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Effect on human monocyte killing of tumour cells of antibody raised against an extracellular monocyte cytotoxin

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Summary. Human monocytes can kill or inhibit the growth of certain tumour cell lines. Amongst the possible mediators is a cytotoxin synthesized in readily detectable amounts by endotoxin-stimulated monocytes.

A neutralizing antiserum to the cytotoxin has been used to assess the cytotoxin's contribution to monocyte killing of tumour cells. The antiserum was tested for possible inhibition of monocyte killing of three tumour cell lines—L929, K562 and A549. Inhibition was complete with L929, partial with K562 and insignificant with A549. Thus the contribution of the cytotoxin to monocyte killing of tumour cells depends upon the tumour line under test.

Antibody against endotoxin-induced cytotoxin also neutralized cytotoxin induced in monocytes by other agents including BCG, *Corynebacterium parvum*, pokeweed mitogen and zymosan.

Cytotoxin could be quantitatively removed from monocyte supernatants by Sepharose-bound, anti-cytotoxin antibody. Recovery of the cytotoxin from the immunoadsorbent was difficult because of its lability in the solutions commonly used for desorption. The best recovery has been achieved with 2 M urea, 2 M $MgCl_2$ giving a 25% yield and a forty-fold increase in purity in a single step.

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INTRODUCTION

Macrophages can kill or inhibit the growth *in vitro* of certain tumour cell lines. The mechanisms involved are as yet ill-defined but several macrophage products with anti-tumour properties have been described including arginase (Currie, 1978), the C3a complement component (Ferluga, Schorlemmer, Baptista & Allison, 1978), cytolytic factor, a serine protease (Adams, Kao, Farb & Pizzo, 1980), H_2O_2 (Nathan, Brukner, Silverstein & Cohn, 1979) and tumour necrosis factor (TNF) (Männel, Moore & Mergenhagen, 1980; Matthews, 1978, 1981a). The contribution of each of the macromolecular mediators to the total anti-tumour effect of the macrophage is unclear, but could be resolved if neutralizing antisera to the mediators were available.

Macrophage anti-tumour mediators have been studied mainly in animal systems but recently we described the production of an anti-tumour cytotoxin by human monocytes. This cytotoxin is newly synthesized by stimulated monocytes, macrophages or myelomonocytic leukaemic cells, it is specific for certain tumour cell lines, has a molecular weight of 34,000 on gel filtration and slow electrophoretic mobility (Matthews, 1981b). Of the mediators described above, the cytotoxin most closely resemble rabbit TNF. A number of agents can stimulate human monocytes to produce the cytotoxin including endotoxin, zymosan, BCG, *Cornyebacterium parvum* and pokeweed mitogen (Matthews, 1982).

This paper describes the raising of an antiserum to

the cytotoxin and the application of the antiserum to (i) purification of the cytotoxin by immunoadsorption and (ii) assessing the contribution of the anti-tumour cytotoxin to the total anti-tumour effect of the monocyte in three experimental models.

MATERIALS AND METHODS

Cytotoxin production

Monocytes were prepared from either the heparinized blood of laboratory staff or from fresh buffy coats from blood donors. In each case the mononuclear cells were isolated using hypaque ficoll, washed twice and suspended at 5×10^6/ml in Eagles minimum essential medium supplemented with 10% foetal calf serum (MEM/FCS). The cell suspension was added to plastic Petri dishes (1 ml/5 cm^2 surface area) and incubated for $1\frac{1}{2}$ 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 monocyte-enriched (>80%) adherent cells were supplemented with the original volume of MEM/FCS containing 10 μg/ml endotoxin (lipopolysaccharide B from *E. coli* 026-B6, Difco). After further incubation overnight the cytotoxin-containing supernatant was collected, centrifuged to remove cells and stored in small portions at −70°.

In experiments using blood from myelomonocytic leukaemic patients the mononuclear cell preparation was used without further purification by adherence.

Cytotoxin assay

The mouse L929 tumour cell line was used as the target cell. Seventy-five microlitre amounts of target cell suspension (3×10^5/ml in MEM/FCS) 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/FCS with 2 μg/ml actinomycin D (Sigma) were added to give three or four replicates/dilution. After overnight incubation at 37° the supernatant containing the dead cells was discarded and the adherent viable cells were fixed for 5 min with 5% formaldehyde and stained with crystal violet. After drying, 100 μl 33% acetic acid was added to each well to dissolve and evenly distribute the dye. The amount of dye bound is proportional to the number of viable cells and was quantified photometrically using a Titertek Multiskan photometer. Reproducibility was within the range 5% to 10%. The percentage cytotoxicity was calculated for each supernatant dilution from the formula $100\ (a-b)/(a-c)$ where a, b and c are the absorbance of wells with respectively L929 cells + medium, L929 cells + monocyte supernatant and no cells. The titre (defined as dilution causing 50% cytotoxicity) was then calculated from the graph of cytotoxicity *v.* $\log_{10}$ dilution using the least squares method with the aid of a programmable calculator. The presence of actinomycin D in the medium expedites and increases the sensitivity of the assay (Matthews, 1981b).

Partial purification of the cytotoxin

Cytotoxin preparations for immunization were partially purified by either gradient polyacrylamide electrophoresis (PAGE) or ion-exchange chromatography. Using PAGE, after electrophoresis the gel was chopped into thirty fractions, proteins were eluted as described (Matthews, 1981b) and active fractions were pooled.

For purification by ion-exchange chromatography monocyte supernatants were dialysed against 0·01 M phosphate buffer pH 5·8 with sufficient NaCl added to give a conductivity of 2·6 mS. Typically, 125 ml supernatant was applied at a flow rate of 60 ml/hr to a CM-Sepharose column (9·0 × 0·9 cm) equilibrated in the dialysis buffer. The unbound material containing more than 90% of the protein was discarded and the partially purified and concentrated cytotoxin was subsequently eluted with 0·2 M phosphate buffer pH 7·3 containing 20% glycerol at a flow rate of 20 ml/hr.

Antiserum production

A 2·5 kg New Zealand White rabbit was immunized according to the schedule shown in Table 1. For the first seven injections the cytotoxin was prepared by PAGE from the supernatants of endotoxin-stimulated myelomonocytic leukaemia cells. For subsequent injections the cytotoxin was purified by ion-exchange chromatography from supernatants of endotoxin stimulated normal monocytes (from buffy coats). Test bleedings were taken 1 week after injections. The antiserum used in this paper was from bleeds after the injections at 15, 16 and 17 months. The separated serum was adsorbed twice with insolubilized human serum and twice with a mixture of washed, packed human blood cells (group A and B) and mouse erythrocytes. The antibody-rich fraction was isolated by precipitation with 18% w/v Na_2SO_4, extensively dialysed against isotonic phosphate-buffered saline (PBS) and frozen in small portions at −70°. Serum

from an unimmunized rabbit was similarly treated and was employed as a control.

Anti-cytotoxin immunoadsorbent

Fifty milligrams of the immunoglobulin fraction of the anti-cytotoxin serum were dialysed overnight against 0·2 M $NaHCO_3$ and then coupled to 2·5 ml CNBr-activated Sepharose 4B (Pharmacia) by incubation for 2 hr at room temperature. The gel was incubated for a further 2 hr with 1 M ethanolamine, washed five times sequentially with acid and alkali and then equilibrated with PBS.

Usually, 20–50 ml crude supernatant was applied to a 3 ml immunoabsorbent column at a flow rate of 15–25 ml/hr at room temperature. The column was washed with PBS and then eluted in the opposite direction with a solution of 2 M $MgCl_2$, 2 M urea (adjusted to pH 7 with Tris). Protein concentrations were estimated by the method of Read & Northcote (1981).

Monocyte anti-tumor cytotoxicity

Monocyte enriched preparations in 96-well microtitre trays (Sterilin M29 ARTL) were prepared by incubation of 75 μl suspensions of blood mononuclear cells (hypaque ficoll-purified) for $1\frac{1}{2}$ hr at 37°. The adherent monocytes were washed three times with warm medium to remove non-adherent cells. Medium was completely aspirated from the wells and 150 μl of tumour cell suspension ($2{\cdot}5 \times 10^4$/ml) was added with 10 μl antiserum or medium as appropriate. Antiserum dilutions are expressed as the final dilution/160 μl culture volume and all tests were performed with four replicates. The plates were incubated for 3 days at 37°. The three tumour cell lines used were mouse L929, human leukaemic K562 cells, and A549 from a human lung adenocarcinoma. In experiments with the plastic adherent cells (L929 and A549), after 3 days incubation with monocytes, the plates were processed and the results calculated as for the cytotoxin assay. Dye uptake by the monocytes in the wells was ignored as it was $< 10\%$ of the uptake of the target cells.

When investigating the effect of antiserum on monocyte cytotoxicity, percentage cytotoxicity was calculated using the formula, $100\ (a-b)/(a-c)$, with a, b and c representing respectively the mean absorbance of wells with tumour cells and monocytes + antiserum dilution, tumour cells + antiserum dilution, and no cells. With non-adherent K562 cells as targets, after 3 days incubation an estimate of the cell numbers in each well was made by counting the number of cells/high power field. The percentage cytotoxicity was calculated as above with cell number/high power field substituting for absorbance values.

The effector cell in these assays is probably the monocyte on the bases of adherence to plastic or cotton wool and the capacity to phagocytose iron carbonyl. With K562 target cells, non-adherent cells (NK cells?) also had cytotoxic potential. However, in 4 hr ^{51}Cr release assays to detect NK activity, the monocyte-enriched preparations had minimal activity.

Monocyte killing of candida

The method was essentially that of Leijh, Van Den Barselaar & Van Furth (1977) except that a final culture volume of 160 μl was employed.

Lymphocyte stimulation with phytohaemagglutinin

Suspensions (75 μl) of blood mononuclear cells at 5×10^6/ml in MEM/FCS were incubated for 3 days with an equal volume of a 1/250 dilution of phytohaemagglutinin (Reagent grade, Wellcome) plus 10 μl antiserum. [^{3}H]-thymidine (0·25 μCi/well) was added for the last 24 hr of culture and the cells were collected for counting using an automatic sample harvester.

RESULTS

Antiserum to the monocyte cytotoxin

A rabbit was given multiple injections of endotoxin-induced cytotoxin over a period of 17 months (Table 1). Neutralizing antibody of sufficient potency was not produced until at least 6 months after commencement of the schedule. The antibody used in the studies described here was the IgG-enriched fraction prepared from bleeds taken at 15–17 months. The capacity of the antibody to neutralize endotoxin-induced cytotoxin is shown in Fig. 1. Neither normal rabbit serum nor a hyperimmune rabbit antiserum to whole human serum neutralized the cytotoxin (Fig. 1).

Agents other than endotoxin can also stimulate cytotoxin production by human monocytes and Table 2 shows that the antiserum raised against endotoxin-induced cytotoxin also neutralized cytotoxin induced by pokeweed mitogen, zymosan, *C. parvum* and BCG.

Immunoadsorbent

Preliminary experiments showed that the cytotoxin was a labile, trace component of monocyte supernatants. Purification using standard protein chemical

Table 1. Immunization schedule

Months after initial injection	Route*	Adjuvant†	Antiserum titre‡
0	i.d.	C	
1	i.d.	"	
2	i.m.	I	4
3	i.m.	"	4
4	i.m.	"	
8	i.m.	None	4
9	i.m.	"	
10	i.m.	"	
15	i.m.	"	128
16	i.m.	"	512
17	i.m.	"	64

*i.d., Intradermal, i.m., intramuscular.

†C, Freund's complete adjuvant, I, Freund's incomplete adjuvant.

‡Antiserum dilution required to neutralize 50% of the activity of a standard preparation of monocyte cytotoxin.

For the first seven injections, 500–1000 units of cytotoxin were given and for subsequent injections 5000–12,000 units.

techniques gave low yields even with minimum separation times and the reduction of non-specific losses by the use of plastic columns and tubes and the inclusion of 20% glycerol in buffers. Accordingly, an immunoadsorption technique was investigated as an alternative purification procedure.

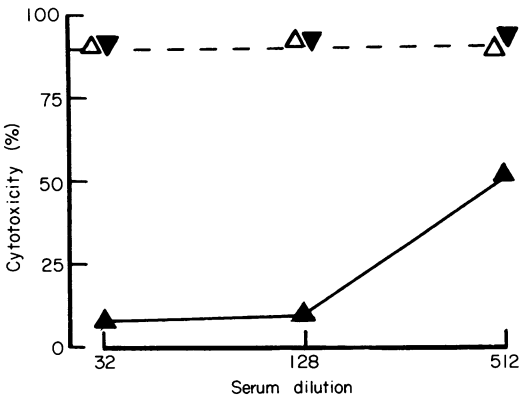

Figure 1. Effect on monocyte cytotoxin killing of L929 cells of rabbit anti-cytotoxin serum (▲), normal rabbit serum (△) or polyvalent rabbit antiserum to human serum proteins (▼). The dashed line represents the cytotoxicity (%) in the absence of serum. Equal volumes of the cytotoxin (1/20) and serum dilution were incubated for 2 hr at 37° before addition to the L929 cells.

An immunoadsorbent, made by coupling anti-cytotoxin antibody to CNBr-activated agarose, was effective at removing the cytotoxin from monocyte supernatants (Table 3). Typically, a 3 ml column of immunoadsorbent could remove >95% of the cytotoxin activity from 20–50 ml of monocyte supernatant. However, desorption of the cytotoxin proved more difficult as the cytotoxin was inactivated by many of the agents commonly used for desorption namely 0·05 M glycine, HCl pH 2·8; 3·5 M KCNS; 4–8 M urea or 2·5 M KI but not by 2 M $MgCl_2$ or 2 M urea. Of the various agents tested, a solution of 2 M urea, 2 M $MgCl_2$ was the most effective in desorbing the cytotoxin from the immunoadsorbent giving a yield of 25% and forty-four-fold purification in one step (Table 3). It is unlikely that the immunoadsorbent acts by a non-specific mechanism as cytotoxin did not bind to a similar column prepared with rabbit antibody to foetal calf serum proteins.

Immunoadsorbent-purified cytotoxin was eluted on gradient PAGE with an apparent mol. wt of 150,000, similar to crude cytotoxin under these conditions (Matthews, 1981b). When large volumes of cytotoxin were applied to the gel several protein-staining bands were detected. The major bands were albumin and α_2 macroglobulin (from the foetal calf serum in the culture medium) but in the region of the gel corresponding to the cytotoxin 3 faint, closely-spaced bands were evident. Further work will be needed to show whether or not one or more of these bands represents the cytotoxin.

Inhibition of monocyte killing of tumour cells by anti-cytotoxin antibody

An estimate was made of the cytotoxin's contribution to the total anti-tumour effect of the monocyte by measuring the extent to which the anticytotoxin serum inhibited monocyte killing of the three tumour cell lines L929, K562 and A549. With L929 and K562 both cytotoxicity and cytostasis is seen but with A549 cells only cytostasis is observed. For the sake of simplicity the term cytotoxicity will be used subsequently. For each of the tumour cell lines percentage cytotoxicity increased with increasing monocyte: tumour cell ratios up to a plateau and in testing the effect of anti-cytotoxin serum the lowest ratio giving maximum cytotoxicity was chosen.

Figure 2 shows that the antiserum markedly inhibits monocyte cytotoxicity against L929 cells. This was repeatedly seen and in some experiments there was complete inhibition of cytotoxicity even with anti-

Table 2. Neutralization by antibody against endotoxin-induced cytotoxin of monocyte cytotoxin induced by other agents

		Cytotoxicity (%) by supernatants* induced by				
Serum	Dilution	Endotoxin	Pokeweed mitogen	Zymosan	*C. parvum*	BCG
Medium	—	90	91	92	94	91
Anti-cytotoxin serum	32	9	−1	3	6	3
	128	11	2	−2	1	9
	512	53	48	56	78	49
Control serum	32	92	91	92	93	90
	128	93	91	92	93	89
	512	91	90	92	95	93

*Supernatants were from human monocytes incubated for 16 hr with 10 μg/ml endotoxin, pokeweed mitogen, zymosan or *C. parvum* or with BCG suspension (1 vial Glaxo percutaneous/10 ml medium). The supernatants had cytotoxin titres of approximately 100 u./ml. Equal volumes of supernatant dilution (1/10) and antiserum were incubated for 2 hr at 37° before addition to L929 target cells.

Table 3. Immunoadsorbent purification of the cytotoxin

		Protein		Cytotoxin activity		
Fraction	Volume (ml)	Conc. (mg/ml)	Total (mg)	Conc. (u./ml)	Total (u.)	Specific activity (u./mg)
Original*	20	1·47	29·4	3019	60,380	2054
Unbound	22	1·43	31·5	2	44	1·4
Bound, eluted with $MgCl_2$, urea	5	0·03	0·154	2800	14,000	90,900

*Supernatant from endotoxin treated monocytes separated on a 3 ml immunoadsorbent column at a flow rate of 15 ml/hr.

serum dilutions of 1/64. With K562 targets the antiserum had less effect on monocyte killing (Fig. 2) giving consistent but less than complete inhibition even at the highest concentrations of antiserum tested. The antiserum had no effect with A549 as the target cell. These results indicate a major role for the cytotoxin in monocyte killing of L929 cells, a lesser role against K562 cells and no contribution to growth inhibition of A549 cells. This is consistent with the relative susceptibility of the cell lines to endotoxin-induced monocyte supernatants—L929 are the most susceptible K562 less so and A549 are resistant.

It may be that the anti-cytotoxin antibody does not inhibit monocyte killing of tumour cells by neutralizing the cytotoxin but by other mechanisms, e.g. a direct toxic effect on monocytes or inhibition of monocyte function by immune complexes in the antibody preparation. This is unlikely because: (i) monocyte killing of L929 cells was inhibited to less than 10% by either immune complexes of foetal calf serum proteins and rabbit antibody to foetal calf serum or heat-induced aggregates of normal rabbit IgG; (ii) the anti-cytotoxin antibody did not prevent monocytes phagocytosing and killing candida. In the presence of medium or 1/16 dilutions of anticytotoxin or control antibody the percentage candida killed by monocytes were 85%, 86% and 80%, respectively; (iii) the proliferative response of mononuclear cells to phytohaemagglutinin was not reduced by the anti-cytotoxin serum. The responding cells are predo-

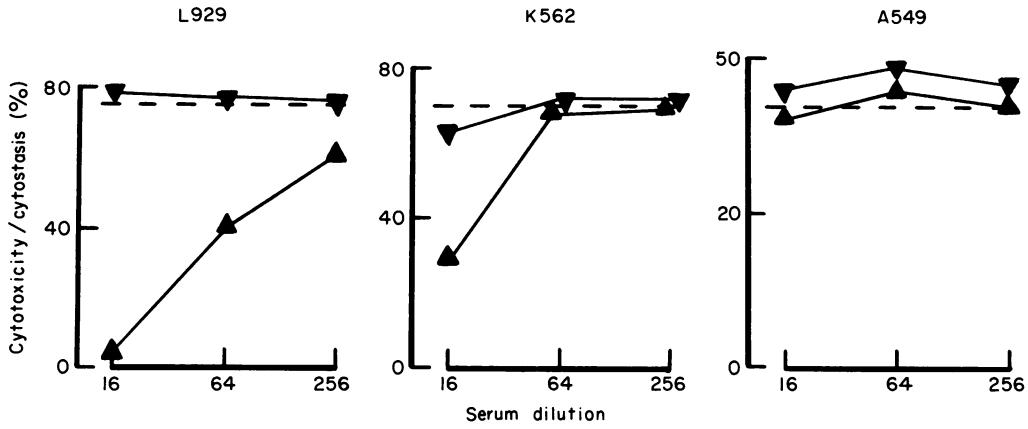

Figure 2. Effect of anti-cytotoxin (▲) or normal rabbit serum (▼) on monocyte cytotoxicity/cytostasis *v.* L929, K562 or A549 cells. The dashed line represents the percentage cytotoxicity/cytostasis in the absence of serum.

minantly T lymphocytes but monocytes are necessary in a helper capacity. The stimulation increments for cultures with medium or 1/16 dilutions of anti-cytotoxin or control antibody were respectively 18,109 ± 921, 27,850 ± 2540 and 33,140 ± 2859 c.p.m.; (iv) the failure of the anti-cytotoxin antibody to prevent monocytes killing A549 cells also argues against it being non-specifically toxic to monocytes.

DISCUSSION

The monocyte cytotoxin is a trace component of monocyte supernatants and this may explain why multiple injections of the cytotoxin over a period of 15 months were necessary to raise a useable antiserum. The antiserum had additional reactivity with certain foetal calf and human serum proteins which could be removed by adsorption with insolubilized human and foetal calf serum without loss of anti-cytotoxin activity. This suggests that the cytotoxin is not a normal component of human serum and this is supported by the observation that a hyperimmune antiserum to human serum proteins failed to neutralize the cytotoxin.

As well as endotoxin, other agents can induce monocytes to synthesize a cytotoxin. Because of similarities in physicochemical characteristics and dose–response curves it was concluded that it was the same monocyte cytotoxin induced by endotoxin, BCG, *C. parvum*, zymosan, pokeweed mitogen, lymphokines and phorbol myristate acetate (Matthews, 1982). This conclusion is strengthened by the observation that cytotoxin induced by the different agents could be neutralized by antiserum raised against endotoxin-induced cytotoxin.

An immunoadsorbent made by coupling the anti-cytotoxin serum to agarose was effective in removing the cytotoxin from crude monocyte supernatants. However, desorption from the immunoadsorbent has proved more difficult because of inactivation of the cytotoxin by the solutions commonly used for desorption. Of the desorbents tested, a solution of 2 M urea, 2 M $MgCl_2$ gave best results with cytotoxin recoveries in the range 20% to 25% and a forty-fold increase in purity in a single step. Other methods of desorption will be tried in the hope of improving recoveries.

The antiserum was raised against a crude fraction and despite removal of contaminating antibodies to serum proteins is unlikely to be cytotoxin-specific. Two approaches are being used to produce more specific antisera. Firstly rabbits are being immunized with more purified cytotoxin preparations prepared by a combination of physicochemical and immunoadsorbent techniques. Secondly, attempts are being made to produce a mouse monoclonal antibody to the cytotoxin. As yet this has been unsuccessful since the cytotoxin seems to be even less immunogenic in mice than rabbits.

Anti-cytotoxin serum had no effect on two monocyte functions, namely, killing of candida and monocyte help in mitogen-induced lymphoproliferation, but it did affect monocyte killing of tumour cells. The most pronounced effect was noted with L929 target

cells, a lesser effect with K562 and no effect with A549 target cells. This suggests that the cytotoxin contribution to monocyte killing of tumour cells depends upon the particular tumour cell line under test. In the mouse, Männel, Falk & Meltzer (1981) have shown that an antiserum against a mouse macrophage cytotoxin partially inhibits macrophage killing of the syngenic 1023 tumour cell line. Comparable data from many more macrophage/tumour combinations will be required before any conclusions can be made about the relative importance of the different macrophage mediators.

Care was taken in the assays described here to select culture media that themselves did not induce cytotoxin production and therefore the cytotoxin inducer in monocyte/tumour cell mixtures may well be a tumour cell product as is the case with cytolytic factor in the mouse (Johnson, Whisnant & Adams, 1981). Thus tumour cell resistance to monocytes may be expressed at two levels. Firstly, failure of the monocyte to produce one or more mediators on interaction with the tumour cell and secondly, intrinsic resistance of the tumour cell to the mediators.

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