without further division, they differentiate into macrophages (van Furth & Cohn, 1968; Crofton *et al.*, 1978; Diesselhoff-den Dulk *et al.*, 1979; Blussé van Oud Alblas & van Furth, 1979; Blussé van Oud Alblas *et al.*, 1981).

The study of mononuclear phagocytes in man has

been hampered for a number of reasons. Human tissue macrophages cannot be obtained easily in large quantities, and kinetic studies with a radioactive marker are not feasible for ethical reasons. Human bone marrow mononuclear phagocytes are more difficult to maintain in long-term culture than are murine marrow mononuclear phagocytes (van der Meer *et al.*, unpublished). Therefore, most investigators use the mononuclear phagocyte most readily available in man, the blood monocyte. However, even the study of the monocyte has been hampered by poor survival of these cells *in vitro*. Quantifiable data on the survival of monocytes in culture are scarce (Johnson, Mei & Cohn, 1977), but it is assumed that a high proportion die during the first few days. There is also little information available about the functional state of monocytes cultured *in vitro* as compared with uncultured monocytes, because during culture the cells are usually adherent to a glass or plastic surface and cannot be recovered in suspension without damage, which would preclude further experimentation.

The method we developed to culture murine mononuclear phagocytes in Teflon bags, which permits recovery of the cells in an undamaged state, is described elsewhere (van der Meer, van de Gevel, Elzenga-Claasen & van Furth, 1979). In the present study we wanted to find out whether the Teflon system was suitable for the culture of human monocytes, and whether quantifiable data of survival could be obtained. We also investigated a number of cytochemical characteristics and the functional state of the cultured monocytes, both in relation to some aspects of the uncultured monocytes.

MATERIALS AND METHODS

Monocytes
Monocytes were isolated from heparinized peripheral blood (20 u./ml heparin) obtained from healthy volunteers. A monocyte-lymphocyte rich suspension was prepared by Ficoll-Isopaque density centrifugation as described elsewhere (van Furth, van Zwet & Raeburn, 1979). After cells were washed they were counted in a haemocytometer and incubated at a concentration of 1×10^6/ml in culture medium composed of medium 199 (Microbiological Associates Inc., Bethesda, Md) and 10% (v/v) inactivated newborn calf serum (Grand Island Biologicals Co., Grand Island, New York), 50 μg/ml streptomycin (Mycofarm, Delft, The Netherlands), and 2000 u./ml penicillin G (Mycofarm). Unless stated otherwise, the cul-

tures were not refed. The cell suspensions were incubated in Teflon culture bags (TCB) as described elsewhere (van der Meer *et al.*, 1979). All cultures were held at 37° in a water-saturated atmosphere of 10% CO_2 in air. The cells were harvested from the TCBs as already described (van der Meer *et al.*, 1979).

Morphology
During incubation the cultures were examined at intervals with an inverted phase contrast microscope (Carl Zeiss, Oberkochen, W. Germany). The morphology of the cells was also studied in Giemsa-stained cytocentrifuge preparations (Crofton *et al.*, 1978).

[^3H]-thymidine labelling
For the studies on DNA synthesis, medium containing 0·1 μCi/ml (specific activity 6·7 Ci/mmol, New England Nuclear Corp., Boston, Mass.) was added to the culture during the last $7\frac{1}{2}$ hr of incubation. Autoradiography was performed as described elsewhere (Crofton *et al.*, 1978).

Enzyme assays
Cytocentrifuge preparations were stained for α-naphthyl butyrate esterase according to Ornstein, Ansley & Saunders (1976) and stained for peroxidase according to Kaplow (1965); details for both staining procedures have been described elsewhere (van Furth *et al.*, 1979).

Lysozyme was measured in the cell-free supernatants by electrophoresis in antibody-containing agarose gel (Laurell, 1972). Anti-human lysozyme antibody was prepared by immunizing rabbits with human lysozyme. The human lysozyme was isolated from the urine of a patient with acute monocytic leukaemia as described by Johansson & Malmquist (1971).

Angiotensin-converting enzyme
Angiotensin-converting enzyme (ACE) was measured with a fluorimetric assay essentially as described by Friedland & Silverstein (1976), except that the phosphate buffer was replaced by a borate buffer (0·1 M Na-burate and 0·3 M NaCl pH 8·3). This change gave a slightly increased conversion of the synthetic substrate. Samples of supernatants (100 μl) were incubated for 60 min. As control, medium 199 with 10% inactivated newborn calf serum was tested for ACE activity. To make certain that ACE was responsible for the measured activity, samples were incubated in the presence of captopril (Squibb, Rijswijk, The Netherlands), an inhibitor of ACE.

Phagocytosis and intracellular killing of bacteria

The capacity to phagocytose opsonized *S. aureus* was assessed microscopically after addition of lysostaphin to lyse the extracellular bacteria (van Furth & Diesselhoff-den Dulk, 1981).

The rate of phagocytosis of *S. aureus* was studied as described in detail elsewhere (van Furth, van Zwet & Leijh, 1977). The rate of intracellular killing of these micro-organisms was determined independent of the rate of phagocytosis, as described elsewhere (van Furth *et al.*, 1977). For these experiments the cells were cultured in culture medium without antibiotics and washed twice with Hanks's balanced salt solution before use.

Locomotion

The assay to evaluate directional locomotion (chemotaxis) and non-directional locomotion (chemokinesis) was performed in a Millipore chamber according to Wilkinson (1974). The cells recovered from the TCBs were washed three times and suspended in Gey's solution (Difco, Detroit, Mich.). Approximately 0.8×10^6 monocytes were then brought onto the upper surface of a cellulose nitrate filter (pore size 8 μm; Sartorius, Göttingen, W. Germany). The attractant casein (Merck, Darmstadt, W. Germany) in a concentration of 1, 2, or 3 mg/ml was added either below the filter (to study chemotaxis) or on both sides of the filter (to study chemokinesis). The chambers were incubated at 37° for 2 hr and the filters were then detached, dehydrated, stained and mounted. The distance of migration was measured according to the leading-front method (Zigmond & Hirsch, 1973). For the calculation of the distances cells are expected to move on the basis of chemokinesis, calculation was performed as described by Zigmond & Hirsch (1973).

RESULTS

Efficiency of Ficoll-Hypaque isolation of monocytes from blood

Differential counts in Giemsa-stained smears of whole blood showed that on average monocytes accounted for 7·1% (SD $\pm$ 1·6%) of the cells in this series of experiments. Per millilitre of whole blood, there were 0·35 (SD $\pm$ 0·09) $\times 10^6$ monocytes. The interphase layer comprised 26·3% monocytes (see Table 1, day 0), representing a yield of 77%.

Table 1. Results of differential counts during cultivation*

Day of culture	Monocytes (%)	Lymphocytes (%)	Granulocytes (%)
0	26·3 $\pm$ 5·8	69·6 $\pm$ 5·7	4·3 $\pm$ 2·2
1	12·2 $\pm$ 5·5	86·3 $\pm$ 4·9	1·5 $\pm$ 0·9
2	10·6 $\pm$ 4·1	88·4 $\pm$ 3·6	0·9 $\pm$ 0·4
7	15·4 $\pm$ 7·1	83·6 $\pm$ 7·7	0·9 $\pm$ 1·0
14	39·2 $\pm$ 13·8	60·8 $\pm$ 13·8	0
21	57·3 $\pm$ 13·9	42·8 $\pm$ 13·9	0
28	84·6 $\pm$ 9·3	15·7 $\pm$ 9·3	0
35	100	0	0
49	100	0	0
56	100	0	0

* Determined in Giemsa-stained preparations; mean values $\pm$ 1 SD.

Cells present in the cultures

Since human monocytes hardly adhere to hydrophobic Teflon film, they cannot be separated from lymphocytes by adherence to the culture substrate, as is the case for cultures on glass or plastic. Therefore, lymphocytes remain present in the culture for up to 28 days, but their number decreases during incubation. Granulocytes, which are present in low numbers in the interphase layer after Ficoll-Isopaque centrifugation, remain present up to about day 7 (Table 1).

The recovery of monocytes from the TCBs after various periods of incubation is shown in Fig. 1. The number of cells is expressed as a percentage of the number of monocytes incubated at day 0. During the first 2 days of incubation there is a considerable decrease in the number of monocytes. The consistent small increase in the absolute number of monocytes recovered from the TCBs on day 21, seems to be due to some adherence of the monocytes to the Teflon surface during the preceding weeks and failure to recover them from the TCB, as confirmed by observations with the inverted microscope and was not due to proliferation (see below).

Morphology

Despite their poor adherence to Teflon, the monocytes in culture show little spreading. After an initial decrease, the monocyte diameter increased approximately two-fold during 4 weeks of culture (Table 2). It is occasionally difficult in Giemsa-stained preparations of the cultured cells to judge whether a certain

cell is a monocyte or a lymphocyte. In general, however, we found concordance with esterase activity and phagocytosis (see below). Less than 1% of the cells became giant cells even with prolonged culture.

[³H]-thymidine labelling

No monocytes in mitosis were encountered in these cultures. The labelling index of monocytes was less than 1% at all time points. Occasionally, labelled lymphocytes were seen.

Esterase and peroxidase

In Fig. 1 the esterase-positive and peroxidase-positive monocytes during the culture period are indicated. Judged by Giemsa stain, the number of monocytes in the Ficoll-Isopaque interphase is a slight underestimation compared with the esterase staining. During culture, the number of esterase-positive cells agrees very well with the number of cells considered to be monocytes in Giemsa-stained preparations. The intensity of the esterase activity increased with the time in culture. Not all monocytes in the Ficoll-Isopaque

preparation had peroxidase-positive granules (Fig. 1). The number of monocytes with peroxidase-positive granules decreased strongly during the first week of culture.

Lysozyme

With the method used, no lysozyme was detectable during the early days of culture (Fig. 2). From day 14 onward, lysozyme activity in the supernatants increased strongly.

Angiotensin-converting enzyme

Over 21 days of culture, ACE activity in the culture supernatants increased more than six-fold (Fig. 2). The ACE inhibitor, captopril, inhibited the activity completely.

Phagocytosis and intracellular killing

At the light microscopical level, more than 90% of the monocytes ingested *S. aureus* throughout the culture period. The rate of phagocytosis (Fig. 3a) and of
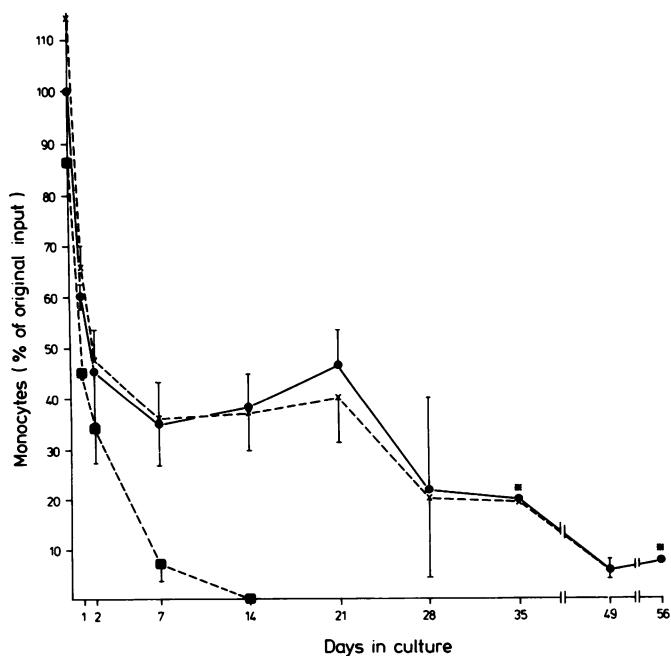

Figure 1. Monocytes recovered from the Teflon culture bag (●————●) expressed as the mean percentage of the original input. (×————×) Esterase-positive monocytes; (■————■) peroxidase-positive monocytes. Bars indicate standard deviation of experiments, asterisks that only results of two experiments were available.

Table 2. Monocyte diameter during culture*

Day of culture	Mean diameter (μm)$\pm$SD
0	11·6±1·1
1	10·7±1·6
2	12·1±1·4
7	12·7±1·8
14	16·7±3·1
21	17·1±4·7
28	22·3±5·9

* Measured with a calibrated ocular at a magnification of × 800.

intracellular killing (Fig. 3b) of *S. aureus* by monocytes cultured for 7 days was of the same order as those of uncultured cells.

Locomotion

Chemotaxis and chemokinesis were studied in cell suspensions obtained after Ficoll-Isopaque centrifugation and suspensions recovered from TCBs on days
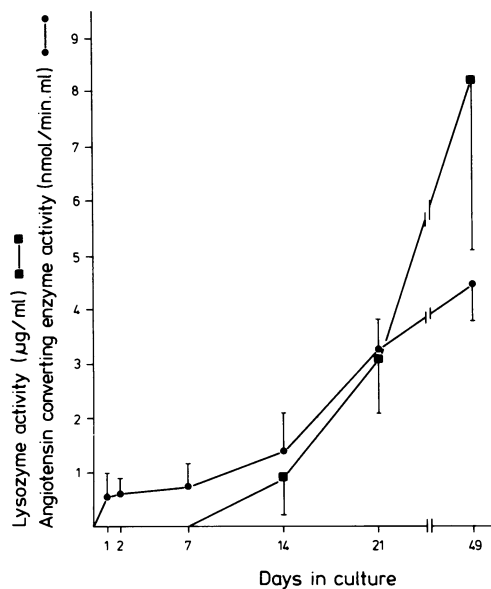

Figure 2. Course of activity of lysozyme (■——■) and angiotensin-converting enzyme (●——●), for 10^6 monocytes. Values are mean and standard deviation of three experiments.
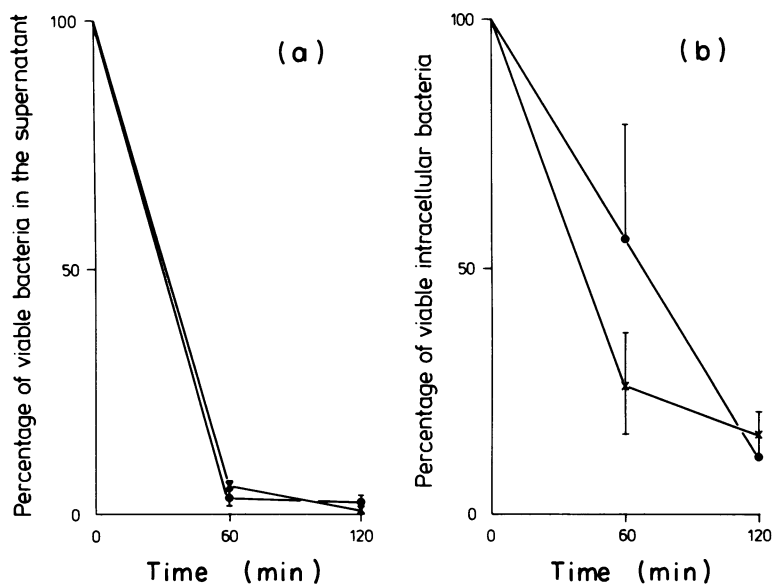

Figure 3. Phagocytosis (a) and intracellular killing (b) of *S. aureus* by monocytes cultured for 7 days (●——●; mean and standard deviation of four experiments), compared with uncultured monocytes (×——× ; mean and standard deviation of ten experiments).

Table 3. Migration of cultured and uncultured monocytes

Concentration of casein in cell suspensions (μg/ml)	Distance of monocyte migration (μm in 2 hr)				
	Concentration of casein below the filter (μg/ml)				
	0	0·2	1	2	3
a. Migration of uncultured monocytes					
0	38	57 (43)	78 (57)	100 (64)	106 (68)
0·2		63	76 (66)	89 (69)	103 (72)
1	55 (70)	62 (71)	74	85 (76)	91 (79)
2	59 (77)	65 (78)	72 (80)	82	89 (84)
3	63 (82)	70 (82)	69 (84)	74	89
b. Migration of monocytes cultured for 4 days					
0	51	75 (61)	82 (84)	90 (91)	99 (94)
0·2		89	89 (92)	98 (96)	114 (98)
1	86 (94)	96 (95)	98	102 (100)	113 (103)
2	93 (98)	101 (99)	97 (100)	102	117
3	97 (104)	103 (104)	99 (107)	111 (112)	120
c. Migration of monocytes cultured for 12 days					
0	44		79	92	
1	74		89	91	
2	86		88	97	
d. Migration of monocytes cultured for 12 days (fresh medium on day 11)					
0	31		49	72	
1	56		70	74	
2	60		60	96	

Between parentheses the calculated distances assuming chemokinesis alone (no chemotaxis) are given. The calculations were performed according to Zigmond & Hirsch (1973).

4 and 12 of culture (Table 3). With non-cultured monocytes maximum migration was towards the highest concentration of the attractant (Table 3a, first line). That this indicates chemotaxis is confirmed by the calculated distances for chemokinesis alone. After 4 days of culture migration increased with higher concentrations of casein, but in this case the maximum migration was observed at the highest concentration, i.e. when 3 mg/ml casein was present on both sides of the filter (Table 3b). This indicates only chemokinesis. The calculated distances for chemokinesis alone are in agreement with this. A similar pattern was observed on day 12, when more chemokinesis occurred than chemotaxis (Table 3c). It was also found that chemotactic activity was not restored by replacement of the culture medium by fresh medium 1 day before the assay (Table 3d).

DISCUSSION

The present paper describes a simple method for long-term culture of monocytes that offers the possibility of recovery of the monocytes in suspension. One advantage of this approach is that the number of cells in culture can be estimated easily. Since most papers concerning the *in vitro* culture of monocytes do not give either quantifiable data on monocyte survival or such data obtained by indirect means, e.g. protein and DNA measurements (Rocklin, Winston & David, 1974; Zuckerman, Ackerman & Douglas, 1979), data on survival of human monocytes in culture are rare (van der Meer *et al.*, 1979). Johnson *et al.* (1977) studied monocyte survival in cultures on plastic and used various media and sera. Their optimal results were obtained by using Newman Tytell medium with

10% fresh autologous serum, but even then survival was less than 40% after 7 days of culture. Similar survival data were obtained in the present study with medium 199 containing 20% newborn calf serum in the Teflon system. The use of animal sera in culture instead of autologous sera is advantageous with respect to standardization and costs (Zuckerman *et al.*, 1979).

That the monocytes can be recovered in suspension after culture is also advantageous for experiments of monocyte function. In our hands, the kinetic assay that measures phagocytosis independent of intracellular killing (van Furth *et al.*, 1977) gives better reproducibility of the results when cells in suspension are used. Phagocytosis and intracellular killing of staphylococci proved to be well preserved during culture. Others have studied phagocytosis and killing of microorganisms such as *Toxoplasma gondii* (Anderson & Remington, 1974; Borges & Johnson, 1975), *Listeria monocytogenes* (Rocklin *et al.*, 1974; Cline, 1970), *Mycobacteria* spp. (Samuel, Godal, Myrvang & Song, 1973), and cryptococci (Diamond & Bennet, 1973) by cultured human monocytes adherent to glass or plastic, in the presence or absence of lymphokines. Although some authors investigated the effect of duration of culture on ingestion and intracellular killing, they did not compare their findings with the behaviour of uncultured cells.

The studies on locomotion could not have been performed without having the monocytes in suspension. It is remarkable that the chemotactic activity of the monocytes became impaired during culture, whereas chemokinesis did not. We have no explanation for this phenomenon, but it seems to suggest loss of receptor or could for instance be due to inhibition of transmethylation reactions (Snyderman, Pike & Fudman, 1981).

A number of our observations concerning the characteristics of monocytes are in agreement with the findings of others, i.e. the uptake of [^{3}H]-thymidine (van Furth *et al.*, 1979; Zuckerman *et al.*, 1979), the absence of proliferation (van der Meer *et al.*, 1979; Zuckerman *et al.*, 1979), increment of cell diameter during culture (van der Meer *et al.*, 1979; van Furth *et al.*, 1979; Zuckerman *et al.*, 1979), loss of peroxidase activity (Johnson *et al.*, 1977; van Furth *et al.*, 1979; Gordon, Todd & Cohn, 1974), increased lysozyme activity in the supernatant despite the decrease in monocyte number (Johnson *et al.*, 1977; Zuckerman *et al.*, 1979; Gordon *et al.*, 1974), and preservation of phagocytic function (as already discussed). In our

culture system, the increase in monocyte diameter cannot be explained by attachment to the culture substrate. Probably, the formation of secondary lysosomes due to continuous pinocytosis of culture medium contributed to the increased size. Unlike other reports (Johnson *et al.*, 1977; Zuckerman *et al.*, 1979; Goldstein, 1954; Berman & Stolberg, 1962), in which up to 70% of the nuclei occurred in giant cells, we did not find more than 1% giant cells with prolonged culture. It has been shown that certain culture substrates (e.g. cellophane, Melinex®) induce the formation of giant cells (Goldstein, 1954; van der Rhee, Hillebrands & Daems, 1978), but this does not seem to hold for the Teflon membrane.

Recently, there has been considerable interest in the production of angiotensin-converting enzyme (kininase II) by mononuclear phagocytes. Friedland, Setton & Silverstein (1978) found a strong increase of this enzyme in both monocytes and culture supernatants of cultures maintained for 6 or 7 days. Although we have studied only culture supernatants, we measured on day 7 enzyme activity of the same order as these authors (Friedland *et al.*, 1978) and found a further increase in ACE after prolonged culture. Production of ACE by the lymphocytes present in the culture has been ruled out by Friedland *et al.* (1978). Since we saw ACE induction in cultures on Teflon membrane it seems unlikely that induction of ACE is due to strong adherence to the culture substrate, as these authors postulated.

The presence of lymphocytes in our culture system may be a disadvantage for certain studies. However, the combined culture of monocytes and lymphocytes can be useful in certain experiments (de Vries, Haasnoot, van der Weij & Cats, 1982). Inclusion of a separation step, e.g. by elutriation centrifugation (Sanderson, Shepperdson, Vatter & Talmage, 1977) or by using a density gradient (Loos, Blok-Schut, van Doorn, Hokbergen, Brutel de la Rivière & Meerhof, 1976; de Vries, Caviles, Bont & Mendelsohn, 1979) would provide monocyte cultures of greater purity.

ACKNOWLEDGMENTS

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