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Growth inhibition of DMBA-induced rat mammary carcinomas by UK 114

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Abstract A perchloric acid-soluble protein extracted from goat liver and designated as UK 114 is known to be expressed over the cell membrane of (some) human cancer cell lines. This protein is antigenic, and specific antibodies elicit complement-dependent cytolysis of neoplastic target cells. In this study we demonstrate that administration of UK 114, either pure or as a crude extract (designated UK 101), inhibits the growth of mammary carcinomas induced in female Sprague-Dawley rats by dimethylbenzanthracene (DMBA). The mechanism of the tumour inhibitory activity of UK 114 is probably related to induction of immunosurveillance.

Key words Immunosurveillance · Cytotoxicity · Neoplasms · Dimethylbenzanthracene · Rats

Introduction

A perchloric acid-soluble extract of goat liver (designated UK 101) [3] consists of three main components: a 10-kDa fraction corresponding to ubiquitin, a 50-kDa fraction consisting of a glycoprotein and a 14-kDa protein (labelled UK 114), representing approximately 30% of the crude extract [5]. The 137-amino-acid sequence of this novel protein has recently been identified [8]. Previous studies have already proved that UK 114 is antigenic in experimental animals [3]. Specific monoclonal antibodies and rabbit antisera were developed and used in immunocytochemical studies to determine the cellular location of the antigen. These studies revealed the constitutive expression of UK 114 inside the cytoplasm, but not on the surface, of normal cells (e.g. liver) of various mammals [3].

Biological interest in this protein was enhanced by the recent observation that an anti-UK 114 monoclonal antibody (P_3 mAb) and rabbit antisera localized the antigen on the cell membrane of several human tumour cell lines. Moreover, these antibodies elicit complement-dependent cytolysis of target cells in vitro and induce significant inhibition of growth of human carcinoma cells xenografted into nu/nu mice [6]. Moreover, we have recently demonstrated that administration of UK 101 to cancer-bearing patients is soon followed by an increase in the serum of specific anti-UK 114 antibodies of different Ig classes, showing cytotoxic and tumour-inhibitory properties similar to those of the experimental anti-UK 114 antibodies [7].

These findings suggest that UK 114 protein might induce immune responses affecting the growth of carcinomas in autologous recipients. To test this hypothesis, we planned experiments based on the mammary adenocarcinomas induced in Sprague-Dawley rats by dimethylbenzanthracene (DMBA) as our model. In this experimental model, administration of the carcinogen to 50-day-old female rats is followed 60–90 days later by the appearance of adenocarcinomas in one or more mammary glands in up to 90% of the animals [1, 17]. In our experiments, upon appearance of the tumours the crude liver extract (UK 101) or the UK 114 pure protein was administered subcutaneously. Tumour growth and serum anti-UK 114 cytotoxic antibodies were evaluated.

Materials and methods

The crude liver extract UK 101 and its main component, UK 114 protein, were obtained as previously described [5]. Briefly, goat liver was homogenized with distilled water (1:1) using a stirring-blade blendor. 2 M Perchloric acid was added (at 4°C) to give a final ratio of 1:1. The suspension was then centrifuged at 10,000 *g*

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Table 1 Plan of the experiments. Animals were killed by decapitation at the 4th week (*s.c.* subcutaneous injection)

Group	No. of rats	Dose[a] (μg)	Treatment
1 Controls	38	vehicle	Weekly×3 s.c.
2 UK 101	36	30	Weekly×3 s.c.
3 UK 101	10	300	Weekly×3 s.c.
4 UK 114	27	10	Weekly×3 s.c.

[a] Each dose of UK 101 was administered in 5 ml saline; UK 114 was administered neat

for 20 min and the supernatant dialysed overnight against water. Solid KCl was added to give a final concentration of 3 M. After centrifugation (100,000 *g* for 1 h), the supernatant was dialysed against phosphate-buffered saline solution (PBS). This final product, referred to as UK 101, contains the 14-kDa protein (UK 114) and two proteins of low antigenic potential [6], i.e. ubiquitin and a glycoprotein of 50 kDa.

To purify UK 114, the liver extract UK 101 was chromatographed in TSK DEAE 5PW (Waters, Milan, Italy). Proteins collected in the break-through material were chromatographed in TSK SW 3000 columns (Waters) and subsequently purified by hydrophobic interaction chromatography using TSK phenyl 5PW (Waters). The eluted product was a protein of 14 kDa, as monitored by SDS-PAGE (Pharmacia Biotech, Uppsala, Sweden) at over 90% purity, called UK 114.

The proteins were dissolved in saline solution and injected subcutaneously.

Lack of toxicity of these proteins had already been confirmed in tests on experimental animals [4].

Mammary tumours were induced in 50-day-old Sprague Dawley female rats (purchased from Charles River, Calco (Milan), Italy) with oral doses of 10 mg DMBA (Sigma Chemical Milan, Italy) in olive oil once a week for three times, according to established protocols [17]. When the tumors appeared the rats weighted 200–250 g.

A total of 111 rats were used in the experiments (see Table 1).

Rats that developed mammary tumours (just palpable, with a diameter of approximately 1 mm) were allocated at random to 4 groups. Control rats were treated with subcutaneous injections of 0.5 ml/rat of saline, given weekly for 3 times. The three test groups followed the same schedule. In group 2 (see Table 1) each animal received individual doses of 30 μg UK 101 (roughly corresponding to the amount used in rabbits to induce antibodies) [3] in 5 ml saline, while in group 3 the dose (300 μg/rat) was 10 times as much. Rats in the fourth group were treated with neat UK 114 (10 μg/rat per dose). Physical appearance, behaviour and body weight were controlled weekly in all animals. In each of 28 rats, two mammary tumours appeared simultaneously in different glands: in these cases both tumours were measured and included in the evaluation. In some animals additional mammary tumours appeared in the course of the experiment, but these were disregarded.

The length and width of the tumours were measured every 6–7 days with calipers, and the tumour area was determined according to Jordan [11]. Animals were anaesthetized with Ketavet (ketamine hydrochloride) (Farmaceutici Gellini, Aprilia, Italy) and killed by decapitation on day 21 (a week after the last injection). Blood samples were collected from all rats for determination of serum oestradiol and progesterone levels and for immunological studies. Tumours and relevant organs were removed, fixed, and processed for histological examination.

Serum oestradiol and progesterone levels were determined by radioimmunoassay (RIA; Estradiol Double Antibody and Coat-A-Count Progesterone, Diagnostic Product Corporation, Los Angeles, Calif.). The oestradiol kit was equipped with standards that had oestradiol values ranging from 5 to 500 pg/ml. The intra-assay coefficient of variation at 23 pg/ml was 5% and the inter-assay coefficient at 163 pg/ml, 3.5%. The calibrators of the progesterone kit had progesterone values ranging from 0.1 to 40 ng/ml. The intra-assay and the inter-assay coefficients of variation were 36% and 3.8%, respectively, at intermediate levels of progesterone.

Lymphocheck Immunoassay Control Serum Levels 1, 2 and 3 (Bio-Rad Laboratories, Milan) were used to validate the assays.

Primary cells of DMBA-induced rat mammary carcinomas were isolated for immunofluorescence tests. Cells were mechanically dispersed and then incubated with Dulbecco's modified Eagle's medium DMEM; Sigma Chemicals, St. Louis, Mo.) containing collagenase (10 mg/ml, type II, Sigma). After 20 min incubation at 37°C, the cell suspension was centrifuged for 5 min at 200 *g* and the pellet was resuspended in DMEM+10% fetal calf serum (FCS; Biological Industries, Israel). Cultured cells were identified as tumor cells by staining with anti-rat milk fat globule membrane (MFGM) antiserum prepared as previously described [16].

The human gastric carcinoma Kato III cells and the colon adenocarcinoma HT-29 cells were obtained from the American Type Culture Collection (ATCC) and were used as targets for anti-UK 114 antibodies for cytotoxicity studies. These cell lines express UK 114 antigen on the cell membrane and react strongly with the P_3 mAb (specifically recongizing the UK 114 antigen) and rabbit immune sera (RIS) against UK 114 [6]. T47D breast cancer cells (from ATCC), which are known to be UK 114 negative [6] and Chang immortalized hepatocytes (from ATCC), which presented the antigen inside the cytoplasm but not on the cell membrane (unpublished observation) were used as negative controls. Kato III, T47D and Chang cells were grown in DMEM. HT-29 cells were cultured in RPMI 1640 medium (Biowhittaker, Walkersville, Md.). Both media were supplemented with 10% heat-inactivated FCS and 50 μg/ml gentamicin (Schering-Plough, Milan, Italy). The cultures were maintained at 37°C in humidified 5% CO_2 atmosphere.

In a standard indirect immunofluorescence (IF) procedures primary cells of DMBA-induced rat mammary carcinomas were grown on glass coverslips for 5 days, washed in PBS, treated with pig normal serum (diluted 1:50 in PBS) for 20 min, and finally incubated first with rat MFGM antiserum (diluted 1:20 in PBS); with P_3 mAb 1 μg/ml in PBS or with RIS (diluted 1:20 in PBS) at 37°C for 60 min, and then with the appropriate fluorescein-labelled or rhodamin-labelled secondary antiserum (Sera-Lab, Sussex; diluted 1:10 in PBS) for 30 min at room temperature. At the end of the reaction, cells were examined with a Leitz Orthoplan fluorescence microscope.

Sera from control and test animals were collected for complement-dependent cytotoxicity (CDC) testing at the end of the experiments and were treated at 56°C for 1 h to inactivate the complement. Kato III, HT 29, T47D and Chang cells (5×10^5 cells) were labelled with 3.7 MBq of $Na^{51}CrO_4$ for 1 h at 37°C and then washed three times to remove the extracellular ^{51}Cr. Aliquots of labelled cells were placed in 96-well microtitre plates (5×10^4 cells/50 μl) and incubated for 1.5 h at 37°C with 50 μl of the collected rat sera diluted 1:10 in DMEM, in the presence of 10 μl of guinea pig complement (Sigma). Every test was performed in triplicate. After centrifugation, release of ^{51}Cr in the supernatant was counted. The percentage of specific cytolysis was calculated from the count of experimental release, total release and spontaneous release. The P_3 mAb and rabbit anti-UK 114 antisera were used as positive controls (see our previous study [6]). To demonstrate the complement dependency of the reaction, control tests were performed in the absence of guinea pig complement.

In order to evaluate the specific cytolytic activity of UK 114 antibodies, positive rat sera were retested after preabsorption with UK 114-purified antigen (1 μg/ml). After preabsorption (1 h at 37°C with constant stirring followed by overnight incubation at 4°C), UK 114–anti-UK 114 immune complexes were removed by affinity chromatograpy on protein A-Sepharose (Sigma). Preabsorption with an irrelevant antigen (bovine serum albumin, Sigma) was used as control.

Data within different experimental groups were analysed by one-way analysis of variance (ANOVA) with Newman-Keuls' multiple comparison test or by Chi square tests where appropriate. Statistical significance was assumed at $P<0.05$.

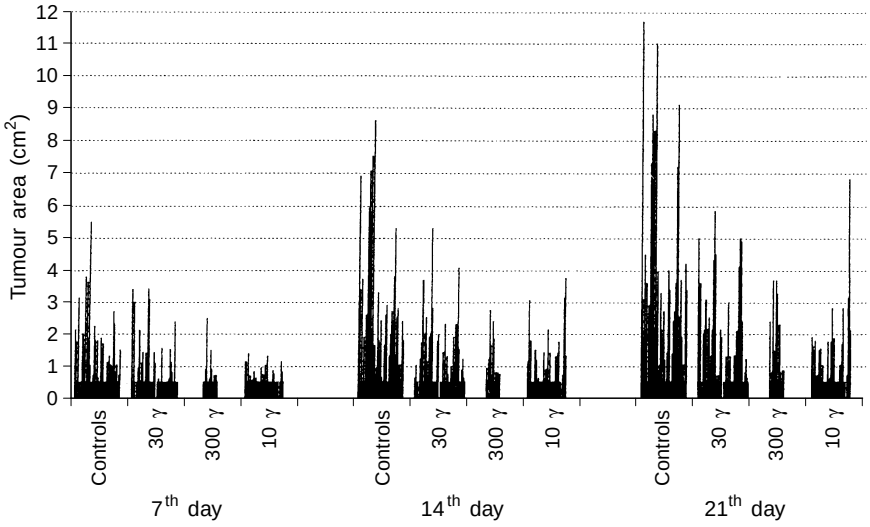


Fig. 1 Effects of 30 μg/rat of UK 101, of 300 μg/rat of UK 101 and UK 114 (10 μg/rat) on the growth, measured at weekly intervals, of mammary adenocarcinomas induced in female Sprague-Dawley female rats by DMBA administration. The tumour area at day 0 (not exactly measurable) was considered to be 0.3 cm^2. The area of each mammary tumour (cm^2) is represented. Variation in the growth of single tumours is a known phenomenon in this experimental model

Table 2 Statistical analysis of tumour growth in rats treated with UK 101, 30 or 300 μg/rat or UK 114, 10 μg/rat (*ND* no determinable)

Group (no of tumours)	Mean tumor area (cm^2) at the following days of treatment			
	0	7	14	21
Controls (43)	ND	1.4±1.1	2.5±2.1	3.4±2.9
UK 101 30 (47)	ND	0.98±0.8	1.5±1.2*	1.8±1.5*
UK 101 300 (14)	ND	0.90±0.6	1.2±0.9*	1.6±1.2*
UK 114 10 (35)	ND	0.70±0.3*	1.1±0.8*	1.3±1.2*

* Significantly different from controls values ($P<0.05$ according to Newman – Keuls's test)

Results

Figure 1 shows the effects of two different doses of UK 101 (30 or 300 μg/rat) and of UK 114 (10 μg/rat) on mammary tumour growth, at weekly intervals.

Table 2 demonstrates that both doses of UK 101 significantly affected the growth of DMBA tumours. The effect of UK 101 on tumour growth was statistically significant from the 14th day after the beginning of the experiment, and the tumour area increased to a lesser extent and more slowly. When we compared UK 101 with UK 114, we observed that UK 114, when used at a dose corresponding to the lower dose (30 μg/rat) of UK 101, was able to slow down and to decrease tumour growth even at an early stage. The statistical analysis among the four groups showed a significant difference between controls and each treated group; no significant difference was evident between treated groups.

By an alternative evaluation procedure (Table 3), we considered the number of tumours that reached or exceeded an area threshold and every relative latency time. The value of 2.5 cm^2 was taken as the threshold area, since it corresponded to the mean value of control tumours at 14 days. A significant difference in tumour takes was observed between controls and tumours treated with either UK 101 (30 μg) or UK 114 (10 μg). The difference from tumours treated with higher doses of UK 101 (300 μg) did not reach statistical significance, possibly because of the low number of cases. No significant difference in latency times was evident among the different groups.

Table 3 Number of tumours in each group that reached or exceeded an area threshold of 2.5 cm^2 and every relative latency time

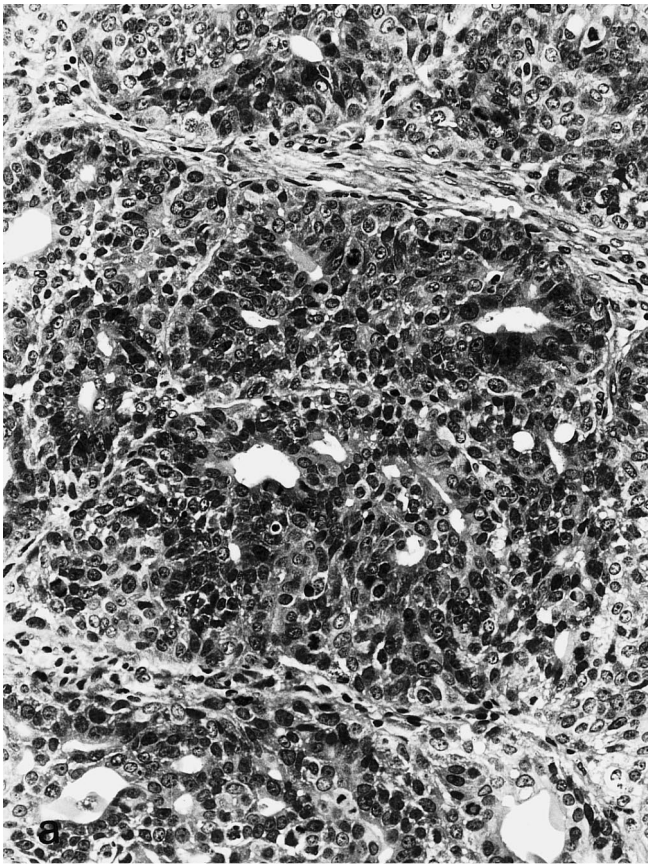
Group	Tumor takes[a]	Latency time[b]
1 Controls	24/43	11±4.5
2 UK 101 30 μg	10/47*	10±4.6
3 UK 101 300 μg	3/14	13±5.0
4 UK 114 10 μg	4/35**	15±4.0

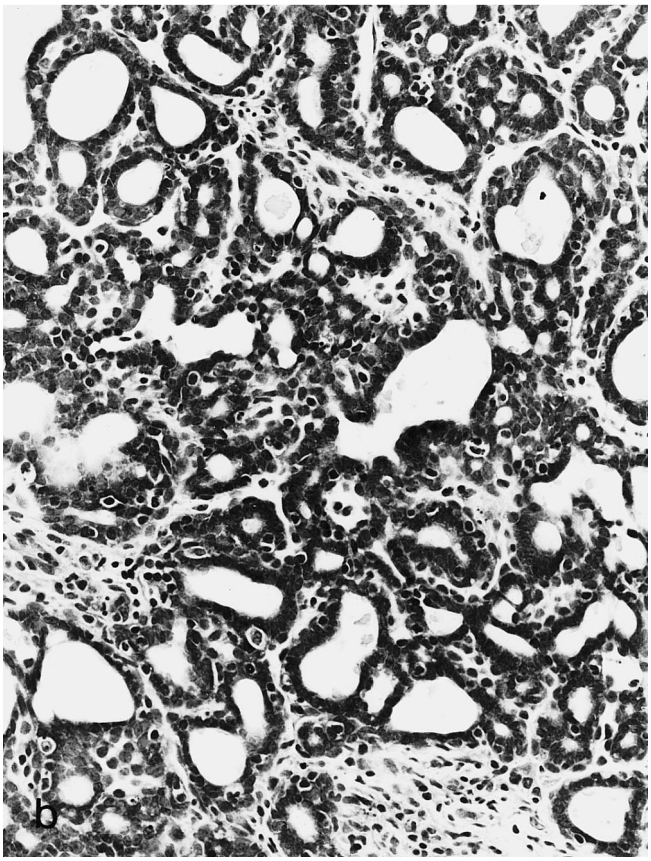
[a] Tumours that exceeded the 2.5 cm^2 threshold.
[b] Period in days required to pass from an area of 0.3 cm^2 to the threshold of 2.5 cm^2
* $P = 0.04$; ** $P = 0.008$ (Chi square, difference from control values)

The effect of UK 101 or UK 114 on tumour growth did not seem to be influenced by oestradiol or progesterone in the circulation, since the plasma concentrations of the two hormones (31±30 pg/ml for oestradiol and 14±13 ng/ml for progesterone) lay in the same range in treated and control groups and no significant differences were found.

The treatment was not toxic, and no drug-induced changes were seen in the physical appearance, behaviour or body weight.

Histological examination of the relevant organs (liver, kidney, thymus, spleen) did not disclose any significant differences between tests and controls. Tumours from control animals were adenocarcinomas showing moderately differentiated areas merging with nondifferentiated foci, infiltrating the stroma and the surrounding muscles. Areas of necrosis were prominent, especially in larger





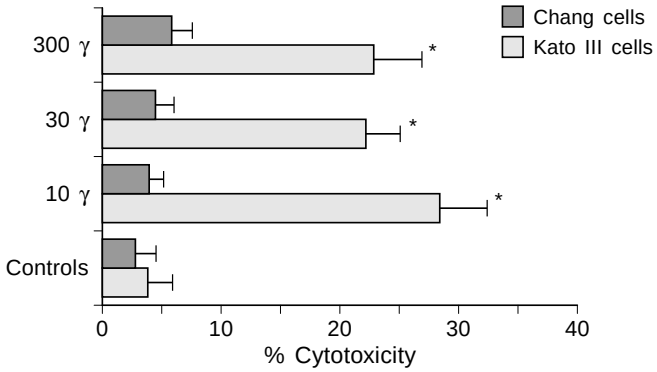


Fig. 3 Complement-dependent cytotoxicity induced by sera obtained from rats treated with 10 μg UK 114 (*10 γ*) or 30 μg UK 101 (*30 γ*) or 300 μg UK 101 (*300 γ*) or by sera from untreated rats (controls). Kato III IF-positive cells and CHANG IF-negative cells were labelled with ^{51}Cr and incubated for 1.5 h with heat-inactivated sera, diluted 1:10, in the presence of 10 μl of guinea pig complement. ^{51}Cr release was used as a measure of cell death. The percentage of cell-killing was calculated from the count of experimental release, total release and specific release. ANOVA with Newman-Keuls' multicomparison test was performed between untreated animals versus animals treated with different doses of UK 114 (* = $P<0.05$) or between groups treated with the different doses of UK 114. Data are mean±SEM of values obtained from sera of all the animals in each treatment group tested in triplicate in three different experiments

tumours. More highly differentiated, cribriform patterns were observed in some of the tumours. However, features of poorly differentiated adenocarcinoma were prominent in the majority (32 of 43; over 70%; Fig. 2a).

Carcinomas from rats treated with UK 114 (10 μg) and with the lower dose of UK 101 showed more highly differentiated, cribriform or even papillary patterns, especially in central areas (Fig. 2b). Such patterns were prominent in 21 of the 35 tumours from UK 114-treated animals. In these tumours, the stroma was infiltrated by lymphoid cells, which appeared more prominent in perivascular areas and between the epithelial cells. Lymphoid infiltration was also observed, but to an apparently lower degree, in control tumours.

The cells used as targets for the cytotoxicity tests were HT 29 and Kato III human carcinoma cells, which are known to express the UK 114 antigen on the cell surface. Chang cells, which are known to express UK 114 in the cytoplasm but not on the surface, were used as controls. Sera from animals treated both with UK 101, at 30 μg and 300 μg, and with UK 114 gave results consistently higher than sera from untreated animals when HT29 and Kato III were used as targets (Fig. 3). No significant difference was observed between the different treated groups. Cytotoxicity was absent when Chang

Fig. 2a, b DMBA-induced rat mammary carcinomas. **a** Control tumour from untreated rat, showing the typical histology of a poorly differentiated adenocarcinoma. **b** Tumour from a UK 114-treated animal. Cells are smaller and arranged in cribriform and low-papillary patterns. Lymphoid cells are present in the stroma and among epithelial cells. H&E, ×250)

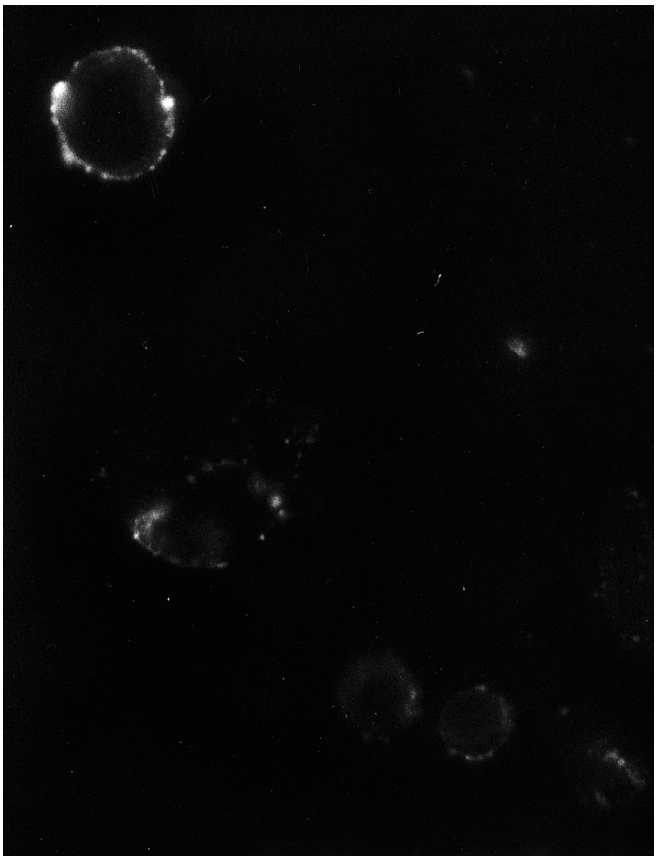


Fig. 4 Cells from primary culture of a DMBA-induced carcinoma. Immunofluorescence test using rabbit anti-UK 114 antibodies reveal the presence of the antigen on the cell surface of most. The immunocomplexes formed appear as a fine chain of multiple fluorescent dots on the cell membrane. ×540

cells were used as targets (Fig. 3). Complement-dependency of the reaction was proved by suppression of the reaction in the absence of complement. Preabsorption of the sera with UK 114 abolished the cytotoxic activity (data not shown).

Cells from DMBA-induced carcinomas, obtained by mechanical separation followed by collagenase treatment, were maintained in primary cultures. Immunofluorescence tests using rabbit anti-UK 114 antibodies revealed presence of the antigen on the cell surface (Fig. 4). The immunocomplexes formed appeared as a fine chain of multiple fluorescent dots over the cell membrane.

Discussion

The experiments indicate that subcutaneous administration of the perchloric acid-soluble fraction of goat liver extract (UK 101), and especially of the active component represented by the 14-kDa protein (UK 114), inhibits the growth of a carcinogen-induced mammary carcinoma. This was demonstrated by differences in both the increase in tumour areas (Table 2) and the number of tumours that reached or exceeded the area threshold of 2.5 cm^2 (Table 3).

The mammary carcinomas developing in Sprague-Dawley rats approximately 2–3 months after DMBA administration represent a classic model of autologous carcinogenesis [1, 17]. These adenocarcinomas are known to be oestrogen-dependent, and this model is currently used to investigate hormone responsiveness [2, 11, 22]. However, we can exclude the possibility that the effect described here might have been related to hormonal alterations, since the serum levels of both oestrogen and progesterone were comparable in treated and control animals.

Histological examination of test and control animals showed differences, since in the former cribriform and papillary differentiation was more extensive and intratumoral lymphocyte infiltration was more evident. This suggests that a lymphocyte-mediated reaction might be involved in the observed decrease in tumour growth induced by UK 114 treatment. Further studies and identification of lymphocyte subsets by appropriate immunological reagents will be needed to clarify this point.

Extensive tests in experimental animals had already proved that UK 101 was devoid of toxic activity [4]. Moreover, in our experiments no enhancement of the effect was produced by increasing the dose of UK 101 administered by 10 times (300 μg vs 30 μg).

Experiments with rabbit anti-UK 114 antisera showed that cells obtained from primary cultures of the DMBA-induced mammary carcinomas express this antigen on the cell membrane. The results are similar to those observed in human cancer cells incubated with anti-UK 114 P_3 mAb and rabbit immune sera [6]. In such experiments, when the cells were incubated at 37°C the former immunocomplexes were redistributed on the cell surface, featuring aspects of patching and capping.

Cytotoxicity tests with human cancer cells known to express UK 114 antigen on the surface used as targets [6] yielded relatively high and comparable values in all treated rats (see Fig. 2). Our hypothesis, also supported by indirect evidence from the literature, is therefore that the observed inhibition of tumour growth by UK 114 is related to antibody-mediated cytotoxic effects. However, a concurrent role might be played by cellular immunity.

The structure of UK 114 [8] shows a high degree of similarity (and minor differences in the $-NH_2$ terminus sequence) to that of the perchloric acid-soluble protein described by Levi-Favatier et al. [12] and by Oka et al. [15] in the rat liver and kidney. The protein from goats is therefore not unique to this species. According to Levi-Favatier et al. [12], analysis of this protein shows a high degree of structural similarity between the perchloric acid-soluble proteins (from rat and goat) and the superfamily of heat shock proteins (hsp) [8, 12].

These hsp are produced inside the cytoplasm of normal cells but, like UK 114, are presented on the surface of cancer cells [9, 10, 14]. The acknowledged function of hsp is to chaperone abnormal proteins; it has therefore

been suggested that they might fold antigenic peptides peculiar to cancer cells and present them on the cell surface. The hsp–peptides complex would then constitute extracellular epitopes available to MHC class I and class II antigen presentation pathways.

It has been demonstrated that hsp-associated peptides can elicit specific cancer immunity, since in fact immunization against hsp purified from some rat immunogenic tumours specifically protects the animals from a challenge with these tumours [18–21]. Moreover, Menoret et al. [13] recently showed that hsp-presenting tumours are rejected through immune mechanisms involving the activation of TRC-αβ-bearing T cells.

A similar mechanism might be involved in the effect observed following UK 114 administration to animals bearing DMBA-induced mammary carcinomas. This would imply a major role of our protein in antigen presentation and tumour immunosurveillance.

We conclude that the goat protein UK 114 is an antigen whose administration is associated with a significant reduction of growth of an experimental carcinoma and that it probably acts by inducing immunosurveillance. The model described in this paper represents a reproducible test that can be used for further investigation of the optimal dose, modalities and sequence of treatment with UK 114 and ultimately the mechanism of its tumour-inhibitory activity.

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