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PRODUCTION OF A MACROPHAGE CHEMOTACTIC FACTOR BY CULTURED JAAGSIEKTE TUMOUR CELLS

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ABSTRACT

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The increase of alveolar macrophages in jaagsiekte sheep lungs is not caused by excessive surfactant production but is due to a chemotactic factor secreted by the tumour cells.

This factor has a molecular mass in the region of 13 kilodaltons, is stable at 56 °C but labile at 100 °C and, being sensitive to proteases, indicates that it is a small protein molecule.

INTRODUCTION

Jaagsiekte, or ovine pulmonary adenomatosis, is thought to be caused by a retrovirus (Verwoerd, Williamson & De Villiers, 1980) that specifically transforms surfactant-producing type II alveolar epithelial cells (Payne & Verwoerd, 1984). Concomitant with this transformation is an overall increase in the alveolar macrophage (AM) population.

One of the roles of the AMs is to take up excess surfactant in the lungs (Eckert, Lux & Lachmann, 1983). An initial supposition was that proliferation of transformed type II cells would lead to the presence of higher levels of surfactant in diseased lungs, thereby explaining the AM population increase. On the other hand, the possiblity also existed that the tumour cells were releasing a chemotactic factor that was responsible for the elevated AM count. Bottazzi, Ghezzi, Taraboletti, Salmona, Colombo, Bonazzi, Mangioni & Mantovani (1985) made a study of tumour-derived chemotactic factor(s) from ovarian carcinomas, and put forward a hypothesis concerning the role of these factors in regulating the macrophage content of neoplastic tissues.

The present study was directed at the demonstration, biochemical characterization and physiological analysis of a macrophage chemotactic factor produced by cultured jaagsiekte tumour cells. The evidence presented in this article supports the hypothesis that AM proliferation in jaagsiekte sheep lungs is stimulated by a tumour-derived chemotactic factor.

MATERIALS AND METHODS

Source and preparaton of alveolar macrophages

AMs were obtained by broncho-alveolar lavage performed on the excised lungs from normal, healthy sheep. Endotoxin-free F-12 nutrient medium (Ham, 1965) at 4 °C was used to aspirate the lungs, after which the mixed cell suspension (AMs plus other lymphoid cells) was centrifuged for 30 min at 400 × g, followed by suspension of the pellet in F-12 + 10 % foetal calf serum (FCS). This preparation was incubated for 2 h at 37 °C in plastic tissue culture flasks, after which the non-adherent cells were removed. Adherent AMs were removed from the flasks with the aid of a rubber policeman and suspended in fresh F-12 + 10 % FCS.

Source and preparation of monocytes

Monocytes were isolated from the peripheral blood essentially according to the method of Carlson & Kaneko (1973). Blood collected in 8 heparinized vacutainer tubes was centrifuged for 30 min at 1 000 × g. The buffy coats were pooled and resuspended in 4 mℓ of phosphate buffered saline (PBS). Red blood cells still present were lysed by the addition of 20 mℓ of distilled water, followed by the restoration of isotonicity by adding 10 mℓ of PBS containing 2,7 % NaCl after 30 s of gentle mixing. The white blood cells were centrifuged for 10 min at 400 × g, washed once in PBS and then resuspended in F-12 containing 10 % FCS. The monocytes were subsequently removed from the white blood cell fraction by adherence to a plastic substrate, as described for the AMs above. It was not necessary to remove the monocytes (Mns) from the tissue culture flask with a rubber policeman, as in the former case, since shaking was found to be sufficient.

Tumour cell and normal type II cell supernatants

F-12 conditioned media (containing 10 % FCS, penicillin, streptomycin and mycostatin) were collected (over 24 h periods) from tumour cell cultures (15.4 & 21.3) and from normal type II (nT-II) cell cultures.

The 15.4 epithelial cell line was established from the affected lungs of a jaagsiekte sheep by Coetzee, Els & Verwoerd (1976), using the differential trypsinization method to remove fibroblasts. The 21.3 cell line was likewise derived from another jaagsiekte sheep (Verwoerd, De Villiers & Tustin, 1980).

Since non-transformed type II cells are sensitive to trypsin digestion, elastase was used for the preparation of cultures (Dobbs, Geppert, Williams, Greenleaf & Mason, 1980). The nT-II cells in suspension were separated from other lung cell types by layering onto a discontinuous metrizamide[1] gradient, followed by centrifugation at 400 × g according to the method of Brown, Goodman & Crandall (1984). Gradients were prepared by layering a density of 1,040 (16,6 g in 100 mℓ of buffer) over a density of 1,090 (17,4 g in 100 mℓ of buffer). A broad, diffuse band containing the nT-II cells was found at the interface with a purity of about 60 %, contamination being mainly due to fibroblasts. These fibroblasts were eliminated during the subsequent culturing in tissue culture flasks at 37 °C in F-12 + 10 % FCS, where islands of nT-II cells gradually forced the former cells off the bottom of the flask. Supernatant was collected only when the nT-II islands merged to form a monolayer in each flask.

Electron microscopy of nT-II cell cultures

Cell culture pellets were fixed in 2 % glutaraldehyde (Karnovsky, 1965) for 4 h, followed by post-fixation for 1 h in 1 % osmium tetroxide. Both fixatives contained 0,1 M cacodylate buffer plus 2 % sucrose (pH 7,2) to maintain tissue osmolarity. The samples were then dehydrated in a graded ethanol series (50–100 %) and

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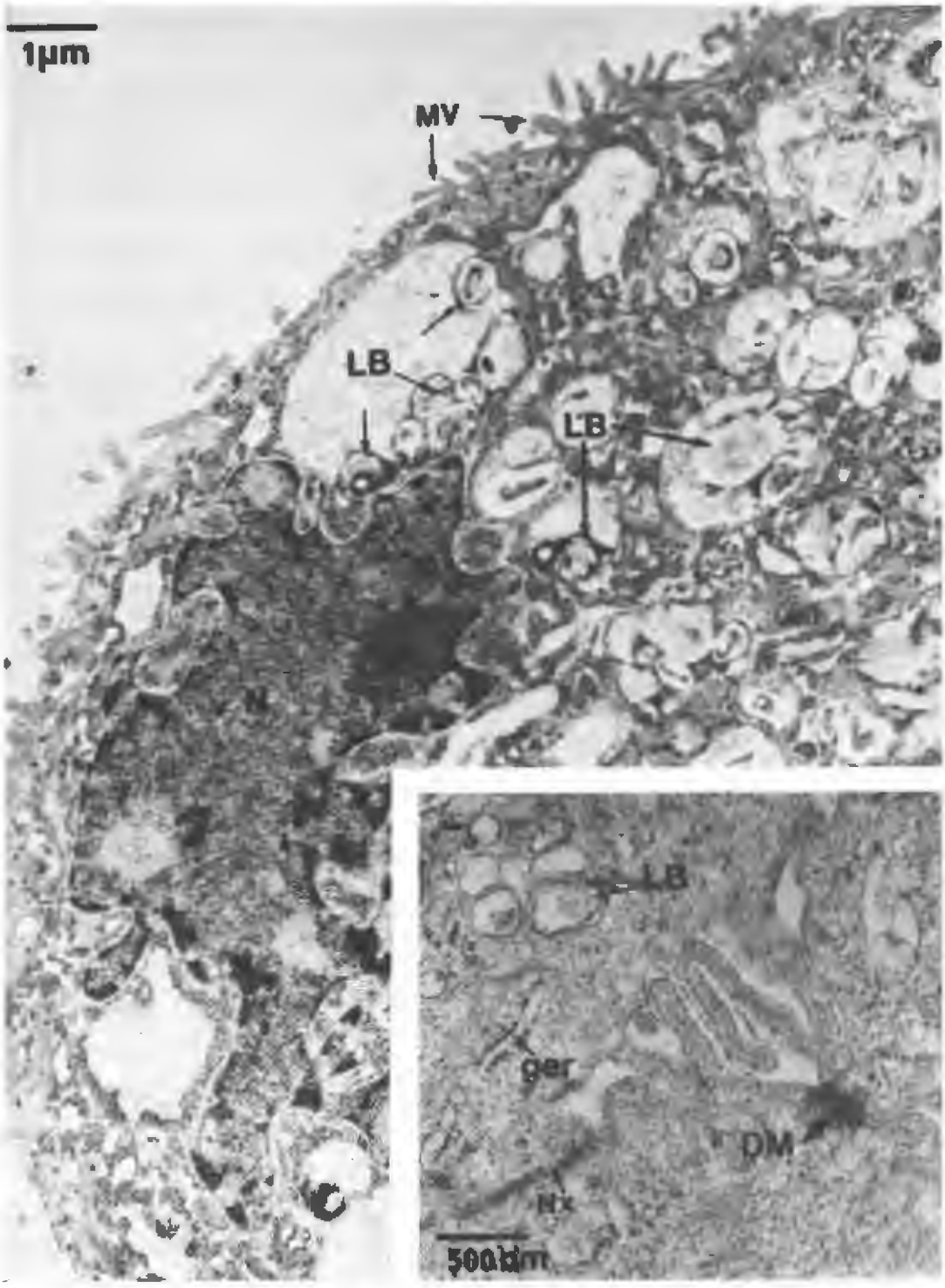

FIG. 1 Electron micrograph of normal type II cell from tissue culture (× 10 000). LB = lamellar body; DM = desmosome; N = nucleus; nc = nucleolus; ger = granular endoplasmic reticulum; Nx = nexus; MV = microvilli (insert × 20 000)

placed in propylene oxide overnight, before being embedded in Epon epoxy resin (Kay, 1965). Thin sections were cut from the Epon blocks, using a Reichert OM U2 ultramicrotome and mounted on 200 mesh copper grids. These sections were then stained with 2 % aqueous uranyl acetate and lead citrate (Reynolds, 1963) and viewed with a Jeol transmission electron microscope.

Chemotaxis assay

Modified Boyden Chambers and polycarbonate membranes were obtained from Nucleopore Corporation. The upper and lower compartments of each chamber were separated by a 10 μm pore size membrane for AMs and 5 μm membrane for Mns, so that these cells could just pass through from the upper to lower compartments if a chemotactic gradient was present in the latter. The AMs or Mns were placed in the upper compartment of each of a series of 4 chambers, where AMs were added at 1×10^5 cells/compartment and Mns at 8×10^5 cells/compartment. Each chamber series constituted a gradient of culture supernatant (from the tumour or nT-II cell cultures) in the lower compartment.

The gradient was established as follows:

lower compartment of chamber 1: 25 % culture supernatant + 75 % F-12 + 10 % FCS

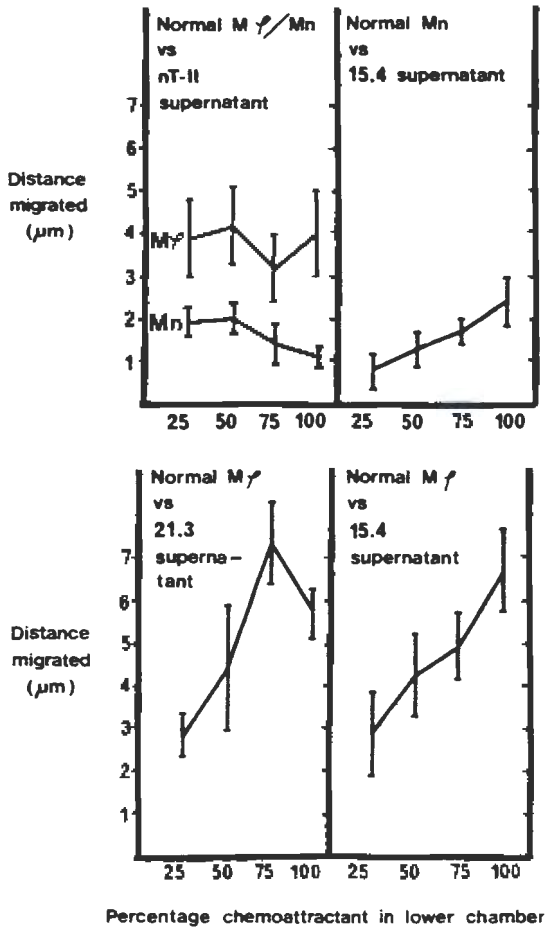

FIG. 2 Macrophage chemotactic response to tumour cell and normal type II cell culture supernatant

lower compartment of chamber 2: 50 % culture supernatant + 50 % F-12 + 10 % FCS

lower compartment of chamber 3: 75 % culture supernatant + 25 % F-12 + 10 % FCS

lower compartment of chamber 4: 100 % culture supernatant

In addition, 25 % of the culture supernatant was also placed in the upper compartment of each chamber in the series, so that chamber 1 effectively had the same amount of culture supernatant on both sides of the membrane. This is necessary for distinguishing chemokinesis or random migration from chemotaxis (directional migration) in response to a chemotactic gradient, which is measured in chambers 2–4 (Snyderman & Goetzl, 1981).

After 3 hours' incubation in a 5 % CO_2 environment at 37 °C, the membranes were removed from the chambers and placed onto glass slides which were coded to remove reader bias. The membranes were first placed face downwards and allowed to air-dry overnight, so as to allow the AMs or monocytes that did not pass into the pores to adhere to the glass surface. The membranes were then mounted right side up in a methanol film on fresh glass slides. The methanol fixes the cells in the membrane and also acts as a mordant for the acid haematoxylin that was used to stain the AMs or Mns within the membranes.

The depth of cell migration for each membrane was measured with a microscopically fine focus in 10 randomly selected oil-fields/membrane, and the results were expressed as a standard deviation about a mean point.

Molecular mass determination

It was necessary to concentrate the serum-free F-12 supernatant from 15.4 cell tumour cultures before fractionation on a Sephadex G-75 column[1] to determine the molecular mass of the chemotactic factor. Serum-free supernatant was collected over a 24 h period from 12 Roux flasks containing 15.4 cell monolayers (180 mℓ in total). This supernatant was concentrated in the following manner: 120 mℓ was reduced to 2 mℓ by freeze-drying, using an Edward's High Vacuum freeze drier, and the remaining 60 mℓ was concentrated down to 5 mℓ by pervaporation from a dialysis bag. Two methods of concentrating the supernatant were used in order to ensure that all molecular species were present, since the dialysis bag had a 10 kilodalton (kDa) pore size, while changes to large molecular mass species can occur during the freeze-drying process.

The 2 mℓ and 5 mℓ of concentrated supernatants were pooled, after which 1 mℓ of the combined sample was placed on a 1,5 × 30 cm Sephadex G-75 column previously equilibrated with F-12 culture medium (no serum). The column was eluted at 4 °C at a flow rate of 2,8 mℓ/h under gravity. One mℓ fractions were collected with an LKB Multirac fraction collector operating in the drop counting mode.

The column had previously been calibrated with the following molecular mass markers: Blue dextran 2000 (Pharmacia) to determine the exclusion volume (Ve), ovalbumin 45 kDa (Miles), Equine myoglobin 17,2 kDa (Seravac), Cytochrome C 13 kDa (Seravac) and ^{3}H-thymidine (Amersham) to determine the elution volume of the column when molecular species below 0,3 kDa appeared.

The chemotactic activity of each of the samples was assayed in duplicate, using Modified Boyden Chambers as previously described, except that a gradient was not used. In this case, the lower compartment of each pair of chambers contained a 10 × diluted sample of each column fraction. It was necessary to dilute each fraction tested 10 × prior to the assay to avoid inhibition of chemotaxis by excessive concentration of the chemotactic factor.

Inactivation of the chemotactic factor

Temperature sensitivity was determined by heating serum-free 15.4 cell supernatant for 1 h to 56 °C & 100 °C. Chemical composition was investigated by treating serum-free 15.4 cell supernatant with the following enzymes: trypsin[1], proteinase K[2], pronase[3] and DNase[3]. All the above samples were tested for activity, using the standard chemotactic assay, where 10 % FCS was added to each treated 15.4 cell supernatant prior to being added to the appropriate compartment of the Modified Boyden Chambers. The influence of pH changes on the activity of 15.4 cell supernatant was investigated in a similar way.

Other effects of 15.4 cell supernatant on normal alveolar macrophages

The possibility that stimulated AMs, in turn, could secrete a factor(s) that would affect the growth of the tumour cells was also investigated. AM cultures, pre-

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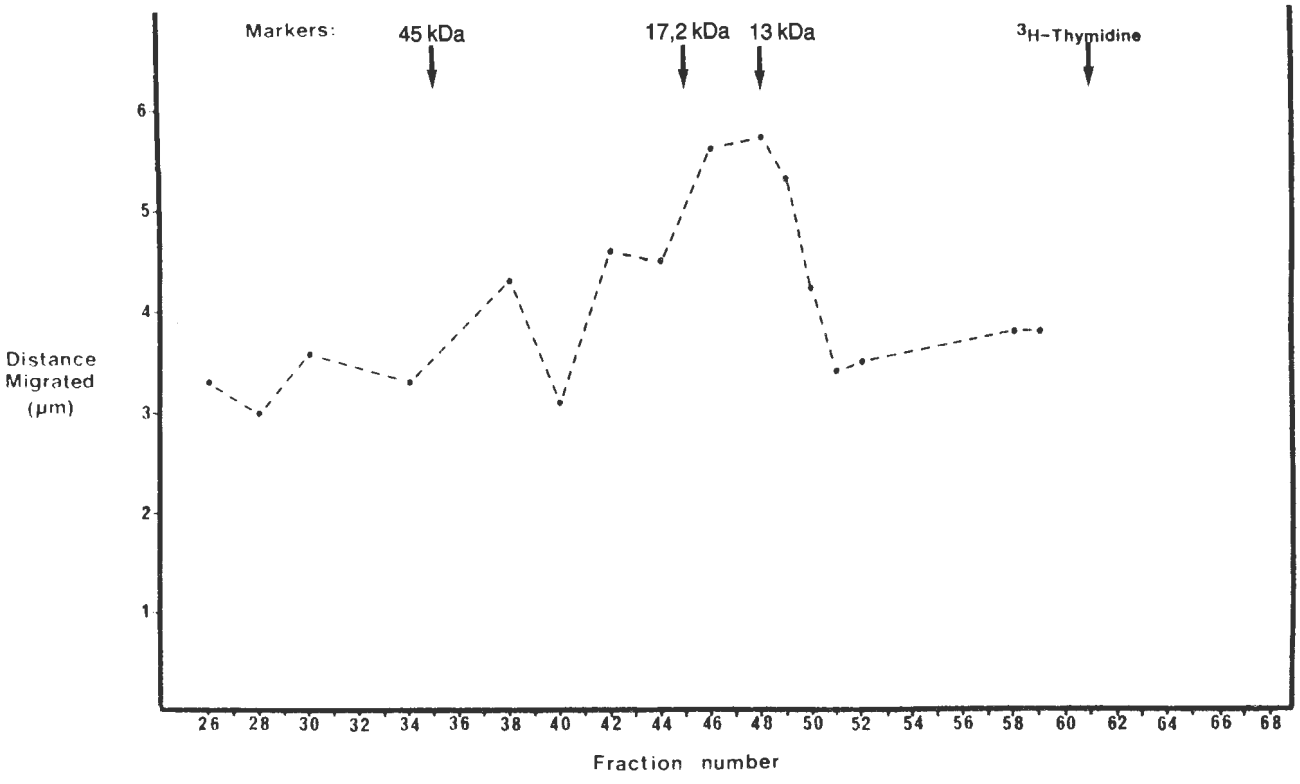

FIG. 3 Chemotactic assay of Sephadex G-75 column fractions

pared from the broncho-alveolar lavages from normal, healthy sheep, were grown in F-12 culture medium containing 25 % 15.4 cell supernatant plus 10 % FCS + penicillin, streptomycin and mycostatin. This supernatant (stimulated macrophage conditioned medium) was collected 24 h later.

As a negative control, a parallel culture of AMs was grown in F-12 culture medium that did not contain 25 % 15.4 cell supernatant (unstimulated macrophage conditioned medium).

Both the stimulated and the unstimulated macrophage conditioned mediums were added at 1:2 dilution to a series of 12 replicates, each of the tissue culture wells having previously been seeded with 15.4 cells at 1×10^4 cells per well. The 15.4 cells were cultured in F-12 medium, containing 10 % FCS and antibiotics (previously mentioned) for 24 h, before the addition of macrophage-conditioned medium. As controls, a further 12 replicates of 15.4 cells were cultured without addition of macrophage-conditioned medium, and another 12 replicates of 15.4 cells were cultured in F-12 medium, containing 25 % 15.4 supernatant with serum and antibiotic additives only.

^{3}H-thymidine was added at the same time to each culture well (1×10^{-7} Ci/well) and the experiment was allowed to run for 72 h in a 5 % CO_2 incubator at 37 °C. The assay was terminated by washing the 15.4 cells in the culture wells 3 times with PBS, followed by lysis of the cells with 250 $\mu\ell$ 1 % sodium dodecyl sulphate (SDS) per well. Each lysed sample was dissolved in Beckman Ready-Solve TM scintillation cocktail and read on a Beckman LS 9000 Scintillation Counter.

RESULTS

Fig. 1 is an electron micrograph of an nT-II cell that is representative of the nT-II cultures used as a negative control in the chemotaxis assays. These cultures were found to consist mainly of type II alveolar epithelial cells, and the one shown in Fig. 1 can be seen to be filled with lamellar bodies, believed to contain surfactant components (Brown *et al.*, 1984) that appear to be secreted into vacuoles within the cell cytoplasm. The presence of granular endoplasmic reticulum (in inset) is also characteristic of a protein secreting cell, while the nexus (electrical junction) and desmosomes shown in the inset confirm the epithelial nature of these cells. Thus, the electron microscopic evidence points to the suitability of these nT-II cultures as controls for the transformed type II cell lines (15.4 & 21.3).

Macrophage chemotactic response to tumour cell and nT-II culture supernatant

The response of AMs and Mns to culture supernatants from tumour and nT-II cell cultures is shown in Fig. 2. Each vertical bar represents the standard deviation about a mean point, which is the distance migrated by the AM or Mn. As can be seen, both AMs and Mns gave a positive chemotactic response to the 15.4 supernatant (no overlap between the standard deviations of the 25 % chemokinesis value with that of the 75 % & 100 % chemotaxis values), while a higher level of chemokinesis at 25 % was observed in the presence of nT-II supernatant. In the latter case, no positive chemotactic response was apparent, thereby negating the role of surfactant (assuming that it had been secreted into the nT-II culture supernatant) in attracting macrophages to the lung alveoli. A surfactant preparation from sheep lungs that was assayed in a separate experiment (results not shown) confirmed this observation. The AMs also showed a positive chemotactic response to the 21.3 tumour cell culture supernatant, but at a concentration of 100 % it was found to be inhibitory to chemotaxis.

Molecular mass of the chemotactic factor

The chemotactic activity of the 15.4 supernatant fractions after passing through a Sephadex G-75 column is shown in Fig. 3. The positions of the markers are indicated by arrows that correspond to their respective fraction numbers. As can be seen, the peak of the chemotactic curve corresponded with the 13 kDa marker, which

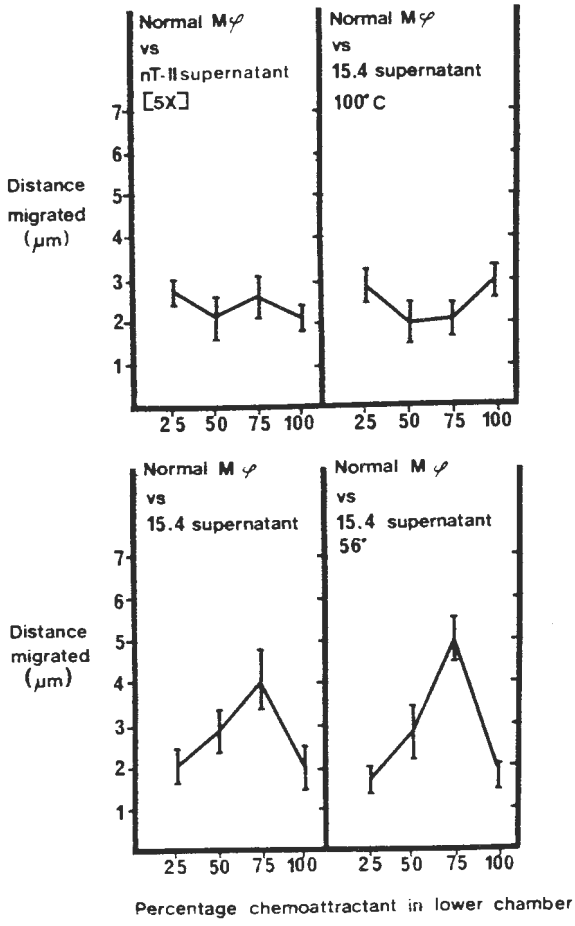

FIG. 4 Effect on AM chemotactic response after heating 15.4 supernatant

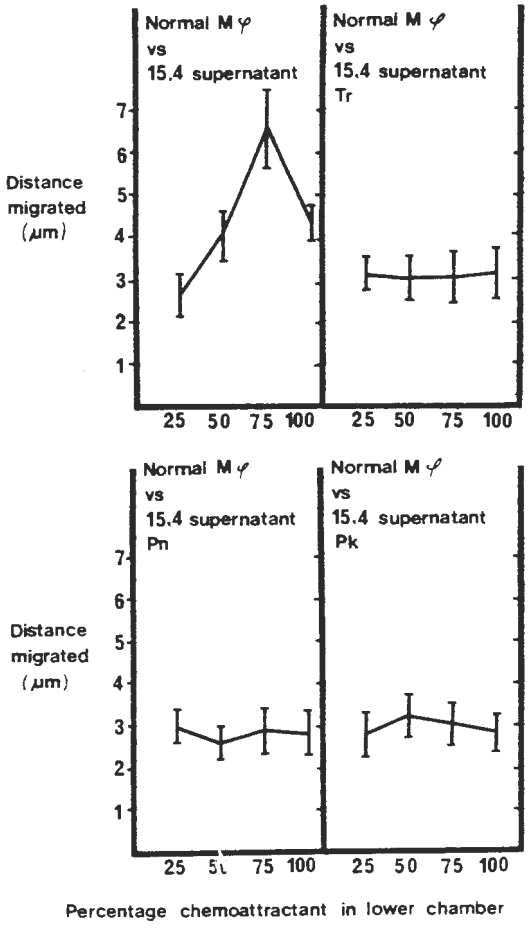

FIG. 5 Effect on AM chemotactic response after treating 15.4 supernatant with protein digesting enzymes. Tr = trypsin; Pn = pronase; Pk=proteinase K

was well before the ^{3}H-thymidine marker. The molecular mass of the chemotactic factor present in the 15.4 cell culture medium is therefore in the region of 13 kDa.

Susceptibility to heating and enzyme treatment

The effect on the AM chemotactic response after heating the 15.4 cell culture supernatant is shown in Fig. 4. The controls in this experiment were the 15.4 supernatant unheated (+ve control) and nT-II supernatant (negative control). As can be seen, the chemotactic factor was stable at 56 °C, but was inactivated at 100 °C. In addition, an inhibitory effect of the 15.4 cell supernatant at 100 % concentration was also apparent (lower 2 graphs).

Fig. 5 shows the effect on AM chemotactic response of treating 15.4 supernatant with proteases. Untreated 15.4 supernatant was used as a positive control, with an inhibitory effect on AM chemotaxis at 100 % concentration. The 3 15.4 supernatants, treated with trypsin, pronase and proteinase K respectively, did not stimulate AM chemotaxis; thus the chemotactic factor present in the 15.4 cell supernatant was shown to be susceptible to proteases. Conversely, 15.4 cell supernatant treated with DNase and pH extremes was unaffected (results not shown).

The results of ^{3}H-thymidine incorporation by 15.4 cells in the presence of macrophage condition medium are tabulated in Table 1. As can be seen, there was no difference in ^{3}H-thymidine incorporation by 15.4 cells in the presence of F-12 culture medium or in medium where 15.4 supernatant had been added, as is reflected by overlap of the standard deviations. Unstimulated macrophage-conditioned medium caused an almost 50 % decrease in isotope incorporation by the 15.4 cells, compared to that in the positive controls, while the stimulated macrophage conditioned medium amplified this effect fourfold.

TABLE 1 ^{3}H-thymidine incorporation by 15.4 cells in the presence of macrophage-conditioned medium

Supernatant	Counts per minute (mean of 12 replicates)	Standard deviation
F−12 only	3 896	± 929
F−12+15.4 supernatant	3 609	± 1 085
Unstimulated macrophage—conditioned medium	2 202	± 351
Stimulated macrophage—conditioned medium	993	± 203

DISCUSSION

The results of this study have shown that cultured tumour cells secrete a chemotactic factor into the supernatant that stimulates chemotaxis in both alveolar macrophages and blood monocytes (Fig. 2). The response of alveolar macrophages to a surfactant preparation (results not shown) resembled the nT-II (negative control) curve shown in Fig. 2. In this regard, it is probable that the nT-II cultures were also secreting surfactant into the culture medium, since electron microscopy confirmed the presence of multilamellar bodies within their cytoplasm (Fig. 1). The presence of increased surfactant levels in jaagsiekte sheep lungs is thus not responsible for the recruitment of alveolar macrophages or monocytes from the peripheral circulation. Rather, on the basis of the *in vitro* findings, it is suggested that the chemotactic factor secreted by the tumour cells will recruit monocytes from

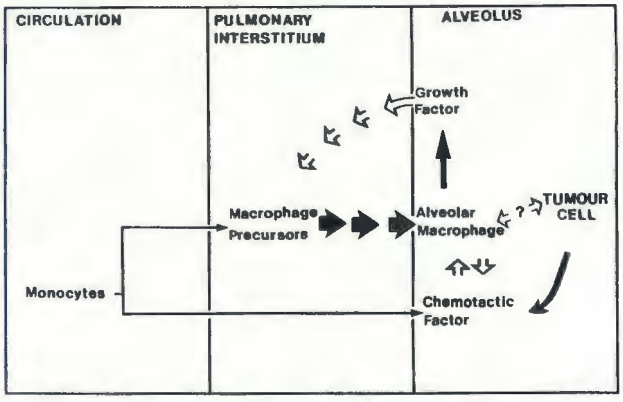

FIG. 6 Postulated sequence of events leading to proliferation of alveolar macrophages in jaagsiekte sheep lungs

the circulation *in vivo* and the alveolar macrophage increase in the lung alveoli is due to an enriched interstitial macrophage population, providing more precursors in response to the effects of the chemotactic factor.

Bowden & Adamson (1978) put forward the hypothesis that alveolar macrophages in the lung alveoli are derived from 2 precursor pools, namely, a primary circulating monocyte/interstitial one and a secondary, static, interstitial pool, where monocytes are supposedly capable of undergoing further mitoses in response to a chronic stimulus. They observed that there was initially a neutrophil response to the instillation of carbon particles (chronic stimulus) into mouse lungs, followed by a long term clearance of the irritant by the alveolar macrophages. The first increase in the number of macrophages was apparently due to recruitment of blood monocytes, while the sustained numbers of free macrophages in the alveoli were associated with division and migration of macrophage precursors in the pulmonary interstitium (Adamson & Bowden, 1980). In addition, it was later shown that *in vitro* incubation of normal alveolar macrophages with carbon resulted in the secretion of a factor into the culture supernatant which, when injected into normal mouse lungs, would stimulate division of the pulmonary interstitial cells (Adamson & Bowden, 1982). In parallel with the Adamson & Bowden (1980; 1982) model, a postulated sequence of events leading to proliferation of alveolar macrophages in jaagsiekte sheep lungs is shown in Fig. 6. The normal pathway of migration of monocytes from the blood stream is via the pulmonary interstitium, where they become macrophage precursors that differentiate into alveolar macrophages under the influence of a growth factor supplied by mature alveolar macrophages. When tumour cells are present, a 2nd pathway for monocyte migration into the alveolus becomes activated. In the latter instance, the chemotactic factor overrides the precursor step in the pulmonary interstitium, and blood monocytes move directly into the alveolus, probably differentiating under the influence of the chemotactic factor to become alveolar macrophages. This primary response to the chemotactic factor would then serve to amplify an already existing pathway, and the sustained proliferation of alveolar macrophages in the neoplastic lungs would, in part, be due to differentiation of more macrophage precursors in the pulmonary interstitium under the influence of increased levels of growth factor, secreted by stimulated alveolar macrophages.

The regulation of the macrophage content of neoplasms by chemo-attractants has attracted the attention of a number of workers. Bottazzi, Polentarutti, Acero, Balsari, Boraschi, Ghezzi, Salmona & Mantovani (1983) found that supernatants from cultured human and mouse tumour cells stimulated chemotactic responses in macrophage target cells. They found that there was a correlation between chemotactic activity and macrophage content in 11 mouse tumours that were studied. Further characterization of this chemotactic factor was done with the aid of a Sephadex G-75 column. Serum-free supernatant eluted as a single chemotactic activity peak in the cytochrome C region (Bottazzi *et al.*, 1985). A similar analysis, carried out in this laboratory, has shown that a chemotactic factor of the same molecular mass (in the region of 13 kDa) is also present in the supernatant of jaagsiekte tumour cell cultures (Fig. 3). This chemotactic factor apparently has similar properties to those described by Bottazzi *et al.* (1985), in that it can be produced under serum-free conditions, is stable at 56 °C but destroyed at 100 °C (Fig. 4), is susceptible to exposure to proteolytic enzymes (Fig. 5), but is resistant to DNase and extreme pH treatment (results not shown). All these characteristics suggest that the chemotactic factor secreted by jaagsiekte tumour cells belongs to the category of polypeptide lymphokines, which also include the growth and tumour cell necrosis factors. In addition, the chemotactic factors also act as migration inhibition factors at high concentration (such as is encountered at the tumour site), and this is borne out by the results of the *in vitro* assays, where 15.4 cell supernatant was found to depress macrophage chemotaxis at 100 % concentration.

Another aspect of this macrophage recruitment system in jaagsiekte sheep lungs that remains to be explored is the effect of the stimulated alveolar macrophages on the tumour cells themselves (see question mark in Fig. 6). A preliminary investigation has shown that normal alveolar macrophages cultured in the presence of 15.4 cell supernatant will, in turn, secrete a factor that causes a fourfold decrease in ^{3}H-thymidine uptake by 15.4 cells. The biochemical characterization and physiological analysis of this 2nd factor will form part of a separate study.

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