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Production of an anti-tumour cytotoxin by human monocytes

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Summary. Human monocytes incubated *in vitro* for 20 hr at 37° release a factor which is cytotoxic to a number of human and murine tumour cell lines: untransformed cells appear to be less susceptible. A similar factor is produced under comparable conditions by myelomonocytic leukaemic cells and by macrophages derived from monocytes by *in vitro* culture for 8 days.

Maximum production of the factor occurred in the presence of foetal calf serum or autologous plasma and endotoxin. The factor is newly synthesized in culture as its production is reduced if the monocytes are treated with cycloheximide or actinomycin D or incubated at lower temperatures. Freshly isolated monocytes do not release the factor on freeze–thaw or hypotonic lysis.

The monocyte cytotoxin has apparent molecular weights of 34,000 on Ultrogel AcA54 gel filtration and 140,000 on gradient polyacrylamide gel electrophoresis; it has β_2–γ_1 electrophoretic mobility in polyacrylamide gel and does not appear to be C3a or arginase.

INTRODUCTION

There is increasing evidence that cells of the macrophage series have an important role in combating neoplasia. *In vitro* macrophages are cytostatic or cytotoxic to a variety of tumour cell lines but spare non-transformed cells (Hibbs, 1974; Holtermann, Klein & Casale, 1973). In some experimental studies it appears that close contact between macrophages and tumour cells is essential for the anti-tumour effect (Stewart, Adles & Hibbs, 1975), whilst in other systems, close contact is not essential since the macrophages release a soluble mediator. These mediators include arginase (Currie, 1978), tumour necrosis factor, TNF (Matthews, 1978) and the complement breakdown product C3a (Ferluga, Schorlemmer, Baptista & Allison, 1978) although others have not found C3a to be cytotoxic (Goodman, Weigle & Hugli, 1980).

In our earlier studies with rabbits we found that monocytes from normal rabbits spontaneously release a factor during incubation *in vitro* which is cytotoxic or cytolytic to some tumour cell lines but has little effect on non-transformed cells (Matthews, 1978). Our preliminary experiments with human monocytes revealed that under comparable conditions much less cytotoxic activity was produced.

This paper shows that with modified culture conditions human monocytes, like rabbit monocytes can produce readily detectable amounts of a cytotoxic factor.

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MATERIALS AND METHODS

Blood mononuclear cells

Peripheral blood from healthy laboratory staff was obtained by venipuncture and collected into lithium

heparin tubes. In all, twelve donors were used in this study. The mononuclear cell fraction isolated using Hypaque-Ficoll (Matthews, 1978) was washed twice and resuspended at 5×10^6 cells/ml in Eagle's minimum essential medium (MEM) supplemented with 10% foetal calf serum (FCS). In some experiments in which various culture additives were being tested the cells were resuspended in MEM alone at this stage. The cell suspension was added to plastic Petri dishes (1 ml/5 cm^2 surface area) and incubated for 1 hr at 37° in 95% air, 5% CO_2. The dishes were washed three times with warm medium to detach the non-adherent cells and the adherent cells were supplemented with the original volume of medium containing 10% FCS and 10 μg/ml endotoxin (lipopolysaccharide B from *E. coli* 026-B6, Difco) unless specified otherwise. When MEM/FCS was used monocytes comprised >90% of the cells as determined by morphology and non-specific esterase staining; with MEM alone, monocytes comprised >80% of the cells.

In some experiments the mononuclear cells after Hypaque-Ficoll isolation were depleted of phagocytic cells by passage through a cotton-wool column (Matthews & Maclaurin, 1974) or by ingestion of iron carbonyl and removal with a magnet (Britton, Perlmann & Perlmann, 1973). After treatment the cells were counted and adjusted to the original volume.

Cell lines

Most of the cell lines are well established lines obtained originally from Flow or Gibco-Biocult and maintained for several years in Cardiff or Gainesville. L/R cells are a TNF-resistant subline of L929 cells (Matthews & Watkins, 1978). The TOL 3 line was induced by transformation of TO strain mouse embryo fibroblasts with L cell virus and shares certain surface markers with L cells (unpublished observation).

Cytotoxicity assay

This was either performed as described in detail previously (Matthews, 1979) or modified slightly as follows when actinomycin D was used.

Seventy-five microlitre amounts of target cell suspension (3×10^5/ml) were pipetted into 96-well microtitre trays and incubated for at least 4 hr to allow the cells to adhere. Dilutions (75 μl) of the monocyte supernatant in MEM with 10% FCS and 2 μg/ml actinomycin D (Sigma) were added to give 3–4 replicates/dilution. After incubation overnight at 37°, the supernatant was discarded and the cells were fixed with 5% formaldehyde and stained with crystal violet or Coomassie blue. The remaining cells were quantified photometrically (Matthews, 1979) by 'photographing' each well in turn with an Orthomat automatic camera using the trial exposure setting; exposure times being measured with a stop watch. The percentage cytotoxicity was calculated from the formula, 100 (b−c)/(a−c) where a, b and c are the mean exposure times of wells with respectively, cells+control medium, cells+monocyte supernatant and no cells. Standard deviations were of the order of 5% of the mean.

Gel filtration

A 76·2 × 1·5 cm column of Ultrogel AcA54 was used at a flow rate of 21·5 ml/hr and 2·15 ml fractions were collected. The buffer was isotonic phosphate-buffered saline, pH 7·2 (PBS) containing 10% (v/v) glycerol.

Polyacrylamide gel electrophoresis (PAGE)

The methods using 7% rod gels or 4%–26% gradient slab gels have been described previously (Matthews, Ryley & Neale, 1980). After slicing, proteins were eluted from the gel by disruption of the slices with a rubber policeman and extraction overnight in PBS with constant agitation.

RESULTS

Preliminary experiments showed that like rabbit monocytes, human monocytes incubated at 37° *in vitro* released a factor into the supernatant which at low dilutions was cytotoxic to mouse L929 cells. The affected cells were shrunken, became detached from the plastic, failed to grow in fresh medium and were stainable with trypan blue. At higher dilutions the monocyte supernatant was cytostatic only. As human monocyte supernatants appeared less cytotoxic than their rabbit counterparts, it was decided to use a more sensitive assay in which the target tumour cells were treated with actinomycin D (Table 1). This modification also had the advantage of reducing the assay time from 3 to 1 day and was therefore used in all subsequent experiments.

Table 2 compares the effect of different culture supplements on the production of the cytotoxic factor by human monocytes. In most, but not all, experiments (e.g. experiment 2) monocytes cultured in MEM alone produced the factor. Addition of endotoxin to the MEM culture medium inconsistently increased

Table 1. Cytotoxicity of monocyte supernatant versus L929 cells. Comparison of assay methods with and without actinomycin D

Actinomycin D present	Duration of assay (days)	Initial L929 cell concentrations	Cytotoxicity (% Supernatant dilution 1/5	1/20	1/80
Yes	1	3×10^5/ml	—	45·9	10·0
No	3	$0{\cdot}5 \times 10^5$/ml	48·5	23·6	6·2

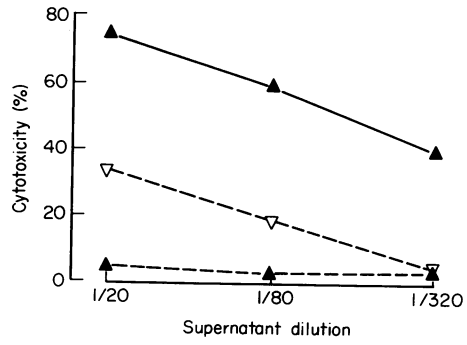

Figure 1. Cytotoxicity versus L929 cells by supernatants of monocytes incubated in MEM + 10% FCS + 10 μg/ml endotoxin for different time intervals, 0–10 hr (—▲—), 10–20 hr (—▽—), 20–28 hr (—△—).

production (compare experiments 1 and 2). MEM plus fresh autologous plasma or FCS gave consistently enhanced production compared with MEM alone (experiment 2). Greatest amounts were produced in the presence of either FCS or autologous plasma supplemented with endotoxin— a wide range of endotoxin concentrations being effective (experiment 2). A complement-dependent effect of endotoxin is unlikely since firstly, the FCS had undetectable haemolytic complement activity and secondly, human plasma heated to 56° for 20 min to inactivate complement was also effective (experiment 3). Like rabbit monocytes, most of the cytotoxic factor was released during the first 10 hr of monocyte culture (Fig. 1) although as there seemed to be some variation between donors a 20-hr incubation period was routinely used.

Table 2. Cytotoxicity versus L929 cells of 20-hr supernatants from monocytes incubated at 37° in MEM with various additives

Exp. no.	Medium supplement*	Endotoxin concentration (μg/ml)	Cytotoxicity (%) Supernatant dilution 1/20	1/80	1/320
1	Nil	0	32·3	19·9	15·3
	Nil	10	37·2	13·5	4·6
	Nil	1	39·7	11·3	7·9
	Nil	0·1	38·4	22·7	6·1
2	Nil	0	−1·3	1·3	5·8
	Nil	10	35·5	20·9	17·8
	Auto plasma†	0	37·8	16·4	7·6
	Auto plasma	10	72·4	40·0	25·3
	Auto plasma	1	71·1	44·4	16·9
	Auto plasma	0·1	66·7	33·3	24·4
	Foetal calf serum	0	66·6	26·7	17·8
	Foetal calf serum	10	72·0	53·3	23·6
	Foetal calf serum	1	74·2	47·6	25·8
	Foetal calf serum	0·1	72·4	43·6	10·2
3	Auto plasma†	10	77·2	76·0	46·4
	Auto plasma††	10	77·9	77·2	55·1
	Foetal calf serum‡	10	79·4	74·2	65·2

* Serum or plasma added to give 10% v/v.
† Fresh autologous plasma.
‡ Heat inactivated, 56°, 20 min.

Table 3. Comparative susceptibility of different cell types to monocyte supernatants

Cells	Species	Transformed	Cytotoxicity (%) of monocyte supernatant dilution of 1/8	1/32	1/128	Susceptibility to rabbit monocyte cytotoxin
Hela	Human	+	71·3	36·2	0·4	—
Hep II*	Human	+	57·0	54·2	42·1	–
Hep II*	Human	+	−13·4	1·5	−1·3	–
HL132	Human	+	75·3	72·4	37·5	NT§
AV3	Human	+	54·6	42·3	22·2	NT
HFF	Human	+	54·6	43·3	22·4	–
MRC 5	Human	–	7·4	−8·0	−2·6	+
Fibroblasts 1†	Human	–	11·2	11·2	16·4	NT
Fibroblasts 2†	Human	–	7·8	6·3	−9·4	NT
Fibroblasts 3†	Human	–	17·0	8·8	−7·1	NT
TOL 2	Mouse	+	71·1	56·9	46·0	+
TO fibros‡	Mouse	–	44·2	21·5	14·2	–
L929	Mouse	+	69·1	55·1	38·5	+
L/R	Mouse	+	8·2	1·0	−8·2	–

* Two different strains of Hep II cells.
† From skin biopsies.
‡ Embryo fibroblasts from TO strain mice.
§ NT, not tested.

The human monocyte supernatants are cytotoxic for a number of tumour cell lines of human or animal origin (Table 3). Of the human cells tested, most of the continuous tumour lines were susceptible with the untransformed fibroblasts being least susceptible. Interestingly, Hep 2 cells from different sources had differing susceptibilities. Of the animal cells tested, untransformed mouse embryo fibroblasts were undoubtedly affected, although their transformed counterparts were more susceptible. The specificity of the human monocytes supernatant differs somewhat from rabbit monocyte supernatant although the L/R subline of L929 cells selected for resistance to the rabbit factor was also resistant to the human monocyte supernatant. The L929 tumour cell line was found to be best suited for assay of the human monocyte cytotoxin and was used in all of the experiments described here although similar results have been obtained with the Hep 2 human cells.

Monocytes were routinely purified from the Hypaque-Ficoll mononuclear cell population by adherence to plastic. The adherent cells comprised >90% monocytes, the contaminating cells being largely lymphocytes. Evidence that monocytes are indeed the cells producing the cytotoxic factor is shown in Fig 2. Hypaque-Ficoll purified mononuclear cells depleted

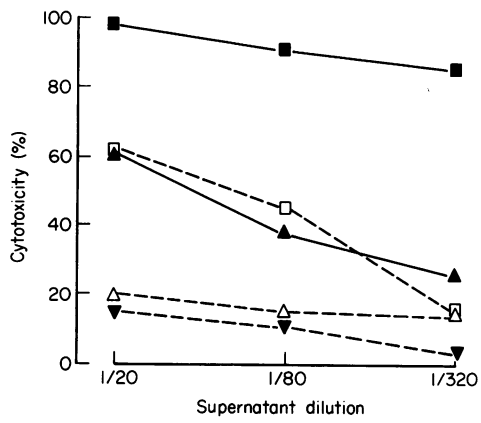

Figure 2. Cytotoxicity versus L929 cells by supernatants of different fractions of Hypaque-Ficoll purified mononuclear cells. Plastic-adherent fraction (-▲-), cells after passage through a cotton wool column (-△-) or the fraction depleted of phagocytic cells by iron carbonyl treatment (-▼-). Also shown is the cytotoxicity by supernatants of either leukaemic cells from a patient with myelomonocytic leukaemic cells from a patient with myelomonocytic leukaemia (-■-) or macrophages derived from blood monocytes (-□-). In all cases the supernatants were from cells incubated for 20 hr in MEM/FCS/endotoxin.

of phagocytic cells by treatment with iron carbonyl or by passage through a cotton wool column were poor cytotoxin producers. Thus the cell producing the cytotoxic factor is a mononuclear, plastic-adhering, phagocytic cell—in all probability the monocyte. There is other evidence for the mononuclear phagocyte origin of the factor. Firstly, leukaemic cells from a patient with myelomonocytic leukaemia were potent producers (Fig. 2) as were the macrophage-rich (60%–80%) cells of human colostrum. Secondly, macrophages derived from blood monocytes after incubation in MEM with 10% autologous plasma produced a cytotoxic factor with similar properties (see below), after challenge with endotoxin. During the 8-day culture period, the contaminating lymphocytes detached from the plastic and the final macrophage population was virtually 100% pure.

Release of the cytotoxic factor could be reduced four to sixteen fold by treating the monocytes with either cycloheximide (an inhibitor of protein synthesis) or actinomycin D (an inhibitor of transcription)—Fig 3. Thus the cytotoxic factor appears to be newly synthesized *in vitro*. Consistent with this is the impaired production at temperatures below 37° and the lack of activity in the supernatant of freshly isolated monocytes which had been frozen and thawed three times (Fig. 4) or disrupted by hypotonic lysis. Impaired production also resulted from increasing the temperature to 40° or 41·5°.

What is the nature of the cytotoxic factor? It is a relatively labile substance and this has hampered attempts at characterization. On gel filtration using Ultrogel AcA54, the activity is eluted as a broad peak

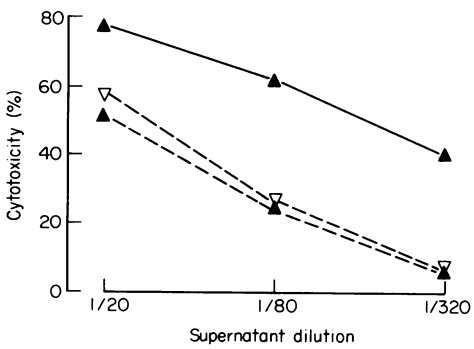

Figure 3. Cytotoxicity versus L929 cells, by supernatants of monocytes incubated for 20 hr in MEM/FCS/endotoxin either without additives (-▲-) or with cycloheximide, 10 μg/ml (–▲–) or actinomycin D, 2 μg/ml (–▽–).

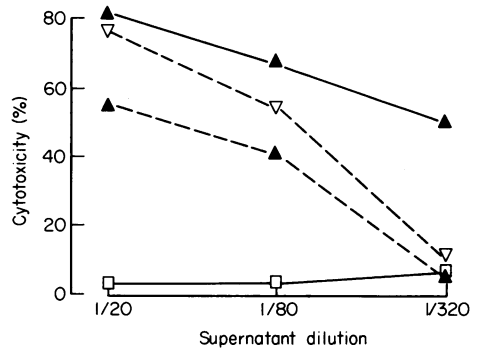

Figure 4. Cytotoxicity versus L929 cells by supernatants of monocytes incubated for 20 hr in MEM/FCS/endotoxin at either 22° (–▲–), 32° (–▽–), 37° (-▲-), or unincubated but lysed by freezing and thawing (–□–).

with an apparent mol.wt of 34,000 (Fig. 5). In most but not all preparations fractions with higher molecular weight actually increased target cell survival (Fig. 5). Although this phenomenon has not been investigated further as yet, as unfractionated supernatants are invariably cytotoxic, it appears that the cytotoxic effect is the overriding one. Molecular weight determination using gradient 4%–26% PAGE (Fig. 6) revealed a much higher molecular weight (approximately 140,000) than obtained with gel-filtration. The cytotoxic factor migrates with β_2–γ_1 electrophoretic

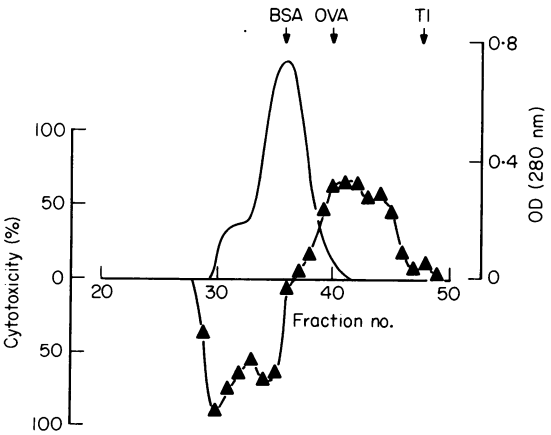

Figure 5. Fractionation of monocyte supernatant on the basis of molecular size by gel filtration using an Ultrogel AcA54 column. The arrows mark the elution positions of the molecular weight markers, bovine serum albumin, 67K (BSA); ovalbumin, 43K (OVA); soya bean trypsin inhibitor, 20K (TI). Percentage cytotoxicity (-▲-); OD 280 nm (—).

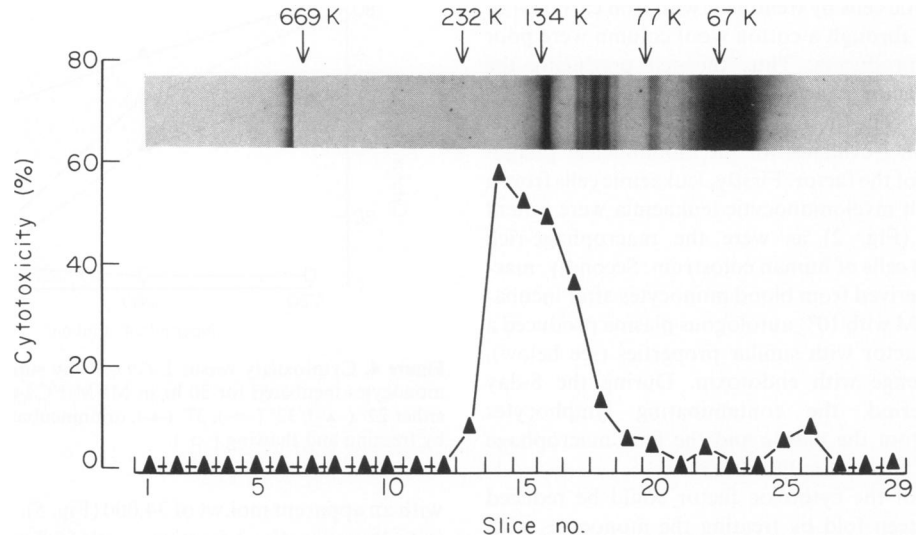

Figure 6. Fractionation of monocyte supernatant on the basis of molecular size by gradient, 4°–26% PAGE. The arrows mark the elution positions of the molecular weight markers, albumin (67K), transferrin (77K), albumin dimer (134K), catalase (232K) and thyroglobulin (669K). The photographic inset is of an amido black stained gel run in parallel, the stained bands are the FCS proteins from the culture medium.

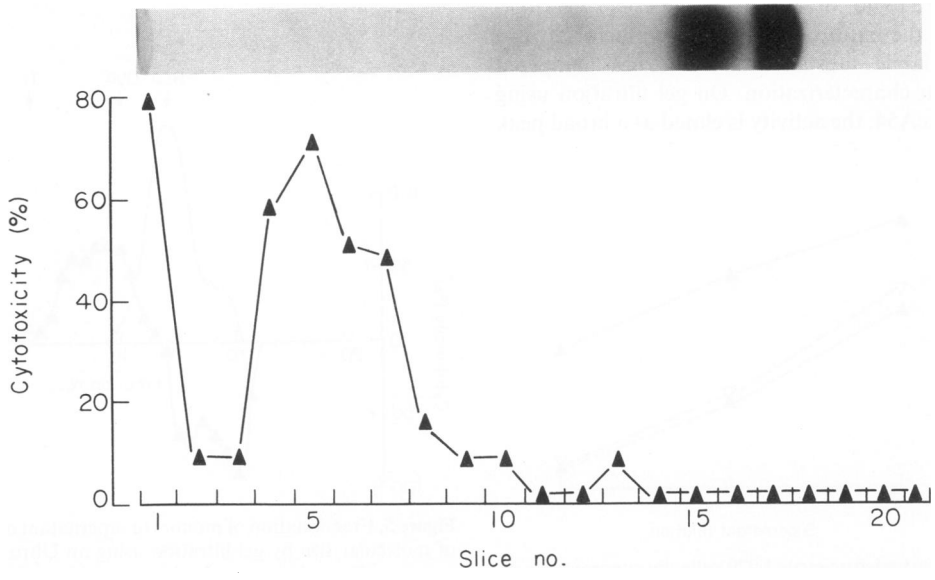

Figure 7. Fractionation of monocyte supernatant on the basis of charge by 7% PAGE; the anode is on the right. In the photograph of the amido black stained gel run in parallel, the dense band is the albumin component of the FCS in the culture medium.

mobility on 7% polyacrylamide gel electrophoresis (Fig. 7).

The cytotoxic factors released by myelomonocytic leukaemic cells and by macrophages were identical to the monocyte cytotoxin in relation to electrophoretic mobility and behaviour on gel filtration and gradient PAGE.

Supplementation of the medium with arginine (2 mg/ml) during the cytotoxicity assay failed to prevent the action of the monocyte cytotoxin suggesting that it is not an arginase.

DISCUSSION

Freshly isolated human monocytes incubated for 20 hr *in vitro* release a factor which is cytotoxic for a number of tumour cell lines but has a lesser effect of untransformed cells. Maximum production of the factor occurred in the presence of serum or plasma and endotoxin at 37°, less being produced at lower temperatures and none at all being released by monocytes which had been disrupted by freezing and thawing or hypotonic lysis. This suggests that the factor is newly synthesized after stimulation *in vitro* and this is confirmed by the reduced production after treatment with cycloheximide, an inhibitor of protein synthesis. The reduction after actinomycin D treatment indicates that the messenger RNA is newly synthesized *in vitro*.

The augmentation of cytotoxin production when the MEM is supplemented with FCS or autologous plasma is unlikely to be due solely to endotoxin contamination as the effect could only be inhibited by about 50% by addition of quite large amounts of polymyxin (unpublished data): polymyxin is an antibiotic which 'neutralizes' endotoxin. It may be that endotoxin operates synergistically with a plasma component.

Using the same assay system, human monocytes release less cytotoxic factor than their rabbit counterparts and to satisfactorily measure the human factor it was necessary to increase the sensitivity of the assay by treating the target cells with actinomycin D. This ploy has proved valuable in *in vitro* assays of lymphotoxin (Reed & Lucas, 1975) and rabbit TNF (Ruff & Gifford, 1980).

The human monocyte factor reported here is unlikely to be arginase since firstly, its cytotoxic effect is not reduced by supplementing the culture medium with arginine (Currie, 1978) and secondly, the various cell lines tested here have a completely different spectrum of sensitivities to the monocyte cytotoxin and to beef liver arginase. The monocyte cytotoxin is distinguishable from C3a by physicochemical criteria and it is probably not a proteolytic enzyme as it is produced and assayed in the presence of large amounts of serum proteins. There has been a previous report of a human monocyte cytotoxin, the factor described by Reed & Lucas (1975) which, although not as well characterized in terms of physicochemical properties, has great similarities to the cytotoxin described here. There are also similarities between the human and rabbit monocyte cytotoxins. Their apparent molecular weights on gel filtration are similar, 34,000 for the human factor and 39,000 for the rabbit, and both give discrepant values when the molecular weight is determined by gradient PAGE, approximately 140,000 for the human and 67,000 for the rabbit (Matthews, 1978 and unpublished observations). Further, both substances are produced under similar experimental conditions and have overlapping but not identical specificities. The consistent discrepancy in molecular weight of the monocyte cytotoxins as determined by gel filtration or gradient PAGE is difficult to explain. One possibility is that the cytotoxins absorb to the gel matrices giving anomalous molecular weight values, low on gel-filtration and high on gradient PAGE.

We noted previously the probable identity of the rabbit monocyte cytotoxin and rabbit tumour necrosis factor, TNF (Matthews, 1978). TNF was first described by Carswell, Old, Kassel, Green, Fiore & Williamson (1975) as a substance found in the serum of animals injected with BCG and endotoxin; TNF was found to induce necrosis of certain tumours *in vivo* and be cytotoxic to certain tumour cell lines *in vitro*. Rabbit TNF, purified by monitoring for *in vitro* activity does appear to be active *in vivo* against the meth A sarcoma in mice (Ruff & Gifford, 1980; Matthews, unpublished results). It is possible that the human monocyte factor may be the human analogue of TNF but this will only be resolved when sufficient amounts of the cytotoxin become available for *in vivo* tests.

Recently, there has been great interest in tumour cell cytostasis or cytotoxicity induced by untreated, endotoxin or lymphokine-treated human monocytes (e.g. Hammerstrom, Unsgaard & Lamvik, 1979; Horwitz, Kight, Temple & Allison, 1979; Mantovani, Jerrels, Dean & Herberman, 1979). Could these effects be due largely to the release of the monocyte cytotoxin described here?

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