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Cytotoxicity on tumour cells of human mononuclear phagocytes: defective tumoricidal capacity of alveolar macrophages

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SUMMARY

Human cells of the monocyte–macrophage lineage were isolated by adherence from peripheral blood, peritoneal exudate, non-neoplastic ascites, benign ovarian cystic fluid and bronchoalveolar lavages. Cytolytic activity was measured as ^{3}H-thymidine release from prelabelled mKSA TU5 tumour cells over 48–72 hr and cytostasis was evaluated in a 72-hr spectrophotometric assay. Mononuclear phagocytes from the various anatomical sites examined, except lung alveolar spaces, were significantly cytolytic and cytostatic on target cells. Unlike other cells of the monocyte–macrophage lineage, alveolar macrophages were not cytocidal, but significantly inhibited tumour cell proliferative capacity. Peripheral blood monocytes and peritoneal macrophages showed enhanced cytotoxicity in the presence of partially purified human fibroblast interferon or of lymphokine supernatants from mitogen-stimulated lymphocytes. In contrast, interferon did not affect the cytotoxic potential of alveolar macrophages, whereas lymphokines augmented their cytostatic activity and rendered them weakly cytolytic.

INTRODUCTION

Human peripheral blood monocytes have significant spontaneous cytolytic and cytostatic activity on tumour cells, different target cells being heterogeneous in their susceptibility to natural monocyte-mediated cytotoxicity (Mantovani *et al.*, 1979a, b, c; Horwitz *et al.*, 1979). Monocytes express preferential cytocidal activity against transformed versus untransformed early-passage human fibroblasts, thus suggesting that they may represent one line of natural defence against human neoplasia (Mantovani *et al.*, 1979c). Natural cytotoxicity on tumour cells is not peculiar to circulating mononuclear phagocytes, as peritoneal macrophages, milk macrophages and *in vitro*-matured monocyte-derived macrophages have spontaneous tumoricidal activity (Mantovani *et al.*, 1980a, b, c; Balkwill & Hogg, 1979). *In vitro* exposure to interferon or lymphokine supernatants from stimulated lymphocytes markedly increases the cytotoxicity of monocytes and macrophages (Mantovani *et al.*, 1980a, b, c; Jett, Mantovani & Herberman, 1980).

Pulmonary alveolar macrophages share many properties with mononuclear phagocytes from other tissues, but have distinctive structural, metabolic and functional characteristics (reviewed by Cohen & Cline, 1971; Golde, 1976). The present study was designed to elucidate the cytotoxic activity on tumour cells of macrophages residing in bronchoalveolar spaces. Evidence will be

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presented that alveolar macrophages differ from mononuclear phagocytes from other tissues in terms of natural cytotoxic activity and responsiveness to activating stimuli.

MATERIALS AND METHODS

Monocytes. Monocytes were isolated from the peripheral blood of adult volunteers (seventeen patients with non-malignant, non-infectious pulmonary diseases) by adherence on microexudate-coated plastic and ethylenediamine tetracetic acid (EDTA) as recently described, with minor modifications (Mantovani, 1980; Mantovani *et al.*, 1979a, 1980a, b). Briefly, mononuclear cells ($1–2 \times 10^6$/ml) separated from peripheral blood were incubated for 45 min on microexudate-coated plastic petri dishes (3003, Falcon, Oxnard, California) in 10 ml RPMI 1640 medium with 20% foetal bovine serum (FBS, GIBCO-BIOCULT, Glasgow, Scotland or aseptically collected FBS, Microbiological Associates, Walkersville, Maryland). Firmly adherent monocytes were recovered by exposure for 10 min to 1 mM EDTA in phosphate-buffered saline. More than 90% of the adherent cells belonged to the monocyte–macrophage lineage as assessed by morphology, avid uptake of neutral red, phagocytosis of latex particles, binding and phagocytosis of antibody-coated erythrocytes and staining for non-specific esterase. Recovery of monocytes was 5–7% of the mononuclear cells seeded on microexudate-coated plastic (Mantovani, 1980; Mantovani *et al.*, 1979a, 1980a).

Peritoneal macrophages. Peritoneal exudate cells were obtained from ten female patients undergoing abdominal surgery for non-malignant gynaecological diseases (ovarian cysts, myomas, etc.) as recently described (Mantovani *et al.*, 1980a). Briefly, peritoneal exudates were collected 24–72 hr after surgery through a silastic drainage tube routinely left in place by the surgeons, after irrigation with 500 ml of saline solution. In addition, ascitic fluid was obtained from one female patient suspected of having gynaecological malignancy and later found to have cirrhosis of the liver. Macrophages were isolated from these exudates by adherence on conditioned plastic, following the procedure outlined above for monocytes, except that serum-free Eagle's basal medium (EBM) was employed for adherence. More than 90% of the adherent cells were mononuclear phagocytes as assessed by morphology, rapid adherence and spreading on plastic in serum-free medium, phagocytosis of latex particles and binding and phagocytosis of antibody-coated sheep erythrocytes. The same procedure was employed to isolate macrophages from the fluid of a benign ovarian cyst.

Alveolar pulmonary macrophages. Alveolar cells were obtained from bronchial washing of fifteen non-smoker patients undergoing diagnostic bronchoscopy as described by Cantrell *et al.* (1973). Only the results of the fifteen patients who were later found not to have infectious or neoplastic diseases are presented here. The patients were anaesthetized by inhaling a mist of 4% lidocaine hydrochloride, and an optical fibre bronchoscope was passed transnasally into a dependent segmental bronchus. With the patient in an upright position, 250 ml of sterile normal saline was instilled in 50-ml portions and recovered by suction. The lavage suspension, which contained surfactant and pulmonary cells (80–97% of which were alveolar macrophages according to the morphological and functional criteria outlined above for peritoneal macrophages), was centrifuged at 180 *g* for 15 min at 4°C. Cells were washed twice in EBM.

Alveolar macrophages were purified by adherence on plastic for 2 hr as described above.

Stimuli. Lymphokine supernatants were obtained from lymphocyte cultures stimulated with phytohaemagglutinin (PHA) as recently described (Mantovani *et al.*, 1980b, c). Briefly, peripheral blood mononuclear cells deprived of firmly adherent monocytes as described above, were cultured in 7–10 ml RPMI 1640 medium with 10% FBS ($2–5 \times 10^6$ cells/ml) in the presence of 10 μg/ml PHA (HA17, Wellcome, Beckenham, England). After 20 hr at 37°C, cells were washed three times with 50 ml of medium and further cultured for 24 hr. Control conditioned medium was obtained from lymphocytes cultured alone. The supernatants were filtered through a 0·45-μm filter (Millipore Corporation, Bedford, Massachusetts) and assayed immediately or stored at −20°C until used. Partially purified human fibroblast interferon (lot 45-10-9) was obtained from HEM, Rockville, Maryland, USA.

Target cells. The murine SV40-transformed mKSA-TU5 (TU5) kidney line (Kit, Kurimura & Dubbs, 1969) was maintained *in vitro* in RPMI 1640 medium with 20% FBS. Non-confluent

cultures were labelled by overnight exposure to [methyl-^{3}H]thymidine (6 Ci/mmol; Radiochemical Centre, Amersham, England) as recently described (Mantovani *et al.*, 1979a).

Cytolysis assay. Cytolytic activity was measured as ^{3}H-thymidine release from prelabelled target cells over a period of 48 hr, unless otherwise specified (Mantovani *et al.*, 1979a, c, 1980a). Briefly, tumour cells (10^4) were incubated in 0·3 ml RPMI 1640 medium supplemented with 10% FBS in 6·4-mm flat-bottomed tissue culture wells (3596, Costar, Cambridge, Massachusetts) using a range of attacker to target cell (A:T) ratios (from 5:1 to 40:1). Tumour cell growth was checked daily under an inverted microscope. Percentage isotope release was calculated as $100 \times A/B$, where A is the isotope release in the supernatant and B is the total radioactivity released by incubating target cells in 1% sodium dodecyl sulphate in water. Specific release was determined by subtracting spontaneous release in the absence of effector cells, which never exceeded 25% of total incorporated radioactivity. The mean total incorporated radioactivity was 4,932 c.p.m. When the effect of interferon or lymphokines was tested, the stimuli were added to the wells together with the effector cells and were present throughout the assay. This procedure was adopted routinely as previous experiments (Mantovani *et al.*, 1980a, b, c) had shown it resulted in better stimulation of cytotoxicity than preincubation. Interferon and lymphokines alone did not affect the viability, spontaneous release and proliferative capacity of target cells (Mantovani *et al.*, 1980a, b, c).

Cytostasis assay. Macrophage-mediated cytostasis was evaluated by a spectrophotometric assay, essentially as recently described (Martin *et al.*, 1978; Mantovani *et al.*, 1980a). Briefly, after 72 hr of culture, the wells were fixed with 5% formalin and stained with 1% methylene blue in 0·01 M borate buffer, pH 8·5. After eluting the dye from cells with 0·1 N HCl, absorbance was read on a spectrophotometer. The percentage of growth inhibition was calculated as $[1-(A-B-C)/(D-C)] \times 100$, where A is the absorbance of cultures of tumour cells and mononuclear phagocytes, B is the value for mononuclear phagocytes alone, C is the absorbance of 10^4 target cells after adhesion for 2 hr, and D is the dye in wells containing tumour cells cultivated for 72 hr.

Statistical analysis. Results presented are mean (±s.d.) of three to six replicates per experimental group. Statistical significance was assessed by Student's *t*-test. Isotope release increases above 5% were usually statistically significant ($P < 0{\cdot}05$, Student's *t*-test) in the cytolysis assay. Growth inhibition > 10% was usually significant.

RESULTS

Table 1 shows that human alveolar macrophages consistently had no cytolytic activity against TU5 target cells. In contrast, mononuclear phagocytes from peripheral blood, peritoneal exudate,

Table 1. Natural cytolytic activity of human alveolar macrophages

Effector cells	Per cent specific lysis*			
	5:1†	10:1	20:1	40:1
Peripheral blood monocytes ($n=17$)	15·0 (3–28)	22·5 (11–35)	28·2 (13–45)	37·0 (17–46)
Peritoneal exudate macrophages ($n=10$)	8·5 (4–27)	11·3 (4–30)	23·9 (6–38)	28·4 (8–45)
Ascitic macrophages ($n=1$)	—	—	50·6	—
Benign ovarian cyst macrophages ($n=1$)	22·3	45·1	48·1	25·3
Alveolar macrophages ($n=15$)	0 (0–0)	0 (0–0)	0 (0–9)	0 (0–0)

* Median with range shown in parentheses.
† A:T ratio.

non-neoplastic ascites and ovarian cystic fluid, tested over the same period of time, were markedly tumoricidal, thus confirming previous data (Mantovani *et al.*, 1979a, b, 1980a, c). It should be noted that the lack of cytolysis of alveolar macrophages is not attributable to chance selection of anergic patients, as most of the peripheral blood data presented in Table 1 were in fact obtained with the same donors as bronchoalveolar lavages. Results presented in Table 1 were obtained after 48 hr of incubation, but a similar defective tumoricidal capacity of alveolar macrophages was observed after 72 hr of interaction with target cells (Fig. 1).

Although no significant cytocidal effect was detected with alveolar macrophages, daily microscopic observation of the wells revealed a clear-cut inhibition of tumour growth in the presence of these effector cells. The capacity of alveolar macrophages to inhibit tumour cell proliferative capacity was confirmed in a dye-uptake test, inhibition levels being comparable to those of peripheral blood monocytes and peritoneal macrophages (Table 2).

In agreement with previous observations (Mantovani *et al.*, 1980a, b, c; Jett *et al.*, 1980) interferon and lymphokines significantly augmented the tumoricidal activity of peripheral blood monocytes (Fig. 2). The same stimuli also enhanced the cytotoxicity of human peritoneal macrophages (Mantovani *et al.*, 1980a, b, c). Unlike other mononuclear phagocyte populations, alveolar macrophages did not show enhanced cytolysis or cytostasis in the presence of interferon (Fig. 2b and Table 3). However, lymphokine supernatants significantly stimulated the growth inhibitory activity of alveolar macrophages on tumour cells (Table 3) and rendered them weakly tumoricidal (Fig. 2b).

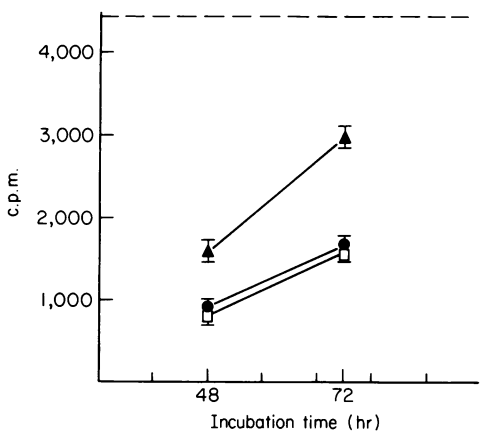

Fig. 1. Kinetics of target cell lysis by human alveolar macrophages (□) and blood monocytes. (▲). Results are presented as counts per minute (c.p.m.). The horizontal dotted line indicates total incorporated radioactivity and closed circles indicate spontaneous release in absence of effector cells. Results are expressed as mean ± s.d.

Table 2. Natural cytostatic activity of human alveolar macrophages

Effector cells	Per cent growth inhibition*			
	5:1†	10:1	20:1	40:1
Peripheral blood monocytes ($n=8$)	0 (0–25)	41 (12–54)	56 (35–78)	82 (68–87)
Peritoneal exudate macrophages ($n=1$)	23	38	77	83
Alveolar macrophages ($n=7$)	26	40	65 (50–89)	71 (40–81)

* Median with range shown in parentheses.
† A:T ratio.

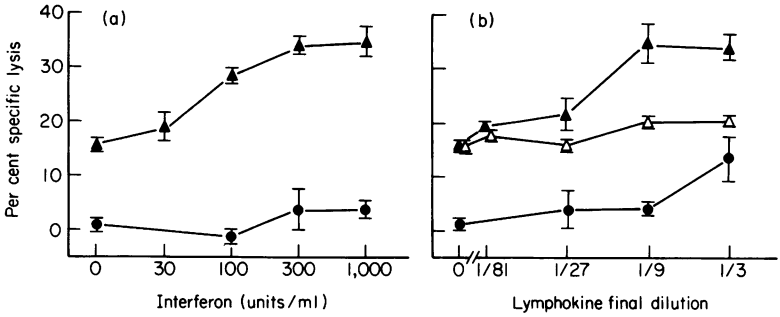

Fig. 2. Effect of interferon (a) and lymphokines (b) on the cytolytic activity of alveolar macrophages (circles) and blood monocytes (triangles). (b) Open and closed symbols indicate cytolysis with conditioned medium or lymphokine supernatant respectively.

Table 3. Effect of interferon and lymphokines on human alveolar macrophage cytotoxicity

Effector cells	Stimulus*	Per cent specific lysis†	Per cent growth inhibition†
Alveolar macrophages ($n=5$)	None	0 (0–0)	72 (40–81)
	Interferon	0 (0–4)	68 (38–71)
	Lymphokine supernatant	8‡ (6–13)	95‡ (90–97)
Peripheral blood monocytes ($n=1$)	None	5	n.t.§
	Interferon	20‡	n.t.
	Lymphokine supernatant	14‡	n.t.

* Lymphokine final dilution was 1/3 and interferon concentration was 1,000 units/ml. Control conditioned medium had no effect on cytolysis or cytostasis.

† Median with range shown in parentheses.

‡ Significantly above cytotoxicity of unstimulated alveolar macrophages, $P<0{\cdot}01$.

§ Not tested.

DISCUSSION

The results presented here confirm that in human cells of the monocyte–macrophage lineage from various anatomical sites (peripheral blood, peritoneal cavity, benign ovarian cystic fluid) appreciable levels of natural tumoricidal activity are found (Mantovani *et al.*, 1979a, b; 1980a, b, c; Horwitz *et al.*, 1979). Natural cytotoxicity of monocytes and macrophages was also reported in mice and rats (Meltzer, 1976; Miller & Feldman, 1976; Keller, 1978a, b; Tagliabue *et al.*, 1979). Unlike other mononuclear phagocyte populations, pulmonary alveolar macrophages expressed no spontaneous cytolytic activity on tumour cells, but consistently inhibited tumour cell proliferative capacity.

Exposure to interferon or lymphokines significantly augments the cytotoxic potential of human monocytes and macrophages (Mantovani *et al.*, 1980a, b, c; Jett *et al.*, 1980), an observation confirmed in the present study. In this respect too, alveolar macrophages differed from mononuclear phagocytes from other anatomical sites in that they were totally unresponsive to interferon. On the other hand, lymphokine supernatants stimulated the growth inhibitory capacity of alveolar macrophages, and rendered them weakly cytolytic.

Lymphokine supernatants from antigen (PPD) or mitogen (PHA) stimulated lymphocytes contain appreciable levels of interferon. In previous studies, in order to elucidate the role of interferon in macrophage activation by lymphokine supernatants, we took advantage of the different biological properties of supernatants elicited by *Corynebacterium parvum* strain CN6134 and strain CN5888 (Mantovani *et al.*, 1980b, c). Strain CN5888 supernatants, which had no interferon, were as stimulatory on monocyte cytotoxicity as CN6134 supernatants which had appreciable levels of interferon (Mantovani *et al.*, 1980b, c). The findings presented here show that, unlike peripheral blood monocytes and peritoneal macrophages, human alveolar macrophages do not show enhanced cytotoxicity when exposed to interferon, but they do respond significantly to lymphokines. Thus, data obtained with anaerobic coryneforms differing in their capacity to induce interferon production, and with alveolar macrophages showing defective natural cytotoxicity and defective responsiveness to interferon but capable of responding weakly to lymphokines, suggest the hypothesis that stimulation of human macrophage cytotoxicity by interferon and lymphokine supernatants involves two distinct biological pathways.

Different, though not mutually exclusive, explanations are possible for the defective tumoricidal capacity of alveolar macrophages. Phagocytosis of selected materials has been reported to inhibit the cytotoxicity of murine macrophages (Weinberg & Hibbs, 1977) and at least some of the mononuclear phagocytes residing in lung alveoli actively phagocytose air-borne particles. On the other hand, prostaglandins of the E series reportedly inhibit the activation of murine macrophage cytostasis by interferon (Schultz *et al.*, 1978), and human and guinea-pig alveolar macrophages produce larger amounts of prostaglandins than peripheral blood monocytes or peritoneal macrophages (Morley *et al.*, 1979). Thus, greater prostaglandin production might at least partially account for the defective tumoricidal capacity of human alveolar macrophages. Experiments to verify this hypothesis are currently under way.

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