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CYCLIC NUCLEOTIDES CONTROL DIFFERENTIATION OF HUMAN MONOCYTES INTO EITHER HIGHLY ACCESSORY CELLS OR MACROPHAGES

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ABSTRACT

Human peripheral blood monocytes have been found to undergo a transitory state of high accessory activity before they fully become macrophages. Time kinetics were done to follow this accessory potential. Studying the regulation of accessory activity, we have found that monocyte derived accessory cells (m-AC) pass through two phases of development, both of which are adversely controlled by cyclic nucleotides. Phase I is positively correlated by intracellular cAMP increase and can be blocked by increase of cGMP, whereas phase II positively correlates with increase of intracellular cGMP and can be completely blocked by cAMP and synergystic agents. In addition to cAMP, non-cyclic adenine nucleotides and adenosine also mimic all cAMP effects. This behavior is explained by the known presence of surface 5´- nucleotidase and adenosine receptors which in turn leads to activation of adenylate cyclase. At phase II, serum is required to convert m-AC into macrophages. In the absence of serum, cells were arrested in the m-AC state. Adenine nucleotides effectively counteract serum induction, leading to the development of m-AC even in the presence of serum. Monocyte/macrophage markers such as Fc receptors and non-specific esterase strictly correlate negatively with the expression of accessory activity, whereas morphologically the appearance of veils positively correlates with all experimental situations of high accessory activity. Therefore, it is evident that serum contains regulatory factors that strongly modify the accessory potency of the m-AC via the cyclic nucleotide system, thus presenting a new immunoregulatory principle at the beginning of the immune cascade.

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INTRODUCTION

Accessory cells (AC) represent a group of sparsely distrubuted but highly active cells with respect to accessory function: a set of functions including antigen presentation and secretion of mediators such as IL-1 for initiating the T-cell and T-dependent B-cell activation. It is known that for T-cell stimulation, cells of high accessory potency such as dendritic cells (DC) described by Steinman[1] and of low potency such as macrophages[1] exist. Therefore immune regulation is not clear at this level and the question remains uncertain as if one major cell population at different stages of differentiation is playing the active role or totally different cell populations can initiate the immune cascade.

Lymphoid DC are well known antigen presenting cells that have a dendritic type of morphology.[2] DC have been described variously as Fc receptor negative, poorly phagocytic and pinocytic, possessing only a small number of lysosomes and lacking reactivity with monocyte specific monoclonal antibodies and peroxidase or nonspecific esterase stain.[3]

We have recently shown that after culturing human monocytes in serum free medium, cells acquire a high functional level of AC like DC after 1-2 days and stay active for long periods of time.[4,5] In the presence of 20% human serum (HS), the "high accessory cell" state is also observed but as a transient phenomenon for monocytes on their way to become macrophages. By down titration of serum in the medium, the state of "high accessority" prolongs. Thus, the circulating monocytes undergo a state of accessority, as high as comparable with DC, before in a next step they become induced by serum factors to become macrophages.[4]

In this paper, we describe signals that favor high AC differentiation from monocytes (m-AC) versus opposing signals favoring macrophage development. We have found that agents recongized to promote an increase in intracellular cyclic AMP (cAMP) support monocytes to develop into m-AC and stabilize this differentiation stage for longer periods of time, whereas in cells treated with cyclic GMP (cGMP) and bibutyryl cGMP, the accessory function was markedly inhibited. The addition of adenosine and adenine nucleotides such as AMP, ADP, and ATP to monocytes appeared to contribute in cAMP increase in the cells, and hence, the cells obtain m-AC criteria. This effect was observed in the presence of serum, thus interfering with its macrophage inducing capacity. It therefore appears that cAMP versus cGMP controls this newly defined process of immunoregulation, the shift between the high AC state of monocyte derived cells and the development of macrophages having a low accessory capacity.

MATERIALS AND METHODS

Chemicals

CAMP, dibutyryl cAMP, cGMP, dibutyryl cGMP, all sodium salts: ATP (disodium salt), NaF, theophylline, ADP, and Adenosine (9-B-D ribofuranosyladenine, were purchased from Sigma (Sigma Chemical Co, St. Louis); AMP (dinitrium salt, E. Merck, Darmstat, FRG); and L-Leucine-Methyl ester (Sigma).

Culture media

The working medium consisted of a combination of 80% RPMI 1640 and 20% medium 199 Earle's salts (Biochrom) (designated as medium 80/20) (Peters et al., 1985) supplemented with penicillin/streptomycin (200 U/ml) plus serum (10% bovine serum or 20% human AN serum). Serum-free medium was MCDB-302 (Sigma).

Endotoxin

All reagents used in purification and culture of monocytes were tested for the presence of endotoxin by a kinetic limulus test,[18], kindly performed by K-P Becker and B. Urbaschek, Mannheim. Buffers, serum free media and Lymphoprep contained less than 20 pg/ml. The combination of medium 80/20 contained 60 pg/ml and medium 80/20 plus HS contained 40 pg/ml of endotoxin. The medium used in the adhesion process was supplemented with 10% bovine serum and contained 1,28 ng/ml.

General purity

"High-yield hybridoma conditions" have been established for our cell cultures.[8] 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, and was therefore kept away from serum-free cultures.

Monocyte preparation

Monocytes were prepared from the buffy coats of healthy blood donors, kindly provided by the blood blank, 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 by Lymphoprep 1,077g/ml (Nyegaard, Oslo) centrifugation. The interphase containing the peripheral blood mononuclear cells (PBMC) was removed and washed 5 to 7 times to remove any thrombocytes. The yield ranged between 3 $\times 10^8$ and 7 $\times 10^8$ PBMC per buffy coat. Cells were resuspended in medium 80/20 supplemented with 10% bovine serum and allowed to attach onto tissue culture plates for 1 hr at 37°C, washed free of non-attached cells and incubated in medium supplemented with 20% HS or serum free medium.

M-AC inducers

12 hrs after incubation of monocytes in medium containing 20% HS, the inducers were added. Dibutyryl cyclic AMP and adenosine as well as the other compounds were used for induction of m-DC. The concentrations of additives are given with the figure legends.

Lymphocyte depletion

PBMC were incubated in petri dishes for 1 hr at 37°C and non-adherent cells were collected, centrifuged and

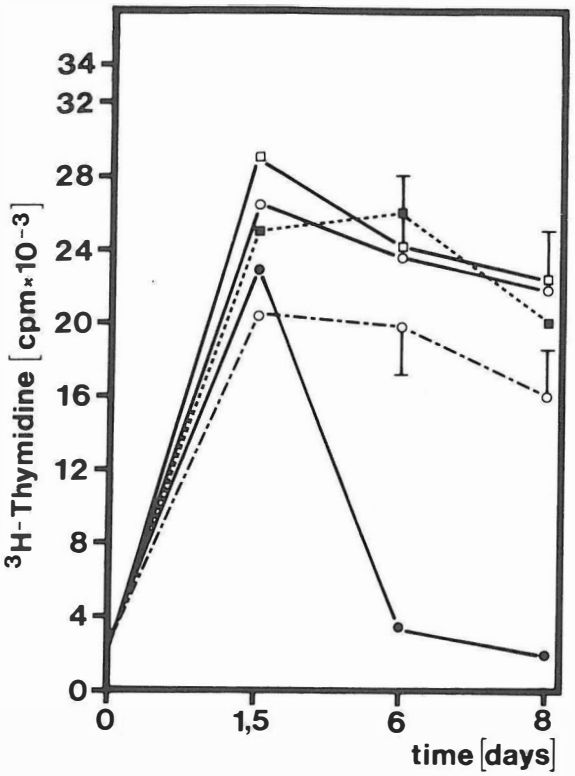


Figure 1. Time kinetics of monocytes cultured in medium containing human serum. Without additives (●--●), dibutyryl cAMP 0.5mM (□----□), cAMP 5mM (■----■), theophylline 0.5mM (○---○), and NaF 0.5mM (●---●). At the time points indicated, (abscissa), the cells were washed free of drugs, pulsed with sodium periodate (see material and methods) and subjected to cooperation by adding cryopreserved human lymphocytes to the culture. The mitogenic stimulation was measured by incorporation of ^{3}H-thymidine added at the last 24 hrs of 48 hrs incubation.

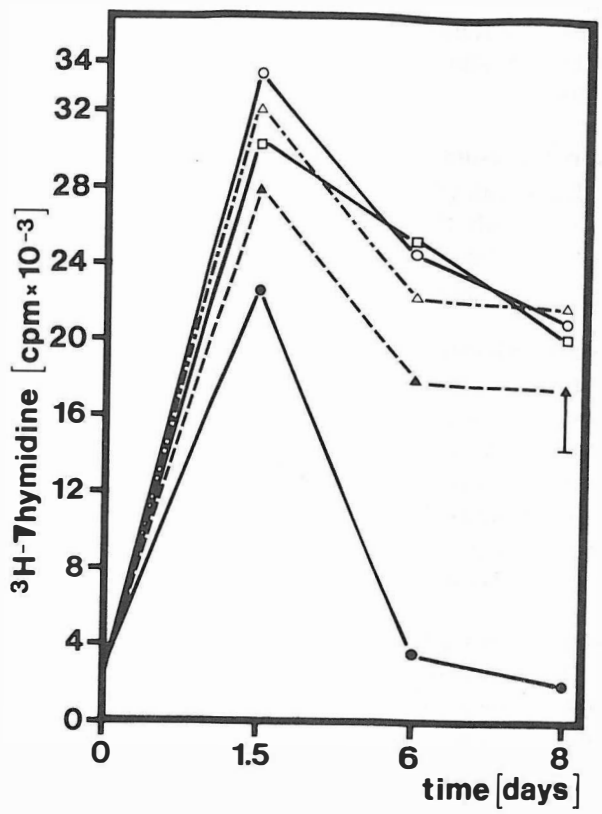


Figure 2. The accessory activity of the cells was tested as indicated in Fig 1. Without additives (●--●), AMP 0.5mM (○--○), ADP 0.5mM (△--△), ATP 0.5mM (▲--▲).

adjusted to 5×10^8 cells/ml of PBS. They were treated with leucine-methyl (Leu-O-Met) in order to further deplete the cells of lysosome rich cells, namely AC, monocytes, macrophages and granulocytes.[20] Leu-O-Met at 25 mM 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 250ml 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 PBD. 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 h 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 100ml 8-channel micropipette (Costar). For fixation, 0.05% glutaraldehyde was added immediately before centrifuging them for 2 min at 200g. Glutaraldehyde was removed and 100ml PBS added per well. Rosettes of four 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 were fixed with 0.05% glutaraldehyde while centrifuging for 2 min at 200g.

Fc-receptor-independent phagocytosis

Indian ink (Pelikan), was incubated at 1:500 final dilution with the cells for 30 minutes at 37°C, the unphagocytized particles were removed with a 100ml 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 200g. The fixative was removed and cells were incubated for 8 minutes at 37°C with the substrate alpha-naphtyl butyrate (Technicon) and washed with PBS. Cells were classified as strongly positive, weakly positive, and negative.

Ac-lymphocyte 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. Accessory cells, monocytes or macrophages were washed five times in microtiter wells and pulsed with the mitogen $NaIO_4$ (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 50ml amount left back in the well. This will prevent any loss of non-adherent cells. The cells were washed and cocultured for 48 hrs 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 last 24 hrs of culture by 0.2 Ci ^{3}H-thymidine per well. They were harvested by an automated cell harvester (Cambridge Technology) and counted by scintillation counting.

Table I. Phenotypic markers of monocytes and their derivatives obtained in cell culture.

PARAMETER	Fc-receptor %			Nonspecific esterase %			Veils	
TIME (day)	0	2	8	0	2	8	0	2	8
Monocyte Treatment									
(-) blood monocytes 90[c]				95		-[d]			
monocytes cultured a) in serum-free medium (MCDB-302)	90	28	10	95	35	18	++		+++
medium 80/20 + 20% human b) serum (mature macrophages)			95			98			+/−
ATP [b]	90	25	30	95	35	45	+[d]		+++
ADP	90	30	20	95	22	30	+		+++
AMP	90	34	30	95	30	40	+		+++
Adenosine	90	26	20	95	18	30	+		+++
cAMP	90	24	30	95	18	30	+		+++
dibutyryl cAMP	90	26	15	95	15	25	+		+++
theophylline	90	28	25	95	12	20	+		+++
NaF	90	32	30	95	25	22	+		+++
cGMP	90	34	67	95	25	75	+		−
dbcGMP	90	38	78	95	38	87	+		−

a) Human peripheral blood monocytes were cultured at serum-free, or

b) Serum containing conditions plus various additives added at 12 hrs after initation of the culture. At time points indicated, cells were tested for the expression of Fc-receptors, non-specific esterase and veils.

c) The numbers indicate the percent of strongly positive cells for the respective markers.

d) − + ++ and +++ indicate the strength of veil expression served by microscopic observation of the living culture.

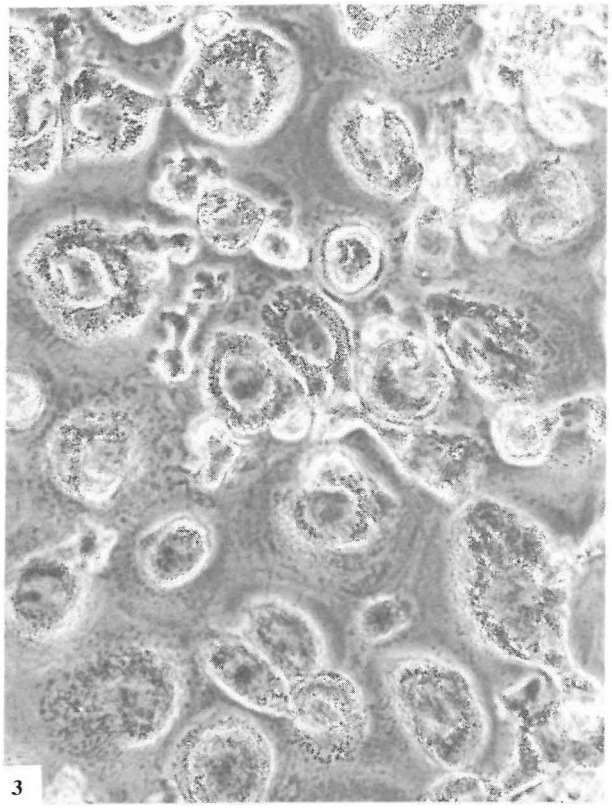
RESULTS

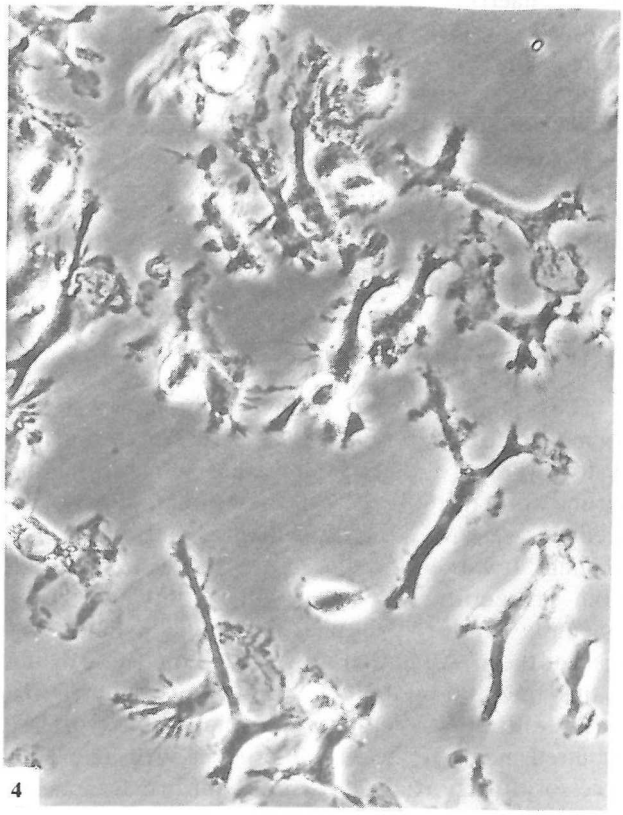
Mode of monocyte development in the presence of human serum

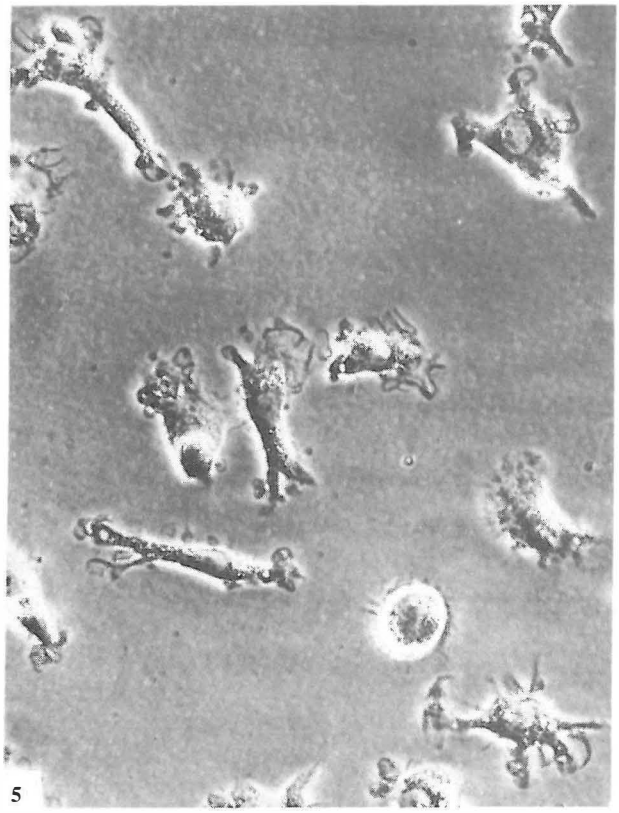
The accessory function of the major cell populations-monocytes, macrophages and m-Ac-was measured by a cell cooperation assay which quantitates the accessory cell-dependent lymphocyte stimulation in a mitogen-triggered system. The resulting mitogenic lymphocyte stimulation was used as a measure of the accessory potency (cooperativity) of various accessory cell populations (AC) and also enabled us to follow the time kinetics of monocyte differentiation.

Freshly prepared monocytes were of low accessory activity. At 1-2 days of incubation, they converted into loosely adherent cells of a high accessory activity (Fig. 4). Fc receptor, Fc receptor-dependent and independent phagocytosis (latter data not shown), and non-specific esterase as the predominant monocyte/macrophage markers, were reduced (Table I). Morphologically, the appearance of veils was significant. This process was referred to as phase I of monocyte differentiation and the cells obtained were designated as monocyte-derived accessory cells (m-AC).

When cells were kept in culture for longer than a week, they retracted the veils and aquired the well-







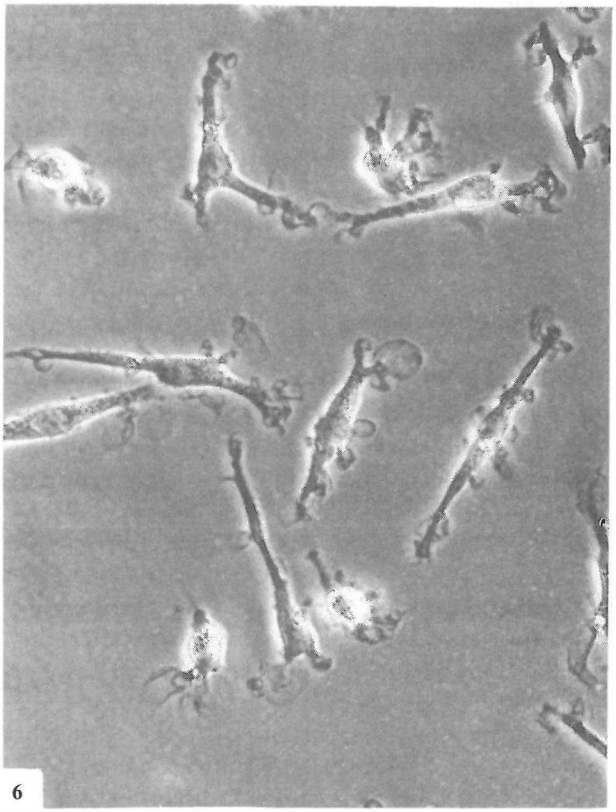


Figure 3,4,5, and 6. Phase contrast appearance of monocyte derived cells after 8 days of incubation in 3) medium 80/20 containing 20% human serum (mature macrophage), 4) serum-free medium MCDB-302, 5) medium 80/20 plus 20% human serum plus 0.5mM dibutyryl cAMP 6) and medium 80/20 plus 20% human serum plus 0.5mM adenosine. The apperance of veils and dendritiform elongations is significant in Figs 4,5, and 6.

known macrophage features such as strong attachment, flat shape, phagocytosis, phase dense granula (Fig. 3), and expression of Fc receptors (Table I) accompanied by low level of accessory avtivity (Fig 1). Conversion from m-AC to macrophages is referred to as phase II of monocyte differentiation.

From these findings, we concluded a developmental sequence whereby monocytes in the presence of human serum first enter the "accessory state" and subsequently convert into macrophages.

M-AC development in the abscence of serum

The best approach to define conditions and signals for monocyte differentiation was the use of a serum free system where regulatory molecules active in human serum were not present. The optimum serum free medium was MCDB-302 which is a protein free medium. It was found that monocytes were inhibited from proceeding to macrophages, but still spontaneously underwent phase I development and reached the m-Ac state (Fig. 8). They could be arrested in this state at a high number and viability for up to 6 weeks witthout developing into macrophages. They acquired most of the m-AC criteria such as a high accessory function (Fig. 8), a reduction in macrophage/monocyte markers (Table I) and development of dendrites (Fig. 4) within a few days of culture. The high cooperativity of the m-AC and the low expression of markers stayed constant for the whole culture period (6 wks). Other serum free media such as Iscove's and medium 199-2 were also effective in m-AC induction (Bru, et al., submitted for publication). The advantage of this system is therefore to define and separate the two phases of monocyte development and to facilitate further study of signals and conditions necessary for m-AC differentiation from monocytes in the absence of possibly interfering serum molecules.

Effect of cAMP on monocyte differentiation into m-AC and macrophages

When we cultured monocytes in medium containing 20% HS, the cells passed through phase I and entered phase II, expressing the macrophage phenotype after 8 days of incubation (Fig 1). At the same culture conditions, we treated monocytes by agents that increase cAMP in the cells. Cells reached an even higher cooperative state at phase I and stayed activated at this phase for a long period of time as a potent stimulator of lymphocyte proliferation (Fig 1). We exposed the monocytes to four different groups of drugs that by different mechanisms increase the intracellular cAMP of the cells: a) DbcAMP causes a direct increase in intracellular cAMP, b) Using cAMP, the optimum effect was obtained at a 10 times higher concentration compared with dbcAMP, c) The third group were drugs that activate adenylate cyclase of the cell membrane, such as NaF, epinephrine and forkskoline, d) Theophylline, an inhibitor of phosphodiesterase, e) The last group of compounds were adenosine and adenine nucleotides such as ATP, ADP, and AMP. When monocytes were treated with any of the above group of compounds, they were strongly reduced in percent of esterase and Fc receptor positive cells at day two (Table I). The cells became mostly detached and appearance of dendrites and veils and irregularly shaped nuclei were pronounced (Fig. 5 and 6). By phase contrast, the cells did not have significant amounts of granula. The low expression of macrophage-specific markers stayed constant even after 10 days of culture (Table I). The accessory activity of these m-AC as measured by the cell cooperation assay was much stronger than that of fully mature macrophages (Fig 1 and 2). This phenomenon was very much similar to phase I of monocyte differentiation but it was evident even after 10 days of incubation. The cooperativity was even higher than that of m-AC obtained in serum-free situation.

Increase in cAMP level of m-AC in the serum-free condition did not have a profound effect on the activity of these cells. In other words, m-AC developed in MCDB-302 were already maximally activated, and an increase in intracellular cAMP did not further increase their accessory activity.

Effect of cGMP on monocyte differentiation in the presence of serum

When cGMP and dbcGMP was added to monocyte cultures containing human serum they first entered phase I but later drastically lost their cooperative activity, even lower than untreated cells (Fig. 7). At day eight of culture, cells were highly viable, attached, strongly granule d, without any veils or dendrites. The percent of esterase and Fc receptor positive cells was higher than in control macrophages (Table I). CGMP favored the cells to strongly express macrophage markers, accompanied by a further decrease in accessory potency (Table I).

CGMP and serum-free medium

When cultured in serum-free medium, cGMP and dbcGMP caused a decline in the accessory function showing that it counteracts with phase I development (Fig. 8). In phase II also, the accessory potency is lowered, showing that cGMP, at least partially, is able to replace serum and thus correlates with the process of phase I. In other words, it seems that cGMP was acting as a cofactor to favor the progression of cells into phase II, but not completely replacing serum for reaching the macrophage state (Fig. 8).

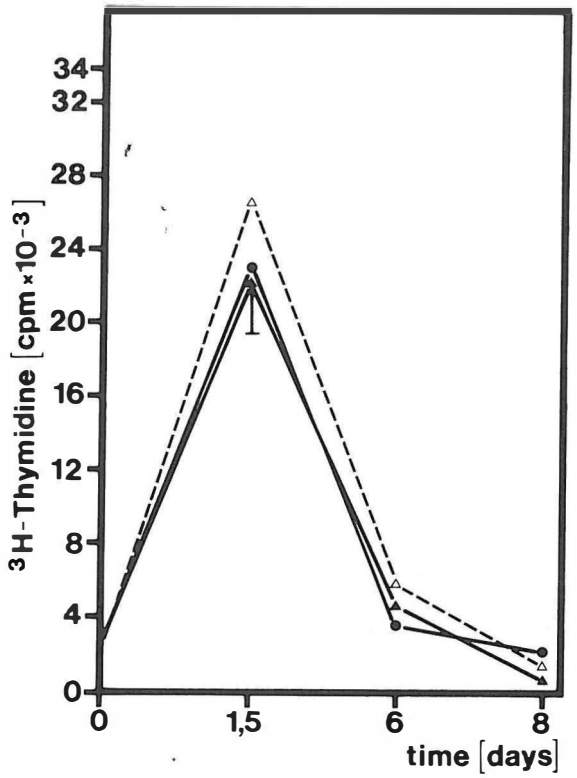


Figure 7. The accessory activity of the cells was tested as indicated in Fig. 1. Without additives (●--●), cGMP 0.5mM (△--△), dibutyryl cGMP 0.5mM (▲--▲).

Effect of indomethacine

When doing the cooperation assay, all the major cell populations including m-AC, monocytes and macrophages were washed free of incubating medium and replaced by fresh medium plus responding lymphocytes. So the idea that the low cooperativity of mature macrophages may be due to PGE or other inhibitors, already existing in the incubating medium, was ruled out. On the other hand we did another control experiment where indomethacine was added at the beginning of the cooperation assay. The results indicated that the accessory activity was similar to nontreated cultures (data not shown).

DISCUSSION

Monocytes after being released from bone marrow circulate for about 24 hrs in the peripheral blood.[10]. Thereafter, they migrate into the tissues and differentiate. So far, macrophages have been considered as the only differentiation products, because monocytes could completely be converted into macrophages in cell culture[11].

It now appears that monocytes follow a more complicated route of differentiation. By doing fine resolution time kinetics we have found that monocytes pass through a transient state of high accessory activity, much exceeding that of monocytes and macrophages (Fig. 1). Arbitrarily it thus appears useful to define two phases of monocyte differentiation: phase I is characterized by an increase in accessory potency and a concomitant decrease of typical monocyte/macrophage markers. Phase II designates the conversion of the cells to acquire the full macrophage phenotype including low accessory potency (Fig 1).

Further analysis revealed the surprising fact that phase I could be experimentally extended by omission of serum. It appeared that the monocyte-derived accessory cells (m-AC) retained their high accessory potency for long periods of time. During the culture they progressively developed dendritiform elongations (day 8) in addition to veils, very much reminiscent of lymphoid dendritic cells as described by Steinman.[2] Therefore, they are designated monocyte derived dendritic cells (m-DC). This paper does not deal with the obvious similarities between the two cell types but rather analyzes mechanisms controlling the biphasic pattern of monocyte differentiation, assuming that these mechanisms could give new insight into the widely unknown processes of immune control at the level of accessory cells.

First, we have found that factors of so far unknown

nature in the serum are responsible for the differentiation of phase II and when the signal strength is reduced the cells persist in the preceding m-AC state. This gives a first clue for intervening with phase II progression.

Second, using serum- free conditions we have been able to study signals at highly defined conditions which are effective on the differentiation steps.

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

Third, it appears that cAMP dependent processes are of a profound effect on the development and maintenance of the accessory potency of the cells. Intracellular cAMP increase was induced directly by dbcAMP and indirectly by corresponding drugs, theophylline as an inhibitor of phosphodiesterase, NaF and forskoline (latter data not shown) for activating membrane adenylcyclase. These agents only variably increased the high AC state of m-AC but completely were able to block progression into phase II, thus preventing development of macrophages even in the presence of serum.

Fourth, two methods are therefore available to block phase II transition. Serum starvation might be taken as artificial; the effect caused by cAMP increase, however, suggest the presence of a regulatory mechanism which also acts in the presence of serum.

There are reports by others showing that an increase in intracellular cAMP in monocytes causes a decrease in phagocytic activity of the cells[12] or inhibits the production of lysosomal enzymes[13] and locomotion. It is possible that these investigators have observed the corresponding partial aspect of m-AC. Putting these data together with our finding suggests that cAMP may function as a second messenger for monocytes to reach and stabilize phase I development of m-AC.

On the other hand, agents that cause accumulation of cGMP cause an earlier differentiation of cells into mature macrophages in the presence of serum. As it is shown in Table I, accessory activity was reduced and a higher percentage of the these cells were esterase and Fc receptor positive in comparison with untreated cultures. Morphologically, they were bigger in size and higher in granula accumulation.

These data suggest that cAMP and cGMP have an opposing regulatory activity in function and, more vigorously, in differentiation of monocytes. CAMP causes the cells to stay in the m-AC state, against the effect of serum, whereas cGMP forces the cells to differentiate earlier into macrophages. (Figs. 1 and 7, Table I). These data correspond with our previous findings that the progress of monocytes to m-AC is not inducible within the culture period. A possible inducer may have acted on the monocytes prior to the culture period. Alternatively, the cells may have been predetermined and do not require exogeneous signals for entering the m-AC state, in contrast with the differentiation process of m-AC into macrophages which requires serum components.

As shown above, a kind of control implies that noncyclic nucleotides and cAMP itself should not exert the dbcAMP effects. However, we have found that adenosine, AMP, ADP, ATP and cAMP equally can enhance the accessory activity of m-DC.

When we tested adenosine, it showed a similar response as did dbcAMP. All reagents could completely mimic the dbcAMP effects and arrest the monocytes in their m-AC state (Fig. 2). This may be explained by a special surface design of the monocyte membrane. The adenosine receptor is found on the outside of the monocyte cell membrane and reacts with the ribose moiety of adenosine.[15] The receptor on lymphocytes is known to consist of two types, termed Al and A2; both of these receptors may be present on the same cell.[16] A1 is a higher affinity receptor which when bound to adenosine, inhibits adenylate cyclase activity, whereas the A2 receptor is of lower affinity, and exerts its effect by activation of this enzyme.[16] Lappin and Whaly [17] have shown that monocytes possess adenosine receptors only of the A2 type, which stimulates adenylate cyclase and hence increases intracellular cAMP. Thus the effect of adenosine, in our monocyte differentiation into m-AC, may be exerted through this receptor, hence increasing the cAMP level in the cell whereas the cyclic nucleotide system can be triggered directly.

Phosphorylated adenine nucleotides such as AMP, ADP and ATP are converted into adenosine by a cell surface ATPase found on monocytes. After being dephosphorylated, adenosine exerts its effect via the adenosine receptor. Thus, it seems that adenosine is a key substance to act as a regulator of cellular function and differentiation in m-AC. The source of adenosine in vivo could be the mononuclear cell itself[18] or it could be the tissue fluid in which the concentration of adenosine may be as high as 2.6 mM.[18] We also donot rule out the possibility that nucleotide effect may be through the conversion to adenosine by membrane 5′-nucleotidase. Actually, the enzyme 5′-nucleotidase catalyzes the phosphorylytic cleavage of 5′-nucleotides, and is present in many tissues.[19] In human lymphocytes, the activity of this enzyme is localized at the external aspect of the cells and involves the dephosphorylation of 5-nucleotides, to which cells are generally impermeable, to the readily transported nucleosides.[20,21] This may be well true since when we added dinucleotides such as AP3A and AP4A to monocytes, they exerted no effect on monocyte dif-

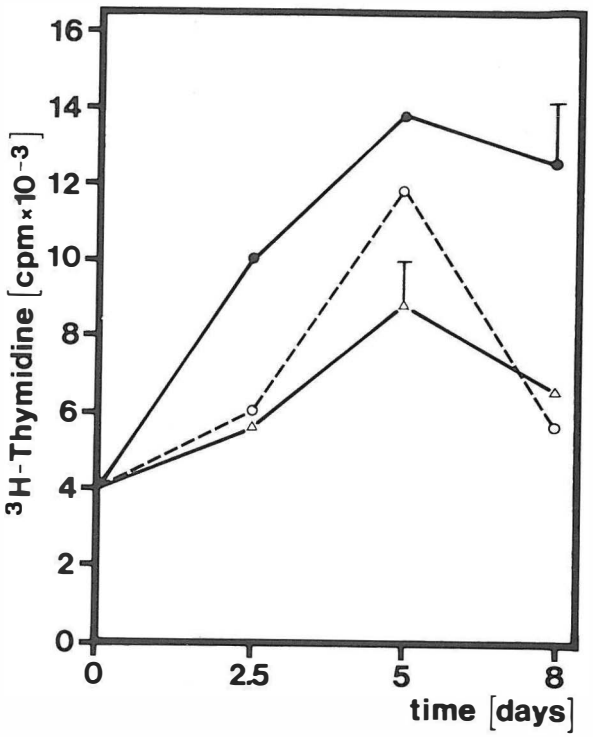


Figure 8. The accessory activity of the cells was tested as indicated in Fig. 1 except the cells were cultured in serum free medium (MCDB-302). Without additives (●--●), cGMP 0.5mM (△--△), dibutyryl cGMP 0.5mM (○--○).

ferentiation into m-AC (data not shown). The enzyme 5-nucleotidase is possibly unable to dephosphorylate these agents since both sides of the molecules are occupied by adenine bases.

By using nucleotides as well as serum as inducers, post-monocyte differentiation can thus be subdivided into two steps, the first being a progression of monocytes into highly accessory cells (phase I). This step is independent of serum factors or exogenously added LPS (see material and methods). During phase I, the accessory potency of the cells increases dramatically, whereas markers typical of monocytes/macrophages such as Fc receptor, phagocytosis, and nonspecific esterase decrease (Figs. 1 and 2, Table 1).

Accessory activity of phase I cells was further increased by cAMP and indirect activation of the cAMP system, accompanied by a further decrease of macrophage specific markers. Monocytes which do not appear to require exogenous signals to enter phase I may well have received signals for this developmental progress during the preceding monocyte phase *in vivo*.

Monocytes, having reached phase I and even kept there as m-AC for several days, can be further converted to become macrophages, thus entering phase II as the second developmental step outlined above. This process depends on a sufficient concentration of serum factor (s) of so far unknown nature. At low serum concentrations, the progression of cells from phase I to II is retarded (5-3%) or prevented (2-1%).[4] CGMP favors this process but is by itself not sufficient to induce it at serum-free conditions (Fig 8). CAMP is able to block phase II and prevent macrophage development even in the presence of serum. This finding makes it likely to believe that also in the body's environment, immunoregulatory processes may be active via the cAMP system to control the balance between the high and low accessory cells.

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