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Growth enhancing property of human monocytes from normal donors and cancer patients

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Summary. Peripheral blood monocytes, but not other leucocytes, from healthy donors, were shown to enhance the DNA synthesis of three cell lines. The effect was very marked on an epithelial (Chang) and lymphoid (CLA-4) cell line derived from normal tissues, and less marked on a carcinoma-derived line (HT-29). The enhancement was demonstrable over a wide range of monocyte:cell line ratios, and some activity was present in supernates from monocytes cultured alone or with a cell line. Furthermore, monocytes from gastric carcinoma patients did not enhance Chang and CLA-4 cells to the same extent, relative to the healthy donors.

INTRODUCTION

Mononuclear phagocytes are known to have a marked effect on the proliferation and differentiation of stimulated lymphocytes in various culture systems (Oppenheim & Rosenstreich, 1976). Macrophages and their products can stimulate or inhibit lymphocyte functions, depending on the experimental conditions (Nelson, 1976). A protein from mouse macrophages has been described which enhanced the rate of [^{3}H]-thymidine incorporation by mouse thymocytes (Unanue & Kiely, 1977). The growth regulating properties of macrophages are not exerted solely on lymphoid cells culture. Rat peritoneal macrophages will enhance the growth of various tumour cell lines and of fibroblasts in explants, when present as the minoritiy population and produce marked cytostatic effects on a variety of 'normal' and 'transformed' cell lines when present in excess (Keller, 1974a; b). The ability of activated macrophages from experimental animals to exert a cytostatic or cytotoxic effect on tumour cells has received considerable attention recently (see Eccles, 1978), leading to suggestions of their role in surveillance against neoplasia (Alexander, 1976). Although it is known that human monocytes produce colony stimulating factors for haemopoietic cells (Territo & Cline, 1976), very little work has been done on their growth regulatory properties and effects on cultured cells.

MATERIALS AND METHODS

Cell lines

Three human cell lines were used. Chang cells, an epithelial line established from normal human liver

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(Chang, 1954), carrying HeLa chromosome markers (Lavappa, Macy & Shannon, 1976), were subcultured twice weekly using vigorous shaking to detach cells from the surface. HT-29, a line established from an adenocarcinoma of colon (gift of Dr J. Fogh, Sloan-Kettering Institute), was similarly maintained except that trypsin–EDTA was used to detach the cells. CLA-4 is a B lymphoid cell line from cord blood lymphocytes, initiated in Edinburgh by Dr C. M. Steel and maintained in suspension culture in Birmingham since 1970 (Steel & Edmond, 1971).

Culture media

Dulbecco's modification of Eagle's minimal essential medium (D-MEM) antibiotic-free, supplemented with 10% foetal calf serum (FCS), obtained from Flow Laboratories, was used for maintenance of cell lines. The same medium was used for the mixed cell cultures. HEPES-buffered D-MEM (D-MEM-H) was used for washing and manipulation stages.

Leucocytes

Peripheral blood, from healthy donors (laboratory personnel) and gastric carcinoma patients (prior to treatment), was defibrinated on glass beads in siliconized bottles. Defibrination was preferred to heparinization of blood because of the cleaner cell separations obtained even though it is known that some monocytes are lost during the defibrination procedure. The blood was centrifuged, the serum removed and replaced by D-MEM-H. Gelatin (3% in saline, one third volume) was then added. After mixing, the blood was allowed to sediment for one hour at 37°. The supernate was removed, the cells spun down and subjected to hypotonic shock (distilled water for 20 s) to remove the remainder of the red cells. The cells were washed twice in D-MEM-H and resusupended in equal parts D-MEM and D-MEM-H supplemented with 20% FCS (10 ml). The cell suspension was transferred to a flat glass bottle which was left for 1 h at 37° for attachment of monocytes to take place. The supernate containing non-adherent cells was carefully poured off and the monolayer gently rinsed twice with D-MEM-H. The non-adherent fractions were used as a source of lymphoctes. The adherent cells were removed by vigorous squirting of PBS at the base of the container. The resulting suspension contained principally monocytes but was contaminated with some lymphocytes (usually 2–3%). The cells were spun down in a plastic tube, washed twice in D-MEM-H and resuspended in D-MEM-10% FCS to 4×10^6/ml. Suspensions containing 10^6/ml and $0{\cdot}25 \times 10^6$/ml were prepared from this. In some experiments, cells were fractionated by centrifugation through a double layer of Ficoll–Triosil (English & Anderson, 1974). Monocytes recovered from the upper interface were separated from lymphoctes by glass adherence as usual; polymorphonuclear cells were obtained from the lower interface.

Assay system

A modification of the method of Nathan & Terry (1975) was followed using flat-bottomed microtitre trays (tissue culture grade). To each well 0·1 ml of a 10^5/ml suspension of the cell line cells under test was added and the monocyte (or other leucocyte) preparation was then added in 0·1 ml. The trays were incubated at 37° for 24 h in a moist, CO_2- enriched atmosphere. Excess [^{3}H]-thymidine (0·05 ml, 2·0 μCi, specific activity 150 mCi/mmol) was then added. Twenty-four hours later the cells were harvested with the aid of a Skatron automatic harvester and the label retained on the glass fibre disc measured by scintillation counting.

Notes on the assay system.

(a) As a check on the efficiency of removal of labelled adherent cells, some of the wells were washed with three drops of 2M NaOH and the washings counted in a Triton-based scintillation fluid. No significant residue of counts was found.

(b) A long period of incubation (24 h) in the presence of low specific activity [^{3}H]-thymidine (150 mCi/mmol) was chosen because this has been shown in this laboratory to give more reproducible results than a short period of incubation with high specific activity thymidine. The thymidine is present at flooding concentrations and is not rate-limiting even for pulses of several days duration. This is in agreement with the more comprehensive studies of Ahern, Taylor & Sanderson (1976).

(c) A cell concentration of 10^4/well in 0·2 ml of medium was optimal for uptake of [^{3}H]-thymidine as shown by the fact that it is on the steepest part of the ascending limb of the thymidine incorporation/cell concentration curve (Fig. 1).

(d) The culture medium used was selected for optimal growth of the cell lines under study. An increase in FCS concentration (to 20%) improved the [^{3}H]-thymidine incorporation up to

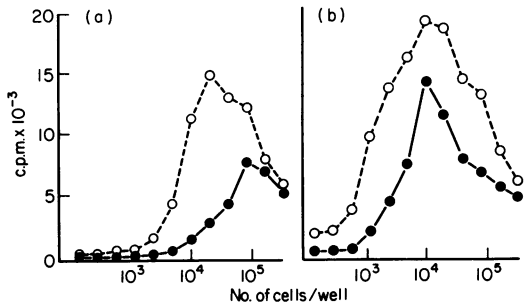

Figure 1. Incorporation of [³H]-thymidine by HT-29 cells as a function of cell density. Cells were plated out in 0·2 ml D-MEM-10% FCS, and cultured for 24 h prior to pulse-labelling for 4 h (●) or 24 h (○) with [³H]-thymidine of (b) high specific activity (5 Ci/mmol) and (a) low specific activity (diluted 1/33·3 with cold thymidine to give 150 mCi/mmol). The cell concentration used for the assays (10^4/well) is on the linear part of the curve using low specific activity precursor. The curves for the other cell lines were essentially identical.

two-fold but this did not influence the monocyte effect described.

Thymidine incorporation (Ti) index.
This was calculated as follows:

$$\text{Ti index} = \frac{\text{(c.p.m. of cell line + monocyte mixture) − (c.p.m. monocytes alone)}}{\text{(c.p.m. of cell line without monocytes)}}$$

RESULTS

Monocytes produced enhancement of the ³H-TdR incorporation of all three cell lines tested (Fig. 2) but the effect was very much more marked on Chang and CLA-4 than on HT-29 cells. The enhanced thymidine incorporation (expressed as $\log_{10}$ of the Ti index in Fig. 2 in order to obtain a linear scale) was evident over a wide range of monocyte/target cell ratios but again the results were different for the three cell lines. The T_i index of Chang altered little over the range 2·5:1 to 40:1 or greater, whereas the T_i index for CLA-4 increased sharply at the higher ratios and that of HT-29 decreased (Fig. 2). Attempts at direct visual assessment of target cell numbers as an alternative to [³H]-thymidine incorporation proved impracticable because of the inaccuracy of visual enumeration of cultures containing up to forty-fold excess of monocytes.

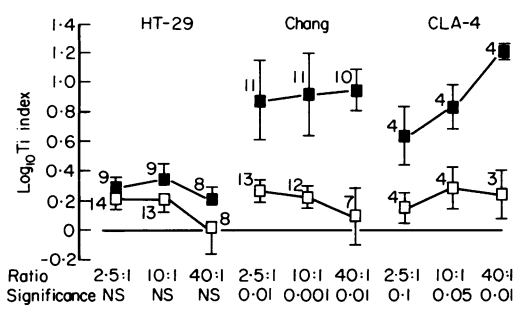

Figure 2. Differences in increased thymidine uptake of three cell lines in presence of monocytes from normal donors (■) and cancer patients (□). Mean values (± standard errors) for three monocyte:target cell ratios. Inset figures show number of donors. Statistical evaluation (*P* values) is by means of a two-tailed Students's *t* test (NS, not significant). Significant differences between normal donors and cancer patients are seen for Chang and CLA-4 cells.

Source of stimulation

Small effects, sometimes stimulatory sometimes inhibitory, were obtained with isolated lymphocytes or polymorphs, but the major source of activity of the leucocytes was in the monocyte fraction (Table 1). Activity was found in the supernates from monocytes cultured alone or with cell line cells, although the results did not closely parallel those obtained with whole monocytes. Supernates harvested after only 1 h of culture of monocytes showed some enhancing activity which diminished over a 3–6 h period and rose again at 24 h (Table 2).

Monocytes themselves were not stimulated to take up precursor by the presence of target cells as shown by the fact that incubation with irradiated (10,000 rads, cobalt source) target cells did not increase the thymidine incorporation of the mononuclear cell preparations.

Variation with source of monocytes

There was considerable variation in the growth promoting properties of monocytes from various normal donors, but repeat determinations on monocytes from three donors did not indicate any consistent intrinsic differences (Fig. 3). However, there were marked differences between the activity of monocytes of normal donors and those from patients with gastric carcinoma; despite an overlap in the spread of enhancing activity (Figs 3 and 4) the monocytes from cancer patients showed significantly less stimulation of Chang and CLA-4 cells (Fig. 2).

Table 1. Effect of different leucocyte fractions on Chang cells: mean $\log_{10}$ thymidine incorporation indices (and standard errors) of four normal donors. Negative values indicate inhibition. The major stimulatory effect resides in the monocyte fraction

	Effector:Target cell ratio		
	2·5:1	10:1	40:1
Total leucocytes	0·2193 (0·55)	0·0229 (0·12)	−0·0356 (0·153)
Monocytes	1·3565 (0·05)	1·2957 (0·027)	1·184 (0·105)
Lymphocytes	−0·0457 (0·169)	0·1048 (0·124)	0·0532 (0·171)
Polymorphonuclear cells	−0·1191 (0·185)	0·0504 (0·075)	0·1683 (0·066)

Table 2. The effect of supernatants from monocyte cultures on Chang cells. Supernatants taken at times specified, diluted 1:1 with fresh medium for 24 h culture prior to labelling. Individual results are shown at two monocyte concentrations from three donors. Stimulatory effects are seen predominantly at 1 and 24 h

	Supernatant times (h)			
	1	3	6	24
Donor A				
Monocytes at $1·25 \times 10^5$/ml	7·0	2·0	2·4	2·5
Monocytes at 5×10^5/ml	4·8	1·7	1·3	2·9
Donor B				
Monocytes at $1·25 \times 10^5$/ml	2·6	1·0	0·6	1·7
Monocytes at 5×10^5/ml	1·1	0·6	0·55	1·6
Donor C				
Monocytes at $1·25 \times 10^5$/ml	1·6	0·7	0·7	1·9
Monocytes at 5×10^5/ml	1·3	0·6	0·5	4·1

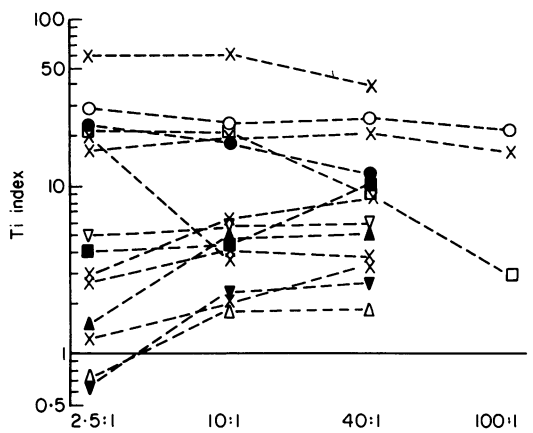

Figure 3. Effect of monocytes from normal donors on thymidine uptake of Chang cells, Ti indices at various monocyte: Chang cell ratios are plotted as individual dose-response curves for eleven donors. The closed square, circle and triangle represent retest values of the corresponding open symbols for three donors retested at 2, 5 and 11 week intervals respectively.

Effect of 2-mercaptoethanol (2ME)

The monocyte effect could not be mimicked by 2ME. When tested over a wide range of concentrations 2ME was either inhibitory to growth or had no effect (Table 3).

DISCUSSION

The effect of monocytes on the three cell lines tested was overwhelmingly one of enhancement of ^{3}H-TdR incorporation over a wide range of monocyte/cell line ratios. This is in contrast to the behaviour of macrophages in a rat system where enhancement was obtained only at low ratios (1:10) with marked suppressive effects at ratios of 1:1 to 10:1 (Keller, 1974;

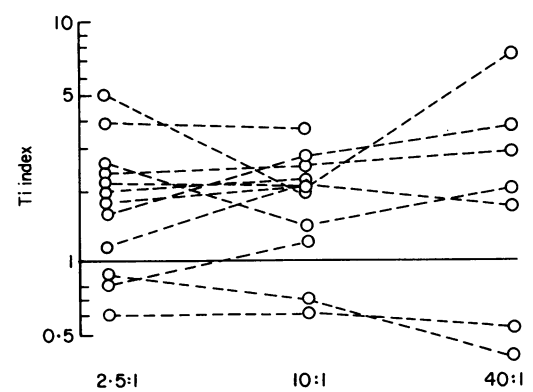

Figure 4. Effect of monocytes from cancer patients on thymidine uptake of Chang cells. Ti indices at various monocyte: Chang cell ratios are shown for twelve individual gastric carcinoma patients.

Table 3. The effect of 2-mercaptoethanol on the thymidine incorporation of cell-lines (results expressed as thymidine incorporation index, Ti, relative to control cultures). No stimulatory effect is apparent

2-ME (final concentration)	Chang	HT-29	CLA-4
6 μM	0·8	0·53	0·9
60 μM	0·9	0·69	0·7
600 μM	0·97	0·81	0·02
6 mM	0·69	0·54	0·01

1976a, b; 1977). The difference is possibly due to choice of species studied, but more likely to the stage of activation of the mononuclear phagocytes and their source. The monocytes responsible for the effect described here were used within 1 h of separation, whereas in the rat experiments 'mature' activated macrophages were used. It is known that the suppressive activity of macrophages is related to their state of activation (Nelson, 1976). This view is supported by the data presented in this paper; cancer patients' monocytes were less stimulatory than monocytes from normal donors. Rhodes (1977) has found significant differences in monocyte activation between cancer patients and healthy donors, and other data suggest that monocytes in gastric cancer patients become inhibitory after *C. parvum* injection (Trejdosiewicz & Dykes, 1977). The extensive animal data (see review by Eccles, 1978) indicate that activated macrophages are cytostatic to cultured cells whereas the scanty human evidence suggests that monocytes from cancer patients are more activated than monocytes from healthy donors.

Macrophages have been extensively studied for their effects on lymphocyte responses to stimuli and more recently for their effects on the growth of tumour cells. Growth augmentation of a lymphoid cell line has also been shown in the present work, but an equally marked effect was obtained on a non-lymphoid (Chang) line. Hence the phenomenon is not specifically of importance in relation to lymphocyte proliferation and over-emphasis may have been placed on this aspect of macrophage activity.

The benefit of macrophages in responses of lymphocytes to antigens may be at least partially substituted by 2ME (Heber-Katz & Click, 1972) whereas 2ME had no monocyte-like effect in the phenomenon described here, in contrast to the murine lymphoma system studied by Nathan & Terry (1975). Other cells (lymphocytes or polymorphs) could not produce the same effect, thus eliminating a trivial 'feeder layer' effect or lymphocyte 'immunostimulation' (Norbury, 1977) as a possible explanation of the phenomenon. Macrophages contain appreciable amounts of thymidine which can cause dilution of label and hence falsely lowered thymidine incorporation levels of the cell lines (Opitz, Neithammer, Lemke, Flad & Huget, 1975). This might account for an artefactual 'suppressor' effect, but could only diminish rather than accentuate an augmentor effect. Thymidine of low specific activity was used precisely to avoid this complication. Thymidine-degrading enzymes which might be released by monocytes would likewise artificially lower rather than raise the thymidine incorporation level.

At least part of the monocyte effect can be produced with monocyte culture supernates. This would be compatible with the release of mitogenic factor similar to that described by Unanue & Kiely (1977) for mouse macrophages or of an important growth factor. The relative freedom of the human system from suppressive factors should ease the investigation of the phenomenon.

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