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Inhibition of monocyte complement receptor enhancement by low molecular weight material from human lung cancers

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SUMMARY

We have studied the effect of dialysates from lung cancer homogenates to alter both the expression of complement (C3b) receptors *per se* and also to inhibit leucoattractant-induced enhancement of complement rosettes on monocytes from healthy individuals. Enhancement and enhancement-inhibition by tumour extracts were compared with material derived from normal lung excised some distance from the tumour. There was no significant difference between tumour homogenate (TH) and normal lung homogenate (NLH) in terms of enhancement of complement rosettes *per se*. In contrast, TH produced a dose- and time-dependent inhibition of leucoattractant-induced enhancement of C3b rosettes which was significantly different from that obtained with NLH. This enhancement-inhibition was observed with four undifferentiated, four squamous and three adenocarcinomas of lung. The degree of enhancement-inhibition was not related to the type of tumour or varying accompanying histological features such as necrosis and the degree of infiltration with inflammatory cells. Following gel filtration on Sephadex G-50 each type of cancer gave a major peak of inhibitory activity which eluted with molecules having an apparent molecular size of approximately 3,000 daltons. A second larger peak (8,000–10,000 daltons) was also detectable with extracts from the undifferentiated and adenocarcinomas. These results support previous findings, mainly from experimental animals, indicating that 'anti-macrophage/monocyte principles' are elaborated from certain tumour types.

INTRODUCTION

It is a widely-held view that cells of the lymphoid series, particularly mononuclear phagocytes, are principal effector cells in tumour immunity. Although the role of lymphoid cells in immunological surveillance of tumours in general, and of macrophages in particular, is controversial, it seems likely that some tumours, at some stage in their development, are susceptible to destruction by mononuclear phagocytes. Macrophages are prominent in syngeneic animal tumours (Evans & Alexander, 1972) and, in mice, can inhibit tumour growth when inoculated together with tumour cells (Bennett, 1965). Macrophages can also enhance the inhibition of tumour progression mediated by BCG (Hopper & Pimm, 1976).

A number of investigators have reported that tumour-derived material from experimentally-induced neoplasms inhibit macrophage function both *in vivo* and *in vitro*. In general, the inhibitory activity has been associated with molecules having a molecular size of less than 10,000 (Fauve *et al.*, 1974; North, Kirstein & Tuttle, 1976; Pike & Snyderman, 1976; Nelson & Nelson, 1978; Normann & Cornelius, 1978). As a result of these observations in animals and studies in man which indicate

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that monocyte locomotion is defective in certain solid tumours such as genito-urinary neoplasms and malignant melanoma (Boetcher & Leonard, 1974; Hausman *et al.*, 1975), we have attempted to establish whether human tumours elaborate 'anti-macrophage' principle(s) comparable to those previously described in mice and rats. We chose to study extracts from lung cancers since fresh material was readily available and because our previous work indicated that the advanced stage of this disease was also associated with a defect in the locomotion of peripheral blood monocytes (Kay & McVie, 1977).

Rather than employing inhibition of monocyte locomotion as our test system we have used a newly developed assay of monocyte function – complement receptor enhancement (Glass & Kay, 1980). 'Complement receptor enhancement' is the term we have given to increased expression (or unfolding) of C3b receptors on human leucocytes following incubation with leucoattractants. The phenomenon was first described with eosinophils and 'eosinophilotactic' agents such as histamine and ECF-A tetrapeptides (Anwar & Kay, 1977, 1978). However, we have also shown that complement receptor enhancement is a property of human neutrophils and monocytes following incubation of these cells with various chemoattractants such as formyl-methionyl peptides, lymphokines and casein (Glass & Kay, 1980; Kay, Glass & Salter, 1979). Thus complement receptor enhancement is induced by leucoattractants and occurs in parallel with cell locomotion. We believe that this apparent increase in the density of complement receptors may be indicative of a general biological phenomenon whereby the degree of adhesion of phagocytic cells to opsonized particles is increased.

In the present report we provide evidence that dialysates from a number of human lung cancer homogenates, when compared to dialysates from normal lung, inhibit casein-induced enhancement of monocyte complement receptors. Furthermore, the inhibitory principles appear to have a similar molecular size to material previously identified from animal tumours and which inhibited macrophage chemotaxis.

MATERIALS AND METHODS

Materials

Materials were obtained as follows: casein (BDH Chemicals Ltd, Poole, England); preservative-free heparin (Evans Medical, Liverpool, England); Ficoll and Hypaque, Sephadex G-50 (Pharmacia, Uppsala, Sweden).

Methods

Homogenates from lung cancer and normal lung controls. Fresh surgical specimens were obtained at thoracotomy and placed directly into sterile plastic bags. A portion of approximately 5 g was removed from the tumour mass in such a way as not to jeopardize the histological diagnosis. A portion of normal tissue of comparable size to the tumour was taken from the periphery of the lobe or bronchopulmonary segment. Portions of the normal lung, as well as the tumour mass, were prepared for routine histopathological sections. The unfixed tissue was either stored at $-80°C$ or prepared immediately in the following way: samples of tumour, or normal lung, were cut into small fragments of approximately 200–400 mg and placed in 5–15 ml of PBS. The materials were frozen and thawed six times, centrifuged at 1,800 ***g*** for 10 min to remove particulate matter and the supernatants dialysed against PBS (twice the volume) for 18 hr at 4°C. The protein concentration of the dialysates was determined by the Coomassie blue method and the samples divided into portions of approximately 1 ml and stored at $-80°C$ until use.

The various histological features of the tumours were assessed by two independent pathologists and graded (0, ±, +, ++, +++) as shown in Table 1. The portions of normal lung from the periphery of the sections were free of microscopically detectable tumour cells.

Preparation of EA, EAC1423b and 'EAC'. This has been described in detail elsewhere (Anwar & Kay, 1977, 1978) and was briefly as follows. Dextrose–gelatin–veronal buffer ($DGVB^{2+}$, pH 7·4) was used for washing sheep erythrocytes (E) during sensitization and coating with complement. The buffer was prepared by mixing equal volumes of isotonic veronal-buffered saline (containing 0·0015

M Ca^{2+}, 0·0005 M Mg^{2+} and 0·1% gelatin–veronal buffer, GVB^{2+}) with 5% dextrose in water containing the same concentration of Ca^{2+} and Mg^{2+}. The IgM and IgG fractions of rabbit antisera to sheep red cells (A) were prepared by Sephadex G-200 gel filtration (Shevach *et al.*, 1972). The IgG fraction was further purified by DEAE-52 anion exchange chromatography (Fahey & Terry, 1978). Either IgG or IgM was used in the sensitization of E for the preparations of EA_G^{rab} or EA_M^{rab} respectively. The antibody concentration of EA_M^{rab} selected was that which gave optimal sensitization for haemolysis but no agglutination. For the preparation of EA_G^{rab}, the antibody concentration was diluted so as to give approximately 30% monocyte rosettes. Functionally pure human complement components were added sequentially to EA_M^{rab} to prepare C3b-coated cells (EAC1423b). The amounts were as follows: 400 effective molecules of C1, 400 of C4, 50 of C2 and 400 of C3. This amount of C4 was insufficient to give EAC14 rosettes with monocytes (Anwar & Kay, 1978). In most experiments fresh human AB serum was also used as a source of complement ('EAC'). Equal volumes of EA_M^{rab} were used at a concentration of 1×10^8 red blood cells/ml. The dilution of fresh serum was adjusted to give between 20 and 30% monocyte rosettes and was usually between 1 in 600 and 1 in 1,000.

Monocytes. Blood from healthy donors was drawn into plastic tubes containing 10 units of preservative-free heparin/ml. Monocytes were separated on Ficoll–Hypaque cushions according to the method of Böyum (1968). The separated cells were washed twice in Dulbecco's PBS (modified) (pH 7·2–7·4), containing preservative-free heparin (2 units/ml) and resuspended in medium 199, pH 7·4, to a concentration of 1×10^6 monocytes/ml.

Complement (EAC) and IgG (EA_G) rosettes and rosette enhancement. For the experiments described in Table 2 equal volumes of monocytes (1×10^6/ml) and various concentrations of casein or medium alone were mixed and incubated in a shaking water bath, usually at 37°C for 30 min. The cells were then washed twice in medium 199 and the numbers readjusted to their original concentration in the same medium. A portion (0·1 ml) of EAC or EA_G (1×10^8/ml) was added to 0·1 ml of cells

Table 1. Histological features of undifferentiated, squamous and adenocarcinomas from human lung

	Accompanying histological features				
Patient	Necrosis	Viable tumour	Fibrous tissue	PMNs	Chronic inflammatory cells
Undifferentiated					
1. Large cell	+	++	+	0	±
2. Large cell	±	++	++	±	+
3. Small cell	+	++	++	0	±
4. Small cell	++	+	±	0	±
Squamous					
5. Moderately well differentiated	++	+	±	±	+
6. Poorly differentiated	±	++	+	±	+
7. Poorly differentiated	±	++	++	+	±
8. Poorly differentiated	++	++	+	++	+
Adenocarcinoma					
9. Well differentiated	±	++	±	++	+
10. Poorly differentiated	±	++	+	+	+
11. Moderately well differentiated	0	+++	++	0	0

0 = Nil; ± = very slight; + = slight; ++ = moderate; +++ = marked. The tumour type was assessed independently by two histopathologists.

Table 2. Enhancement of monocyte complement rosettes by casein

	Rosettes (%)		
	'EAC'	EA_M1423b	EA_G
Medium 199	25±4	23±1	30±3
Casein			
1 mg/ml	34±5	35±1	31±4
2 mg/ml	42±2	41±2	30±4
3 mg/ml	48±6	46±2	34±5

Red cell intermediates were sensitized with IgM and prepared either with whole serum ('EAC') as a source of complement or with purified components (EA_M1423b) or with IgG (EA_G). The results represent the mean ±1 s.e.m. of three experiments.

and centrifuged at 100 *g* for 10 min at 4°C and the pellets incubated at 37°C for 20 min for EAC and 0°C for 30 min in the preparation of EA_G. The pellet was gently resuspended in medium 199 containing 1% formal–saline and smears prepared on clean glass slides in duplicate. These were dried quickly in air, fixed in methanol and stained with May–Grünwald/Giemsa or fixed in 2·5% glutaraldehyde and stained using α-naphthyl acetate histochemistry according to the method of Yam, Li & Crosby (1971). Comparison of these two methods by four independent observers revealed no statistical significance in differentiating monocytes from lymphocytes.

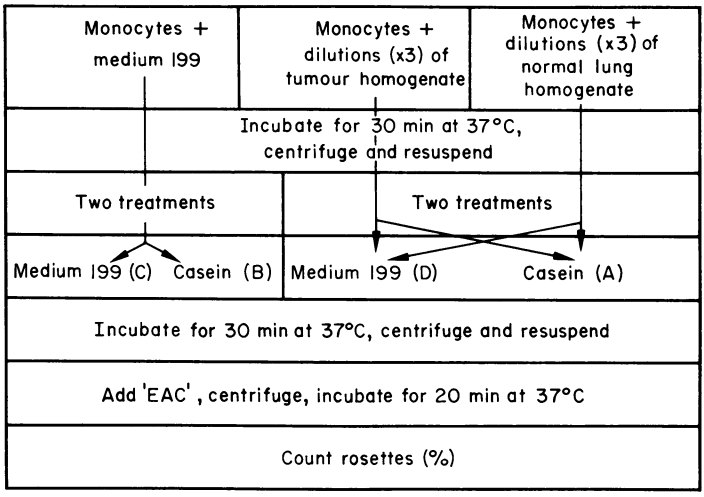
Inhibition of casein-induced enhancement by tumour or normal lung homogenates. Monocytes were prepared as described above and divided into 14 portions of 0·1 ml each containing 1×10^6 monocytes/ml. The experimental design is shown in Fig. 1. Two tubes were required for 'enhancement by casein alone' [(B − C/C) × 100] and this was performed as described for Table 2 (but with the addition of a 30-min incubation period at 37°C, after which the cells were centrifuged (300 *g* for 10 min at 4°C) prior to the addition of 0·1 ml of medium 199 (C) or casein (B) – Fig. 1). Monocyte suspensions were also incubated with dilutions of tumour extract (25 μg protein/ml – two tubes, 50 μg/ml – two tubes, 100 μg/ml – two tubes) and normal lung homogenates (25 μg protein/ml – two tubes, 50 μg/ml – two tubes, 100 μg/ml – two tubes). Three of the monocyte preparations treated with tumour extract (i.e. 25, 50 and 100 μg/ml) and three treated with normal lung (i.e. 25, 50 and 100 μg/ml) were then incubated with either medium 199 or casein (3 mg/ml). Enhancement, and enhancement-inhibition were calculated as described in Fig. 1.

Gel filtration. Samples (0·75 ml) of either tumour or normal lung extract were applied to a column of Sephadex G-50 (1·5 × 100 cm). The column was calibrated with molecular markers (blue dextran, cytochrome *c* and vitamin B_{12}) in phosphate-buffered saline (pH 7·2) and 1-ml fractions collected. Every third fraction was tested for inhibition of casein-induced monocyte complement receptor enhancement using 0·1-ml amounts as described in Fig. 1.

RESULTS

Enhancement of complement rosettes

The capacity of casein, a recognized monocyte leucoattractant, to enhance the percentage of monocyte C3b rosettes is shown in Table 2. The effect was dose-dependent and demonstrable when either 'EAC' or EA_M1423b were used as indicator erythrocytes. In contrast, there was a small but statistically insignificant increase in the percentage of EA_G (Fc) rosettes. When expressed as a percentage increase, enhancement of Fc receptors by the highest dose of casein was only 13%



$$\text{Enhancement by casein alone} = \frac{B-C}{C} \times 100$$

$$\text{Enhancement by tumour or normal lung alone} = \frac{D-C}{C} \times 100$$

$$\text{Inhibition of casein-induced enhancement by tumour or normal lung} = 100 - \frac{(A-C)}{(B-C)} \times 100$$

Fig. 1. Schematic outline of the methodology for monocyte complement receptor enhancement and enhancement inhibition by tumour and normal lung homogenates.

whereas with 'EAC' or EA_M1423b the increases were 92 and 100% respectively. Because of the similar results obtained using either 'EAC' or EA_M1423b in terms of the dose response by casein (and also, as reported elsewhere, as a function of time (Glass & Kay, 1980)) for convenience 'EAC' were used as indicator cells in further experiments.

Inhibition of enhancement

A dialysable extract from a homogenate of an adenocarcinoma of the bronchus was tested both for its effect on the percentage of monocyte complement rosettes and for its capacity to inhibit casein-induced monocyte complement receptor enhancement. The tumour extract, at concentrations of 25, 50 and 100 μg protein/ml, was compared with microscopically normal lung from the same resected specimen. Neither the tumour homogenate (TH) nor the normal lung homogenate (NLH) significantly influenced the percentage of rosettes with 'EAC'. In contrast, the TH inhibited casein-induced enhancement in a dose-dependent fashion which at the highest concentration (100 μg protein/ml) was significantly different from NLH ($P < 0{\cdot}05$).

The time course of inhibition enhancement by TH and NLH from another adenocarcinoma of the bronchus is shown in Fig. 2. The percentage increase was maximal at 30 min with no further inhibition being observed up to 2 hr at which time the experiment was terminated. Inhibition by NLH was considerably less and when expressed as the mean of three experiments was virtually negligible.

Various histological types

Undifferentiated. The effect of TH and NLH from four patients with undifferentiated carcinoma of the bronchus on monocyte complement rosettes and casein-induced enhancement is shown in Fig. 3. The percentage enhancement of the normal donor monocytes by casein was comparable to that achieved in the experiments described in Table 2. Both TH and NLH promoted some complement receptor enhancement *per se* although the differences between them, at the three concentrations tested, were not significant. Furthermore, the dose–response with NLH was 'flat'

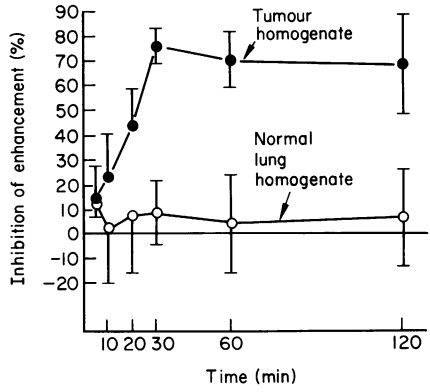


Fig. 2. The time course of inhibition of leucoattractant-induced enhancement of monocyte complement receptors by tumour and normal lung homogenates. Each point represents the mean ±1 s.e.m. of three experiments and the experimental design is shown in Fig. 1. The material was derived from an adenocarcinoma of lung.

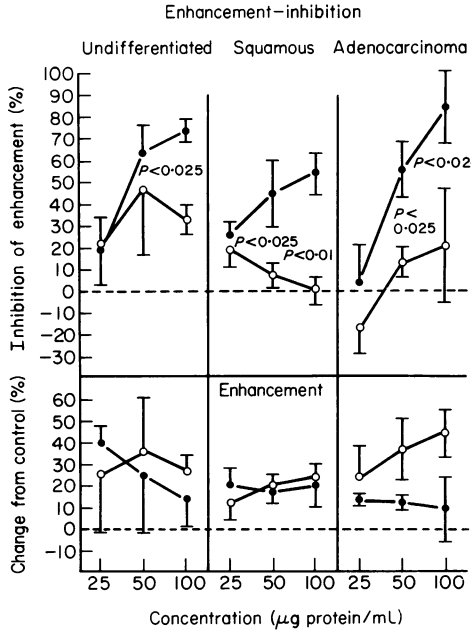


Fig. 3. Inhibition of leucoattractant-induced enhancement of monocyte complement rosettes by homogenates from various lung cancers. (•) Tumour and (○) normal lung homogenate. Each point represents the mean ±1 s.e.m. of four undifferentiated tumours, four squamous and three adenocarcinomas of lung. The *P* values were calculated from Student's t-test. The experimental design is outlined in Fig. 1.

and with TH there was apparent inhibition at higher doses. In contrast, TH when compared to NLH gave significant *inhibition* of casein-induced enhancement at the highest concentration tested. The inhibitory effect of TH increased in proportion to the concentration of TH. Some inhibition of enhancement was observed with NLH but the dose–response was not linear, less effect being observed at 100 μg than with 50 μg protein/ml. The individual data (not shown) indicate that although there was considerable variation in the amount of inhibition achieved by the four undifferentiated tumours, the general pattern of increased enhancement inhibition by TH, as compared to NLH, was sustained.

Squamous. Comparable results were obtained with four squamous carcinomas of the bronchus (Fig. 3). With these tumours the difference between TH and NLH in terms of their effects on receptors *per se* was negligible although with both materials there was a slight increased percentage change from control but this did not appear to be related to the concentration of the extracts. By contrast, *inhibition* of casein-induced enhancement by these tumour extracts increased with dose and, at both the 50 and 100 μg concentrations, was significantly different from NLH. NLH from the squamous tumour resections gave very little enhancement-inhibition, even at the higher concentrations.

Adenocarcinoma. Three adenocarcinomas of the bronchus were tested in a comparable manner (Fig. 3). The NLH from these resections gave a dose-dependent increase in monocyte complement rosettes *per se*, but the effects were not significantly different from TH. With enhancement-*inhibition* there was a marked and statistically significant difference between TH and NLH at the 50 and 100 μg protein concentrations. The individual results (not shown) indicate that the variations in observed effects were wide, but the general pattern of dose-dependent inhibition of casein-induced enhancement by TH, as compared to NLH, was sustained.

Gel filtration of homogenates

TH and NLH from one undifferentiated (patient 1), one squamous (patient 5) and one adenocarcinoma (patient 11) were passed separately through a column of Sephadex G-50, previously calibrated with molecular markers. Fractions were tested for inhibition of enhancement as described in the methodology (Fig. 4).

Virtually all the fractions tested, irrespective of whether they were TH- or NLH-derived, gave inhibition of enhancement although in some the values were below zero. Because of the high 'background' activity with the normal lung homogenates only those peaks having maximal enhancement-inhibition of 40% or more were considered as having appreciable biological activity. Using this 'cut-off' point it could be seen that with the undifferentiated tumour there were two peaks of activity of approximately 3,000 and 8,000 daltons respectively. The squamous tumour also had a

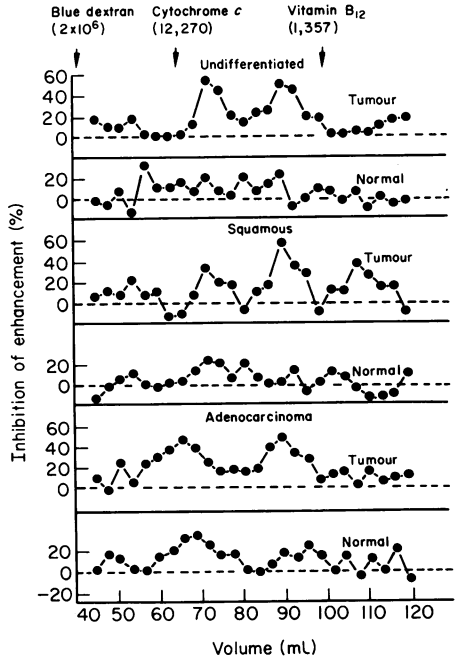


Fig. 4. Sephadex G-50 gel filtration of homogenates from lung cancer and normal lung. The points are the mean of three assays of inhibition of leucoattractant-induced enhancement.

peak at 3,000 and the adenocarcinoma had inhibitory activities at 3,000 and 10,000 daltons approximately. Thus all three tumour types had a peak molecular weight at 3,000 and the undifferentiated and adenocarcinoma had activities between 8,000–10,000 daltons. NLH from each tumour type did not give peaks of greater than 35% and in most measurements it was less than 20% enhancement-inhibition.

DISCUSSION

The main purpose of the experiments reported here was to determine whether factors derived from human lung cancer affected leucoattractant-mediated increase in complement receptor 'density' of blood monocytes from healthy individuals. Although we have provided evidence for the presence of such inhibitory principles we cannot say with certainty whether they are tumour-associated or tumour-derived. It is of note that there did not appear to be an association between the degree of inhibition and the histological features of the tumours (Table 1). For example, tumour nos. 3, 4 and 11 had little or no associated chronic inflammatory cells or infiltration with polymorphonuclear leucocytes but they all gave considerable inhibition of enhancement of complement receptors. Similarly, tumour nos. 2 and 11 had little or no necrosis but considerable inhibitory activity could be derived from the tumour mass. Thus the inhibitory properties of the tumour dialysate would not appear to be directly attributable to the degree of acute and chronic inflammation, necrosis or fibrosis associated with the neoplastic lesion.

We have so far only examined the effect of TH on casein-induced complement receptor enhancement on the blood monocyte and are unable to say whether a similar effect would be demonstrable (a) with other blood leucocytes such as neutrophils and eosinophils and (b) with other appropriate C-receptor-enhancing chemoattractants. Because of limitations in the supply of tumour extract we felt that priority should be given to preliminary studies on the possible heterogeneity of the activities as assessed by gel filtration chromatography. As shown in Fig. 4 each tumour contained at least two peaks of inhibitory activity although NLH did not give inhibition of $>40\%$. Taking 40% inhibition as a reasonable cut-off point it can be seen that the only substantial peaks of inhibitory activity are in the tumour extracts. All three tumour types shared one peak which eluted just before the vitamin B_{12} molecular marker and had an estimated molecular size of 3,000 daltons. The undifferentiated and adenocarcinoma tumour extracts both had a higher peak of inhibitory activity at approximately 8,000–10,000 daltons. The squamous tumour also had a peak at 8,000–10,000 daltons but maximal activity was just $>40\%$. This aspect of the study clearly requires expansion to include more detailed protein-separation techniques.

It is generally agreed that there is no clear association between clinical prognosis and the histological types of various forms of lung cancer. There was no evidence from the present study that the degree of inhibitory activity was related to the histological type.

There is formidable literature on the relation between macrophages and cancer and, as mentioned in the Introduction, there are a number of animal studies which support the view that tumours elaborate anti-macrophage material. Snyderman and his colleagues reported that mouse tumours inhibited macrophage mobilization in the peritoneal cavity and that a number of cell lines produce a factor of molecular weight between 6,000–10,000 daltons which inhibits macrophage chemotaxis *in vitro* (Pike & Snyderman, 1976; Snyderman & Pike, 1976). The agent was heat-stable and had peak inhibitory activity 3 days after administration. When the factor was injected together with tumour cells into syngeneic mice there was an increase in tumour dissemination when compared to the effect of the tumour alone. Fauve *et al.* (1974) have demonstrated that mouse teratocarcinoma and other tumour cells in culture elaborate a factor of between 1,000 and 10,000 which inhibits local inflammation *in vivo*, and these same cells *in vitro* were found to prevent direct contact by normal peritoneal macrophages. Nelson & Nelson (1978) have described a similar factor produced by murine fibrosarcoma cells which depressed delayed-type hypersensitivity reactions *in vivo* and inhibited macrophage chemotaxis *in vitro*. It is believed to be glycopeptide in nature and associated with an RNA fragment. North *et al.* (1976) reported that in the serum of tumour-bearing mice there was a dialysable factor which depressed macrophage-mediated resistance to infection.

In the present report we have described low molecular weight material derived from human lung cancers which inhibits leucoattractant-induced enhancement of complement receptors on normal human monocytes and in this respect may be analogous to factors described in animals which depress macrophage function. Further studies are required to characterize these principles more fully and to explore the possibility that active immunity to them may have therapeutic potential.

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