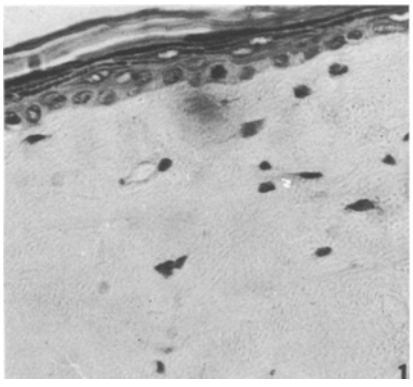
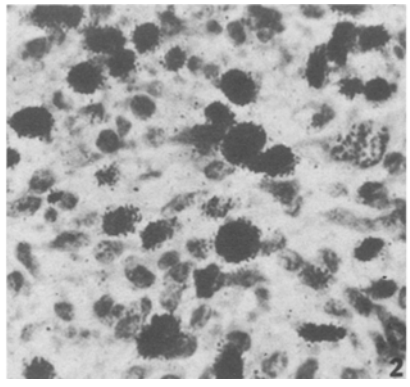
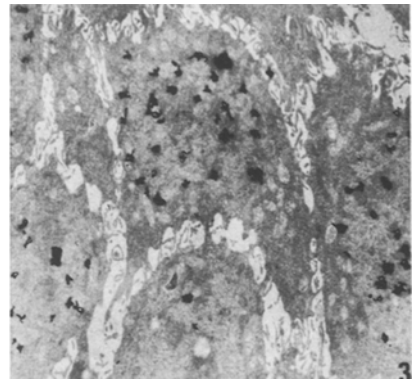

Fig. 1. Light microscope autoradiogram showing the incorporation of ^{3}H-PGF$_{2\alpha}$ in the cell nuclei of mouse control epidermis. × 200. Fig. 2. Light microscope autoradiogram showing a heavy incorporation of ^{3}H-PGF$_{2\alpha}$ only into the nuclei of neoplastic cells of a mouse with squamous cell carcinoma. Mitotic cells (arrow) can be frequently seen. × 200. Fig. 3. Electron microscopic autoradiogram showing a heyvy and specific incorporation of ^{3}H-PGF$_{2\alpha}$ as several developed grains only over the nuclear chromatin (dense chromatin, Dc); of neoplastic cells. No grains are visible over mitochondria (M), endoplasmic reticulum or intercellular spaces (Is). × 4000.

dol X, fixed and washed. Autoradiograms were examined under HS-8 electron microscope.

Observations and discussion. Light microscopic autoradiograms revealed a marked uptake of ^{3}H-PGF$_{2\alpha}$ in the nuclei of the neoplastic cells, as compared to that of mouse control epidermal cells (figures 1 and 2). Frequently mitotic figures with a heavy autoradiographic reaction and an intense reaction is also visible in the multinucleated cells. The autoradiographic reaction is mostly located over nuclei and their nucleoli. No reaction is visible over the cytoplasm. Quantitative analysis revealed significant differences ($p < 0.001$) between control and neoplastic cells (table). Electron microscopic autoradiograms also revealed a heavy concentration of ^{3}H-PGF$_{2\alpha}$ in the neoplastic cells. Several enlarged nuclei of neoplastic cells, heavily incorporated the ^{3}H-PGF$_{2\alpha}$ as developed grains, mostly in dense chromatin (heterochromatin) and at the periphery of the nucleolus (figure 3). No ^{3}H material was found in the loose chromatin (euchromatin) or in the cytoplasmic organelles (mitochondria, endoplasmic reticulum).

The role of PGF$_{2\alpha}$ and PGE$_2$ on the cell growth and proliferation, namely of neoplastic cells has been of considerable interest in recent years[7-9]. However, the mechanism by which prostaglandins exert their effects is still a matter of speculation. In the present studies, we have shown that ^{3}H-PGF$_{2\alpha}$ is strongly bound to the macromolecules of nuclear chromatin of cancerous cells of mouse squamous cell carcinoma. The ^{3}H-PGF$_{2\alpha}$ binding is 9-fold greater than that found in the nuclei of control mouse epidermis, although concentrations of ^{3}H-PGF$_{2\alpha}$ in control epidermal nuclei are still significant. Electron microscopic autoradiograms revealed that ^{3}H is specifically bound to nuclear chromatin, especially dense chromatin (heterochromatin). It is therefore possible that prostaglandins exert their physiological effects, by interfering with DNA synthesis. To our knowledge there are no previous publications which studied the intracellular binding of ^{3}H-PGF$_{2\alpha}$ in cancerous cells as compared to that of control cells from the same tissue. This marked increase of ^{3}H-PGF$_{2\alpha}$ in the neoplastic cells can explain the enhancement of carcinogenesis induced by prostaglandin F$_{2\alpha}$ and indicate the presence of nuclear receptor(s).

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The in vitro effect of aprotinin upon spleen cells from normal and tumour-bearing mice exposed to PPD and tumour cells[1]

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Summary. Aprotinin (Trasylol) is shown to enhance the response of spleen cells from normal and tumour bearing mice to PPD and tumour cells. This enhancement is greater in the tumour-bearing mice.

Protease inhibitors have been shown to inhibit both tumour growth and invasiveness in animal systems[2-5]. It has recently been suggested that these agents may produce their effects by stimulating the hosts immune system[6,7], although they may have other effects.

The effect on the immune system has been further investigated in the studies reported here by assessing the effect of protease inhibitor aprotinin (Trasylol) upon the in vitro activity of spleen cells against the antigen PPD (purified protein derivative of mycobacterium tuberculosis), and

against a tumour cell suspension. The effect of the tumour upon this response has been assessed by repeating the studies in both normal and tumour-bearing mice. The immunological reactions were assessed using the tanned erythrocyte electrophorectic mobility test (TEEM test) which is a measure of an erythrocyte slowing factor, probably a lymphokine, produced by the reaction of lymphocytes to antigen.

Materials and methods. 33 mice (C_3H females), 3 months of age, were used for these studies. 18 acted as a control group and the others as a tumour-bearing group. The tumour used was a mammary adenocarcinoma (SMT C_3H/HE V1) derived from a spontaneous adenocarcinoma in a virgin C_3H/HE mouse. It was raised by injecting the mice s.c. into the right thigh with 5×10^6 tumour cells derived from a propagated tumour line. Tumour growth could be detected within 7 days and most neoplasms were, by then, attached to deeper tissues.

All mice were sacrificed 14 days after tumour inoculation. The spleen was removed and homogenized by passage through a sieve (100 mesh). The cells were washed in medium 199 with Hanks salts and the red blood cells were lysed by the addition of 0.2 ml 10× phosphate buffered saline after which was added 1.8 ml of sterilized distilled water. The cells were centrifuged to remove haemoglobin etc. and washed 3 times in Hanks buffered salt solution. Viability was confirmed by the trypan blue staining technique and was greater than 90%. The spleen cells were washed with Hanks buffered salt solution (BSS) and suspended in the same solution at a concentration of 1×10^6.

A tumour cell suspension was prepared from the animals own tumour by homogenising part of the tumour[3] and after several washings in Hanks BSS the tumour cells were suspended as a single cell suspension at a concentration of 1×10^6/ml Hanks BSS. For each experiment 3 tubes containing 0.5×10^6 spleen cells in 0.5 ml of Hanks BSS were set up. The 1st tube (a) contained no other additive and acted as the control. The 2nd tube (b) had the antigen added to the spleen cells. The antigen being either 0.1 ml PPD in 0.1 ml Hanks BSS or 0.5×10^6 tumour cells in 0.5 ml of Hanks BSS, and the 3rd tube (c) contained the same amount of antigen used plus 10 kallikrein units of aprotinin in 0.6 ml of Hanks BSS. The final volume in each tube was made up to 3 ml with Hanks BSS.

All tubes were allowed to stand for 1 h at room temperature and were then centrifuged to remove all cells. The supernatant was transferred to another tube and 0.2 ml of a suspension of 10^8 tanned sheep red blood cells in 1 ml of Hanks BSS was then added. The tubes were then incubated for a 2nd h at room temperature, and the presence of an erythrocyte slowing factor in the solution was assessed by measuring the mobility of the red blood cells in a Zeiss cytopherometer. Mobility was measured as the mean time taken for 20 cells to move over a set distance in 2 directions. Percentage slowing (%S) was calculated from the following formula:

$$\%S = \frac{(Tb \text{ or } Tc) - Ta}{Ta} \times 100$$

T = mean time taken for 10 cells to move the set distance in tubes a (Ta), b (Tb) and c (Tc). The percentage rise in response after the addition of aprotinin is calculated as follows:

$$\%\text{ rise in response after addition of aprotinin} = \frac{\%S_A - \%S_{Ag} \times 100}{\%S}$$

Where $\%S_{Ag}$ is the percentage slowing of the antigen and lymphocyte interaction and %SA is the percentage slowing of the reaction after the addition of aprotinin. All differences were analyzed statistically using the Mann Whitney U-test.

Results. No change in erythrocyte mobility could be detected either when tanned sheep red blood cells were incubated with aprotinin alone or with supernatant obtained from the tube containing spleen cells and aprotinin.

Table 1 demonstrates the TEEM test results for spleen cells exposed to either PPD or tumour cells. There was no significant difference in the percent slowing seen in response to PPD between the control and tumour bearing animals ($p > 0.05$). However, when tumour cells were used as antigen there was a significant suppression of the spleen cell response ($p < 0.005$) in the tumour-bearing compared to normal mice.

The effects of aprotinin upon the responses are illustrated in table 2. In all groups aprotinin caused increase in the percentage slowing seen in response to PPD and tumour cells. This was a statistically significant difference in the tumour bearing groups but failed to reach significance in the control groups. The greatest increase in response occurred in the tumour-bearing group (96% to PPD and 160% to tumour cells). In relation to the response to tumour cells this nullified the difference between normal and tumour-bearing animals in the observations with aprotinin added.

Discussion. The precise role of protease inhibitors in lymphocyte function is still unclear as some workers have found inhibitory effects while others have found a stimulatory effect[6,8].

Our study shows that aprotinin at the dose used is capable of enhancing the lymphocyte responses studied. From published data aprotinin, at a similar concentration as we used, slightly stimulated the response of lymphocytes to PHA and Con-A using lymphocyte transformation as mea-

Table 1. TEEM test results for mouse spleen cells exposed in vitro to PPD or tumour cells

Animal group	Number	PPD	Tumour cells	Mean % slowing	SEM
Normal (controls)	11	+	−	8.78	±3.4
Tumour-bearers	13	+	−	8.40*	±2.5
Normal (controls)	7	−	+	9.8	±2.4
Tumour-bearers	8	−	+	4.7**	±1.4

* $p > 0.05$; ** $p < 0.005$ when compared to control group.

Table 2. TEEM test results for mouse spleen cells exposed in vitro to PPD or tumour cells after the addition of aprotinin

Animal group	Number	PPD	Tumour cells	Mean % slowing	SEM	% Rise in spleen cell response after addition of aprotinin
Normal (controls)	11	+	−	12.0	3.4	36.6
Tumour-bearers	13	+	−	16.5*	7.2	96.4
Normal (controls)	7	−	+	14.1	3.4	44
Tumour-bearers	8	−	+	12.2*	2.5	160

* p-value not statistically significant when compared to control group.

sured by ^3H thymidine incorporation whereas higher doses were inhibitory[10]. Our own unpublished data using the TEEM test show a dose-dependent stimulatory effect for aprotinin ending in a plateau at higher doses[11].
In the present study aprotinin stimulated the spleen cells from normal and tumour-bearing animals to the antigens used. The magnitude of the responses was greatest in animals whose immune response was suppressed by tumour. In this case the response of spleen cells became equivalent to that of spleen cells from normal animals. The spleen cell stimulation after the addition of aprotinin is not specific to tumour cells since it also occurred with PPD even though tumour cells were not present in the initial suspension.

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Chemical studies on the silver staining of nucleoli

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Summary. The uptake of Ag-ions by isolated nucleoli of rat liver cells was studied. Nucleolar proteins separated by electrophoresis were examined for selective silver-staining. A mechanism for a preferential Ag-staining of the nucleoli is discussed.

Nucleoli of interphase nuclei and nucleolus-organizing regions of metaphase chromosomes (NORs) that were active during the preceding interphase can be specifically stained with silver salt solutions. The high affinity of the nucleolus for silver was first noticed at the beginning of this century[2-6]. The improvement of the silver staining methods[8,9] and the applications of electron microscopy has resulted in the assignment of the material with high silver-affinity to the fibrillar component of the nucleolus[10,11]. Acidic nucleolar proteins are considered to be the most likely candidates responsible for silver staining, since proteolytic digestion abolishes the specific staining of nucleoli and NORs with silver[8-11].
The present study was undertaken to investigate quantitatively the reaction of isolated nucleoli with Ag-ions. Saturation values were determined in isolated nucleoli and under conditions of silver staining. The influence of the pH was studied. Electrophoretically separated components of 3 extracted nucleolar protein fractions were examined for preferential silver staining. Atomic absorption spectrophotometry was used to determine the amount of bound silver.
Materials and methods. For the determination of the amounts of silver taken up by the nucleoli under saturation and staining conditions, the Ag-loaded nucleoli were filtered on acetate filters (pore size: 0.2 µm; Schleicher and Schüll) with hydrophobic border. The loaded filters were disintegrated in acid-digestion bombs (Parr Instrument Co Moline, Ill. USA) with nitric acid (sp. wt 1.4 g/ml) at 160 °C for 75 min. Silver content was determined in an atomic absorption spectrophotometer (AAS 400, Perkin Elmer) by the graphite tube method. Instrumental parameters: Dry(°C/sec) 100/30; ash (°C/sec) 490/20; atomize (°C/sec) 2400/10; wave length: 278 nm; injection volume (µl) 20.
Nucleoli were isolated from fresh rat liver cells[12]. The preparations of nucleoli were analyzed for RNA[13], DNA[14] and protein[15]. Photometric determination of the amount of nucleoli was based on the relationship: 1 mg nucleoli/ml = 10.0 OD at 260 nm[16].
For saturation with Ag^+, the nucleoli were suspended in 0.05 M Na-acetate buffered $AgNO_3$ solutions (pH 5.3; containing 0.17 M sucrose and 5 mM Na-acetate) with different Ag^+-concentrations (figure 1). Saturation values at different pH were determined in 0.05 M Na-acetate (pH 4.0 and 5.0), 0.05 M boric-acetate (pH 6.0 and 7.0) and 0.05 M Na-borate (pH 8.0 and 8.9) buffered 0.28 M sucrose + 5 mM Mg-acetate at a concentration of 150 µg Ag^+/mg nucleoli. The incubation time was 2 min at 0-4 °C (figure 2).
For silver treatment under staining conditions 2.0 mg nucleoli were incubated in 8 ml of a staining solution − 7.0 ml 50% $AgNO_3$ (w/v) + 1.0 ml Na-formate buffer -

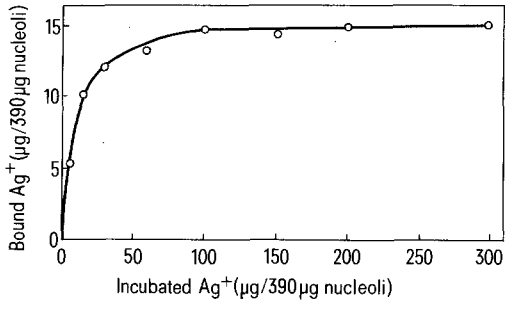

Fig. 1. Saturation of nucleoli with increasing amounts of Ag-ions.