

DIFFERENTIATION OF MONOCYTE DERIVED DENDRITIC CELLS IN SERUM FREE CONDITIONS

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ABSTRACT

Human peripheral blood monocytes (HPBM) were cultured in the absence of human serum and were converted into a state exhibiting a high accessory function expressed by their ability of supporting lymphocyte proliferation. After a prolonged culture in serum free media the monocyte derived cells were highly viable, increased in size and developed veils and dendritiform elongations. Paralleling the increase in accessory function, the cells decreased in the expression of markers typical of monocytes and macrophages (M \emptyset), approaching the phenotype of lymphoid dendritic cells. We here define conditions for reproducibly generating these monocyte derived dendritic cells (m-DC) in various serum free media. This study also shows that acidic conditions prevent M \emptyset development and facilitate m-DC differentiation even under serum conditions.

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INTRODUCTION

Dendritic cells (DC) were shown to act as potent accessory cells (AC) in various proliferative responses of T cells.¹⁻⁷ Steinman et al, 1980, had reported that DC serve as powerful stimulators of both the allogeneic and syngeneic mixed leukocyte reaction (MLR), whereas monocytes and M \emptyset are either weak or inactive.^{2,8}

These DC are different from M \emptyset as they are loosely adherent, nonproliferative, expose veils and dendritiform elongations, lack Fc-receptor, and are weakly or negatively stained with nonspecific esterase.⁹⁻¹¹ HLA-DR is highly expressed in human DC.^{12,13} Since DC are non-phagocytic, it seems likely that they are responsible for the proliferative response observed with T cell cultures depleted of phagocytic M \emptyset .⁵

A problem encountered in studying DC is whether DC represent novel cells with a unique biology or whether they represent subpopulations of M \emptyset in unusual stages of differentiation. The final answers to these questions will only be available when the ontogeny of DC has been clarified.

We have recently reported that human peripheral blood monocytes (HPBM) can be *in vitro* differenti-

ated into veiled dendritiform cells of an increased accessory potency showing reduced M \emptyset markers.¹⁴⁻¹⁶ We have initially detected this phenomenon in cultures supplemented with some selected charges of fetal calf serum. In order to completely define the conditions which facilitate this differentiation, we have studied serum free culture conditions. We here demonstrate that some serum free media are able to allow monocytes to differentiate into monocyte derived dendritic cells (m-DC). After 1-4 days of incubation, the cells will acquire most of DC criteria; functionally, morphologically, and phenotypically. It also appears that the cells can be maintained in culture with a stable phenotype for several weeks without proliferation. In parallel we also define serum and pH conditions for optimal m-DC and M \emptyset development.

MATERIALS AND METHODS

Culture media

The working medium consisted of a combination of 80% RPMI 1640 and 20% medium 199 Earle's salts (designated as medium 80/20),¹⁷ supplemented with

penicillin/streptomycin (200 U/ml) plus serum (10% bovine serum or 20% human AB serum).

Serum supplements added to the medium 80/20 such as 20% human serum (inactivated) or a serum substitute BMS (basal medium supplement, Biochrom) at 10% were used.

Serum free media were FEB (Biochrom), Iscove's medium (Behring), medium 80/20 supplemented with 0.8 g/l sodium bicarbonate up to a concentration of 2.8 g/l as needed, 50% mixture of HB 104 (Hana-NEN) and SFRI-4 (SFRI Lab. Martignas, France), MCDB-302 (Sigma), medium 199-1 (Sigma), Iscove's medium (Boehringer), RPMI 1640 (Biochrom), NCTC-135 (Sigma), 199 Earle's salts (Biochrom) and medium 105 (Sigma).

Cell viability

Cell viability was assessed by trypan blue dye exclusion as previously described.¹⁸ Absolute number of cells was determined by direct microscopic counting.

Endotoxin

All reagents used in purification and culture of monocytes were tested for the presence of endotoxin by a kinetic limulus test,¹⁹ kindly performed by K P Becker and B. Urbaschek, Mannheim. Buffers, serum free media and the Ficoll-Hypaque solution contained less than 3.7 pg/ml. The combination of medium 80/20 plus BMS contained 4.2 ng/ml and medium 80/20 plus HS contained 5.4 ng/ml of endotoxin, while medium 80/20 alone contained 1.2 ng. The attachment medium supplemented with 10% bovine serum contained 1.28 ng/ml.

General purity

"High yield hybridoma conditions" have been established for our cell cultures.¹⁷ Disinfectants have to be kept away from the incubator and the respective utensils. Under serum free conditions a higher purity is needed. We observed that in MCDB-302 penicillin/streptomycin had a toxic effect on the cells.²⁰

pH adjustment

Cells were incubated in a 5% CO₂ atmosphere as indicated. Reagents used to change the pH of the medium were sodium bicarbonate (7.5% solution; Flow), lactic acid (90% pure; Merck), and HEPES (1M; Behring).

Monocyte preparation

Monocytes were prepared from buffy coats of healthy blood donors, kindly provided by the blood bank, University Hospital of Göttingen. Buffy coats were prepared in Biopack bags (Biotest Pharma) using CPDA as an anticoagulant. They were diluted 1+1 in

PBS and separated Lymphoprep 1.077 g/ml (Nyegaard, Oslo) centrifugation. The interphase containing the peripheral blood mononuclear cells (PBMC) was removed and washed 5 to 7 times. The yield ranged between 3×10^8 and 7×10^8 PBMC per buffy coat. Cells were resuspended in medium 80/20 supplemented with 10% bovine serum and allowed to attach on tissue culture plates for 1 hr at 37°C, washed free of non-attached cells and incubated in appropriate media.

Monocyte depletion

PBMC were incubated in petri dishes for 1 hr at 37°C and nonadherent cells were collected, centrifuged and adjusted to 5×10^8 cells/ml of PBS. The lymphocyte fraction was treated with leucin methyl-ester (Leu-O-Met) in order to further deplete the cells of lysosome rich cells, namely Ac, monocytes, macrophages and granulocytes.²¹ Leu-O-Met at 25 mmol was incubated for 3 min at room temperature with the resuspended PBMC. When incubation was completed the cells were washed twice with PBS and either resuspended in fresh culture medium or cryopreserved until use.

Lymphocyte cryopreservation

The cryoprotective solution consisted of 90% bovine serum + 10% DMSO. 4×10^7 lymphocytes were suspended in 250 ul solution and pipetted into a capillary tube (Minitüb, Tiefenbach, West Germany). They were frozen down to -80°C in a styropor box yielding a temperature decrease of approximately 1°C/min and stored at -80°C until use.

When required they were quickly thawed up and transferred to a 10 ml centrifuge tube by a Pasteur pipette. 5ml of medium 80/20 + 10% bovine serum were added slowly over a period of 1 minute. The cells were centrifuged at room temperature for 5 minutes at 400g and washed twice with PBS. They were resuspended in the working medium at a concentration of 3×10^6 lymphocytes per ml.

Fc-receptor

Washed bovine erythrocytes were opsonized by first a pretreatment with trypsin/EDTA (0.005/ 0.002 v/v final concentration in the erythrocyte suspension) for 30 minutes at 37°C and coated with a subagglutinating dose of rabbit anti bovine red blood cell IgG fraction (Paesel, Frankfurt) for 1 hr at 37°C by gentle shaking. 2×10^6 opsonized erythrocytes were incubated for 1 hr at 4°C with 2×10^4 cells. At the end of incubation the unbinding erythrocytes were gently washed away with a 100 18-channel micropipette (Costar). For fixation, 0.05% glutaraldehyde was added immediately before centrifuging them for 2 min at 200 xg.

Glutaraldehyde was removed and 100 ul PBS was

added per well. Rosettes of > 4 erythrocytes were considered as Fc-receptor positive cells.

Fc-receptor-dependent phagocytosis

The cells were treated as above but incubated for 30 minutes at 37°C. Thereafter the suspended erythrocytes were removed. The remaining cells fixed with 0.05% glutaraldehyde while centrifuging for 2 min at 200 xg.

Fc-receptor-independent phagocytosis

Indian ink (Pelikan), was incubated at 1:500 final dilution with the cells for 30 minutes at 37°C, the unphagocitized particles were removed with a 100 μ l Oktapette and cells were fixed as above.

Nonspecific esterase

Cells in microtiter plates were fixed with 1% paraformaldehyde in PBS for 2 minutes and centrifuged at 200xg. The fixative was removed and cells were incubated for 8 minutes at 37°C with the substrate α -naphthyl butyrate (Technicon) and washed with PBS. Cells were classified as strongly positive, weakly positive, and negative.

Immunoperoxidase staining

Binding of monoclonal antibodies, one directed against non-polymorphic Ia-like molecules from the hybridoma line L227 (ATCC-HB96), and 63D3 directed against a 200,000 dalton molecule on human monocytes (ATCC-HB44), was tested by immunoperoxidase staining technique.

Cells were fixed either by air drying or with 0.05% glutaraldehyde and then incubated with the respective antibody. Biotinylated sheep anti-mouse IgG₁ (Amersham R.P.N.1001; 1:200) was used as second antibody, followed by a streptavidin-peroxidase conjugate (Amersham; R.P.N.1231; 1:200). Aminoethylcarbazole was used as substrate.

Cell cooperation

The accessory function of a respective cell population was measured by a cell cooperation assay which quantitates the accessory cell dependent mitogenic lymphocyte stimulation. Cells used as accessory cells were washed five times in microtiter wells and pulsed with the mitogen NaIO₄ (0.3mM) for 30 minutes on ice. On each washing step, the plates were centrifuged at 200g for 2 minutes and sucked off down to a 50 μ l amount left back in the well. This will prevent any loss of non-adherent cells. The cells were washed and co-cultured for 48 hr with 2×10^5 unstimulated allogeneic lymphocytes/well, recovered from the cryopreserved pool of depleted cells as described above. The proliferating cells were labelled during the

TABLE I. Buffers and unusual components contained in three representative media of this study.

ADDITIVES	MCDB-302	199-1	80/20
Linoleic acid	+	-	-
CuSO ₄	+	-	-
D-a-lipoic Acid	+	-	-
Putrescine	+	-	-
NH ₄ VO ₃	+	-	-
Buffer	NaHCO ₃	HEPES	NaHCO ₃
Vit A-acetate	-	+	+
Calciferol	-	+	+

last 24 hr of culture by 0.2 μ ci ³H-thymidine per well. They were harvested by an automated cell harvester (Cambridge Technology) and counted by scintillation counting.

RESULTS

Cell culture

We have investigated several serum free media to establish defined conditions for monocytes to differentiate into AC rather than into M ϕ . Among all tested, Iscove's medium; a 50% mixture of HB 104 and SFRI-4; medium 80/20; FEB; 199-2 and MCDB-302 were optimal for inducing the cells into the accessory state and for staying active for long periods of time. This functional activity was generally measured by the ability of these cells in supporting human lymphocyte

TABLE II. Phenotype and function of monocytes and monocyte derived cells. Phenotypical cell markers and values of accessory activity after four and eight days of culture in MCDB-302 or medium 80/20 containing 20% human serum. Nonspecific esterase staining intensity is defined as weakly (+) and strongly positive (+ +). Markers specific of monocytes and M ϕ reduce maximally at day four in both cultures. Markers typical of DC stay low in cells cultured in MCDB-302, but increase in medium supplemented with 20% human serum. All the cell populations stain positive with the monoclonal antibody 63D3. ³H-thymidine refers to the accessory potency of the cells as tested in lymphocyte mitogenesis (see "materials and methods"). For further details see text.

PARAMETER	MCDB-302		HUMAN AB-SERUM	
	day 0	day 4	day 4	day 8
Nonspecific esterase	75	20	20	30
Fc-reception test	90	23	20	27
Fc-receptor dependent phagocytosis	85	30	15	10
Fc-receptor independent phagocytosis	87	30	10	30
63D3	100	100	100	100
HLA-DR	45	100	100	85
³ H-thymidine (dpm X 10 ⁻³)	3,2	17,3	14	18,4

proliferation (Fig. 1). Other serum free media, such as NCTC-15 (Sigma), medium 199-1 (Sigma), RPMI-1640 alone (Biochrom), and medium 105 (Sigma) did not support the viability and activity of the monocytes.

Most of these media except MCDB-302,²⁰ contained proteins, lipids, and/or some type of serum replace factors which may cause difficulties in later studying signals involved in AC and M ϕ development.

Gel electrophoresis confirmed that MCDB-302 contains no protein. Also nucleosides are not in the constituent of this medium. Unusual constituents of this medium are listed in Table I.

Addition of penicillin/streptomycin to medium 302 will reduce the life span of cultured cells.

Because progressive cell death could lead to selection of specific subpopulations we have carefully controlled cell number and cell viability at various cell culture conditions. It appeared that cell loss in human serum and MCDB-302 ranged at about 3% and never exceeded 12% after four days of culture. At day eight the cell death never exceeded 30% of the starting population.

AC function of m-DC and M ϕ in lymphocyte proliferation induced by NaIO₄

The AC function of m-DC was tested in NaIO₄-induced proliferation of human lymphocytes. m-DC turned out to have effective AC functions. Comparison of the accessory functions of m-DC and M ϕ grown in 20% human serum indicated that the proliferative responses in the presence of m-DC were much stronger than those obtained with M ϕ (Fig. 1). Preincubation of monocytes in medium MCDB-302 activated their accessory potency as early as one day after onset of the culture, reached a maximum at day four and stayed high for long periods of time (Fig. 1). The cells were maintained with high viability in MCDB-302 for several weeks.

Accessory potency of monocytes incubated in 20% human serum also increased at day one and was maximum at day four. It declined later, as cells developed into mature M ϕ .

Time kinetics of the M ϕ development (Table II) led us to conclude that the accessory state is a developmental step of monocytes preceding M ϕ development. Again it could be confirmed that m-DC are more effective AC than differentiated M ϕ in mitogenic activation of human lymphocytes.¹⁴

Surface markers

As demonstrated in Table II monocyte/M ϕ -markers, such as nonspecific esterase, Fc-receptor expression, Fc-dependent and independent phagocytosis were down-regulated in MCDB-302 between day two and four of culture to low levels. In serum free media they stayed so for a long period of time,

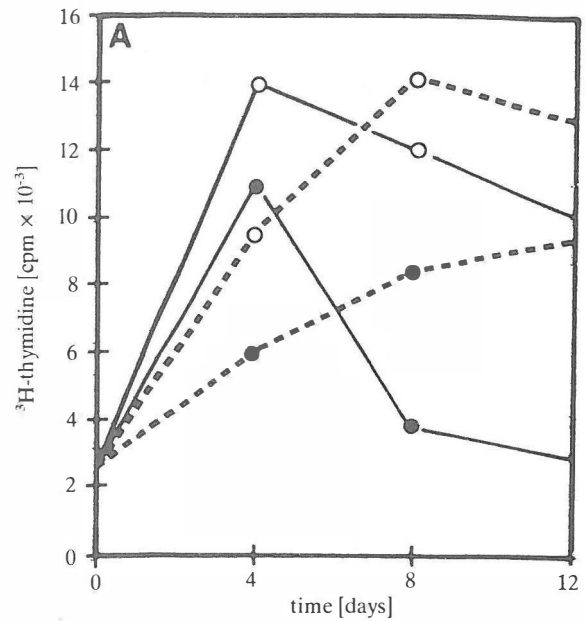


Fig. 1 A. medium 80/20 (●—●—●), MCDB-302 (○---○---○) and 104-SFRI (○...○...○).

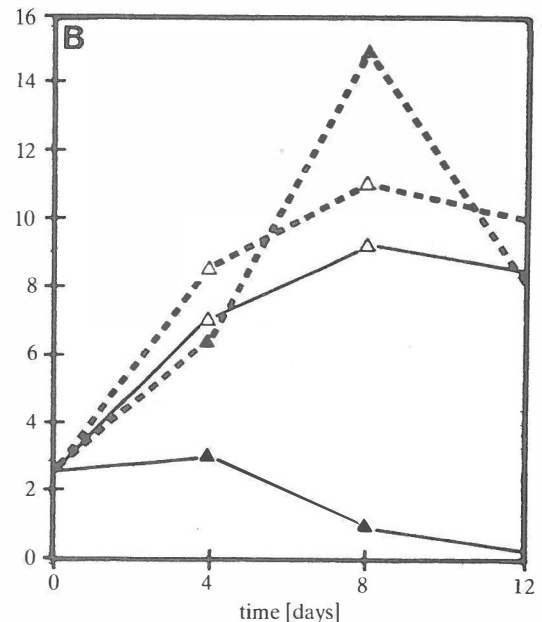


Fig. 1 B. 199-1 (▲—▲—▲), FEB (▲...▲...▲), Boehringer Iscove's (△—△—△) and medium 199-2 (△...△...△).

20% human serum in medium 80/20 (●—●—●), was used to induce M ϕ development according to standard conditions as a control (Fig. 1. A). All monocyte derived cells appeared viable at the end of culture except those cultured in medium 199-1.

Fig. 1. Comparison of different media with respect to their ability to induce differentiation of accessory cells (AC) or M ϕ from monocytes. They were cultured up to 15 days (abscissa) and tested for their accessory function (ability to promote lymphocyte mitogen stimulation) at time points indicated. The resulting lymphocyte stimulation was measured by tritium thymidine incorporation (ordinate) and taken as a measure of the cooperative potency of the AC. Two groups of serum free media were used.

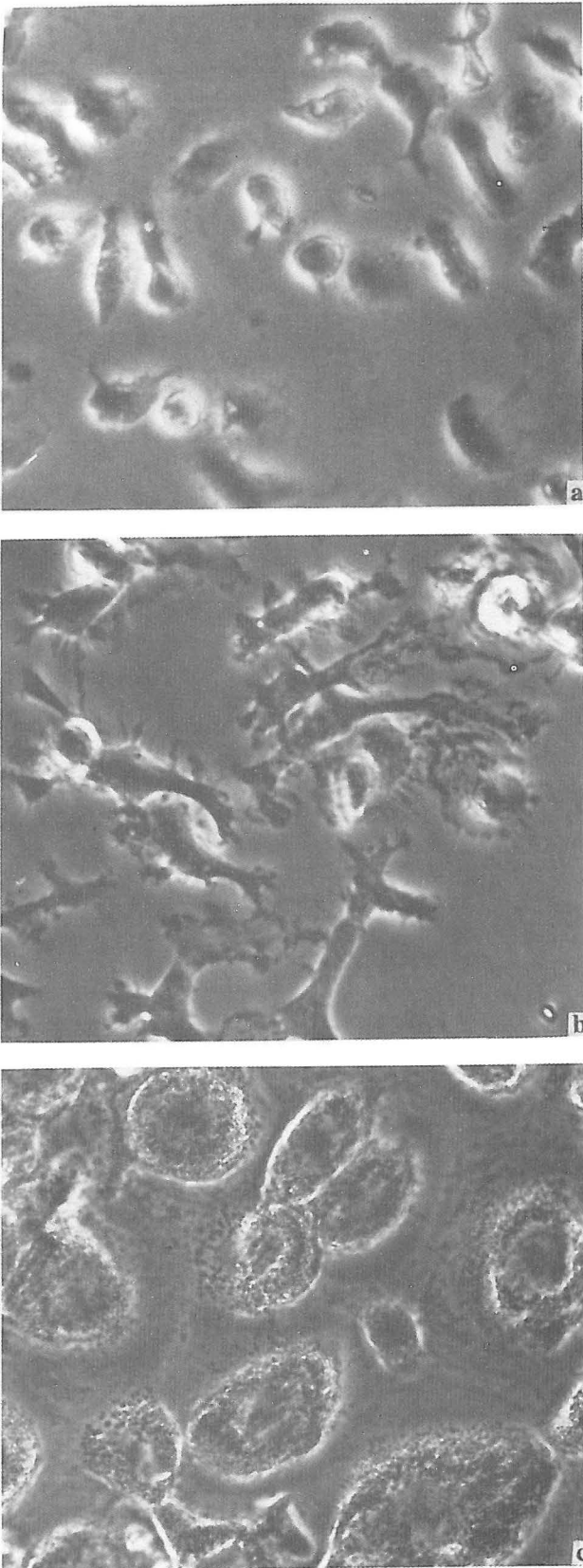


Fig. 2. Phase contrast appearance of fresh monocytes (a) and monocyte derived dendritic cells after 8 days of incubation in MCDB-302 (b) or in 20% human serum (c). The appearance of veils and dendritiform elongations are significant in (b).

approaching the phenotype of DC, described by Steinman.²²

When monocytes were cultured in medium plus human serum, all the above markers were equally poorly expressed at day four of culture, again correlating with the high cooperativity of the cells with lymphocytes. Monocytes recovered those M ϕ -markers after a week of culture, and became typical M ϕ , characterized by high percentages of esterase positive cells, Fc-receptor, Fc-receptor dependent and independent phagocytic cells.

Cells cultured in MCDB-302 stained all positive for HLA-DR. Monoclonal antibody to HLA-DR also reacted with monocytes and M ϕ , but never reached the percentage of positive cells seen in MCDB-302. All cell populations stained with 63D3, a monoclonal antibody known to stain monocytes and M ϕ .¹ Thus, 63D3 binding is the principal difference between monocyte-derived AC and conventionally prepared DC.

Morphological appearance

When monocytes were cultured in MCDB-302, they developed veils and dendritiform elongations after 1.5-3 days (Fig.2). The appearance of processes that extend in several directions and irregularly shaped nuclei was most pronounced after one week of culture than during the first days. The cells were mostly detached and had irregular shape. By phase contrast, the cells did not have significant amounts of granula.

Monocytes cultured in 20% human serum also developed veils after one day of culture. This correlates with a state of high accessory function (see Fig. 1). After seven days of culture they were more adherent, flattened and showed typically oval to reniform nuclei and more prominent organelles, especially endocytic vacuoles and lysosomes, thus resembling M ϕ . No dendritiform elongations were observed at this stage. This correlated with low accessory function.

We conclude that a criterion that distinguishes m-DC from other blood-borne cells is the possession of spiny dendrites, lamellipodiae and/or veils. Appearance of veils in one day culture correlates with the increased accessory potency of cells in our system. As veils became more prevalent at about day 1.5-3, the accessory function of cells increased. When cells were incubated longer in medium MCDB-302, the long dendrites became more prominent than spiny veils. It therefore appeared that veils were best morphological correlates of accessory function; and when veils were abundant in culture, there was always a higher support for lymphocyte proliferation by m-DC.

pH conditions

Monocytes were cultured in 3 different media at varying pH values as shown in Fig. 3, 4 and 5. The alkaline condition was obtained by addition of sodium

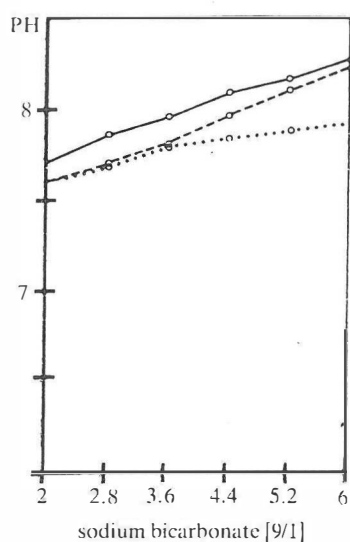


Fig. 3

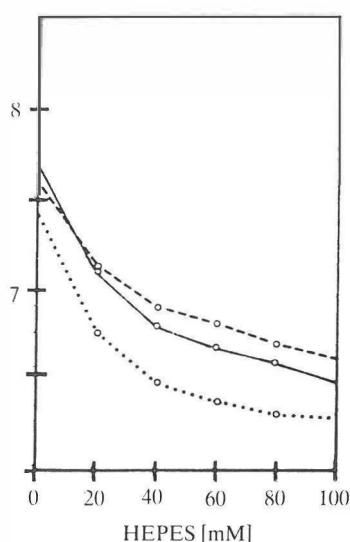


Fig. 4

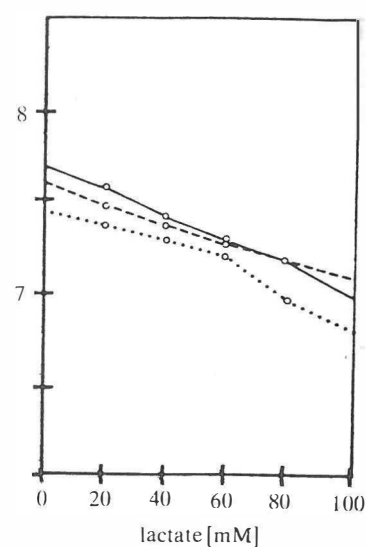


Fig. 5

Fig. 3, 4, and 5. Effect of sodium bicarbonate, HEPES and lactate on the final pH of various media in a 5% CO₂ atmosphere. 20% human

serum in medium 80/20 (—), 10% BMS in medium 80/20 (---) and MCDB-302 (....).

bicarbonate and acidity by addition of HEPES or lactic acid. Development of the cells under these conditions was followed morphologically and functionally.

Monocytes cultured in 20% human serum at alkaline pH became adherent, granulated and flattened earlier than in an acidic milieu. Functionally, they reached a peak of accessory function after 36 hours and then their accessory function decreased progressively (Fig. 6). This combination therefore appeared optimal for M ϕ development.

In contrast, monocytes cultured in 20% human and acid milieu also reached this peak after 36 hours but maintained their high accessory function for at least 10 days as shown in Fig. 7. This was paralleled by a specific morphology.

Acid milieu due to HEPES could be distinguished from lactic acid from the morphological point of view. In HEPES macrophages got heavily veiled, granulated; and detached, while in lactic acid they neither became as veiled, granulated, and detached, nor did they get the typical flattened M ϕ morphology. Functionally this correlated with a higher accessory function in acid milieu due to HEPES (Fig. 7). The only controversial issue here was the presence of granula in acidic conditions which was induced not only by HEPES but also by lactic acid and CO₂. This phenomenon was not observed in DC described by Steinman. Thus, acidic pH was able to prevent the completion of M ϕ development.

The adverse effect of acidity to stimulate AC development and human serum to stimulate M ϕ development were compared as to their dominance. Monocytes were incubated in 20% human serum plus various amounts of lactic acid or HEPES to cover a range of 7.7

to 6.5. It appeared that acid condition of lower than pH 6.8 was able to counteract the M ϕ differentiation effect caused by human serum without impairing cell viability. However, in serum free media acid milieu did not further enhance accessory activity. Optimal viability was obtained in alkaline milieu (pH = 7.7-9.9), without lowering the accessory function.

We concluded that in serum containing media, alkaline condition was most suitable for M ϕ development and acidity was able to counteract M ϕ development, thus arresting the cells at the preceding developmental state, the m-DC.

DISCUSSION

We have found that monocytes develop into a state of high accessory activity on their way to become M ϕ when cultured in human serum. We have shown that they can be arrested in this intermediary state. From there, they further develop to become veiled dendritiform cells closely resembling lymphoid dendritic cells²³ by function, morphology, and most of the markers.

Morphologically, the cells develop veils after 2-3 days, become loosely adherent and lack granula. Phenotypically, they rapidly lose Fc-receptors, nonspecific esterase, and the ability of phagocytosis. Functionally, they acquire an increased capacity of initiating T-lymphocyte stimulation.

From these data it appears that monocytes can acquire the phenotype of an accessory cell distinct of the macrophage phenotype, depending on culture conditions.¹⁴

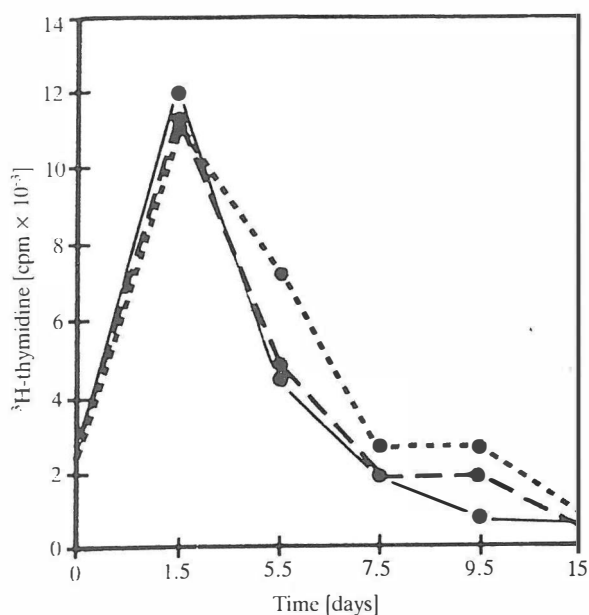


Fig. 6. Effect of sodium bicarbonate on Mø development. Monocytes cultured in medium containing human serum were subjected to three different concentrations of bicarbonate. 2g/l (●—●—●), 3.6 g/l (●- -●- -●) and 5.2 g/l (●...●...●).

Standardized medium conditions are a prerequisite for reproducibly inducing the two states of differentiation but also for studying the signals which may be active for inducing differentiation. We succeeded to replace serum containing media by more defined serum free media.

Even in the most defined medium (MCDB-302) cells were not significantly reduced in viability and recovery (see results). Thus, the vast majority of cultured monocytes underwent the phenotypic changes described here.

With respect to the possible signals involved in induction of m-DC development, the successful use of completely defined media demonstrated that neither serum nor various additives in supplemented media could contain the hypothetical inducers, as they were absent in media 80/20 and MCDB-302.

Analysing these media by a kinetic limulus test and gel electrophoresis confirmed them to be free of endotoxins and proteins.

Besides the standard components of media, these media either contain additives such as linoleic acid, CuSO_4 , D-lipoic acid, putrescine, NH_4VO_3 (MCDB-302), or vitamin A-acetate, and calciferol (medium 80/20). Future research will reveal whether these agents contribute to signals to the m-DC development. Alternatively, the step of monocytes to m-DC development might be an autonomous process preprogrammed in monocytes. This contention is supported by a novel monoclonal antibody raised in our laboratory²⁴ that stains m-DC, but not Mø, and also stains a

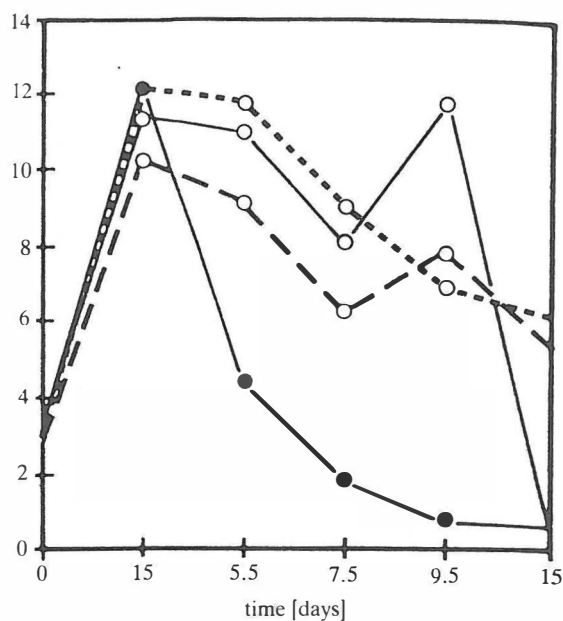


Fig. 7. Effect of lactic acid and HEPES on Mø development. Monocytes were subjected to three different concentrations of lactic acid and HEPES. 20% human serum in medium 80/20 without additives containing already 2 g/l sodium bicarbonate (●—●—●), 120 mM lactic acid (○- -○- -○), 40mM HEPES (○—○—○) and 80 mM HEPES (○...○...○).

They were tested at previous time points (abscissa) for their accessory function on lymphocyte stimulation, as described in Fig. 1.

subfraction of HPBM, which then may represent the oldest monocyte compartment. Thus, a developmental trigger may already have been acquired *in vivo* or may be preprogrammed endogenously.

On the other hand, nucleotides are playing a role in differentiation of Mø to DC, which will be the subject of a forthcoming paper. However, this cannot be exclusive because also media lacking nucleotides were able to support m-DC development.

It remains to be investigated to which extent LPS constitutively present at low levels in human blood, and the media used for preparation of the cells contributes to the effect of DC and Mø differentiation. Even though, in MCDB-302 which contains no measurable amounts of LPS, the high accessory potency remains for a long period of time without medium change. On the other hand, in human serum, containing higher amount of LPS, cells progress into Mø and decrease in accessory potency. Working absolutely free of contaminating endotoxins would nearly be impossible and could even be regarded as unphysiological.

We have not been able to develop serum free conditions for Mø development. The progression of m-DC to macrophages, therefore, seems to be actively induced by factors which are active in human serum and will possibly be defined in the future. The speed of Mø development depends on the strength of the signal, i.e. serum concentration. Lowering the serum concentra-

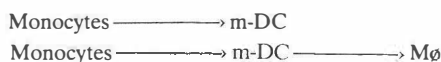


Fig. 8. Scheme of proposed ontogenesis of monocyte derived dendritic cells (m-DC) and macrophages (M ϕ). For details see text.

tion retards the M ϕ and keeps them for a long period of time at the preceding state, which further supports the contention that macrophages differentiating from monocytes, have to pass the intermediate state of increased accessory activity between 24 and 36 hours (Fig. 8).¹⁴

The development from monocytes to DC and M ϕ can be enhanced by physical factors such as pH. Especially, we demonstrate that acidic milieu can counteract serum driven M ϕ development, leaving the cells at the accessory state for several more days.²⁵

Morphologically, the monocytes cultured in this situation develop veils but become granulated like classical M ϕ .

Alkaline condition favors both differentiation steps. Morphologically, monocytes cultured in serum at alkaline milieu progress earlier into the state of M ϕ . In serum free media, cells maintain their high accessory activity and look healthier at alkaline pH. Therefore, a slightly alkaline condition of pH 7.8-8 is optimal for both types of cell culture.

The cell culture conditions defined here will facilitate further studies of signals and conditions necessary for AC differentiation. This is exemplified by our unpublished findings of high amounts of interleukin-1 and tumor necrosis factor produced by these cells.

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