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Immunotherapy with monoclonal antibodies directed against the immunosuppressive domain of p15E inhibits tumour growth

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SUMMARY

Immunosuppressive retrovirus-related proteins, like p15E, are involved in tumour-associated immunosuppression. In the present study we investigated whether such proteins could be used as targets in tumour immunotherapy using MoAbs. Immunotherapy was performed in mice inoculated with the Rauscher virus-transformed myeloid cell line RMB-1. RMB-1 cells express retroviral antigens at their cell surface. In order to obtain constant serum titres of MoAbs over a prolonged period of time during therapy, anti-p15E antibody-producing hybridoma cells were encapsulated in alginate and injected intraperitoneally in tumour-bearing mice. Using this technique, serum antibody titres of 50–100 μg/ml were obtained, which remained constant over a period of at least 3 weeks. Therapy experiments were performed using anti-p15E antibodies 19F8, which recognizes both cell surface-associated as well as circulating p15E, and ER-IS5, which did not react with surface-bound p15E beyond background, but which neutralizes circulating p15E. Inoculation of alginates containing anti-p15E hybridoma cell lines in RMB-1 tumour-bearing mice showed inhibition of tumour cell growth. In survival experiments, 19F8 cured eight of 23 tumour-bearing mice. The p15E neutralizing antibody ER-IS5 caused a significant longer survival, but therapy with this MoAb alone was not sufficient to cure the animals of the RMB-1 tumour.

Keywords immunotherapy p15E monoclonal antibody alginate mouse model

INTRODUCTION

Immunosuppressive factors produced by tumours are often responsible for a disturbed functioning of the patient's immune system [1]. Some of the tumour-associated immunosuppressive factors have a structural relationship to retroviral immunosuppressive proteins [2].

In this respect, p15E is a well known retroviral immunosuppressive protein, responsible, at least part, for the immunosuppression accompanying retroviral infections [3]. Retroviral p15E-related proteins are expressed by several virally as well as non-virally induced tumours and tumour cell lines [4]. P15E and its immunosuppressive conserved domain CKS-17, exert their suppressive function on cells important in the immune response against tumour cells like macrophages, T cells and natural killer (NK) cells.

Immunosuppressive p15E-related proteins are also associated with (non-retrovirally induced) human diseases, mainly tumours (reviewed by Oostendorp *et al.* [5]). Recently, studies have been published describing the presence of p15E-related proteins of low molecular weight in breast cancer [6], and RNA corresponding to the highly conserved immunosuppressive CKS-17 epitope of p15E has been detected in colorectal and gastric cancer [7]. Especially in patients with squamous cell carcinoma of the head and neck (SCC-HN), p15E-related proteins are thought to play a role in the depression of the cellular and humoral immune response [8]. In this context, retroviral p15E-related immunosuppressive factors were identified in serum obtained from patients with SCC-HN [9] and could be detected in tumour tissue of SCC-HN [8,10].

As it becomes more and more evident that immunosuppressive p15E-related proteins are associated with several types of human cancer, it is of great importance to study the potential of p15E as a target for immunotherapy.

In the present study we investigated whether p15E could be used as a target for immunotherapy in a syngeneic mouse leukaemia model. Fast growing, disseminated tumours were induced in mice by i.v. injection of the Rauscher virus transformed myeloid cell line RMB-1. RMB-1 tumours express viral antigens, as was demonstrated by Berends *et al.* [11], and RMB-1 cells produce immunosuppressive factors which could interfere with the immune response against the tumour cells.

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We used MoAbs directed against retroviral p15E (antibody 19F8) and against the immunosuppressive (CKS-17) domain of p15E (antibody ER-IS5) to investigate the potential of p15E as a target in immunotherapy. Both 19F8 and ER-IS5 cross-react with and neutralize the circulating immunosuppressive p15E-related protein [12]. However, only 19F8 binds to retroviral p15E expressed at the cell surface of RMB-1 cells. Both antibodies were compared for their effectiveness in tumour therapy.

Therapy experiments were performed in the pesent study using alginate-encapsulated hybridoma cells. Encapsulated hybridoma cells serve as a continuous source of MoAb production, after i.p. injection in (tumour-bearing) mice.

The present study shows that (i) MoAbs directed against the immunosuppressive protein p15E have immunotherapeutic potential in our mouse leukaemia model; and (ii) alginate encapsulation of hybridoma cells producing antibodies directed against tumour-associated antigens can be successfully applied in tumour therapy models in mice.

MATERIAL AND METHODS

Cell lines

RMB-1 is a Rauscher virus transformed myeloid cell line of BALB/c origin (H-2^d) [13]. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS).

The hybridoma cell lines used were 19F8 (IgG2b), producing MoAbs directed against the retroviral protein p15E, which originated from a 129 mouse × NS1 fusion [14], and ER-IS5 (IgG2b) of BALB/c origin, producing MoAbs directed against the immunosuppressive CKS-17 domain of p15E [12]. Hybridoma 19F8 was a generous gift from Dr R.A.J. Oostendorp (Free University Amsterdam, The Netherlands). Hybridoma cell lines were cultured in RPMI 1640 containing 10% FCS, 0·05 mM β-mercaptoethanol (β-ME) and 40 U/ml IL-6.

The non-antibody-producing myeloma cell line SP2/0 was used as a negative control cell line to exclude a possible effect of i.p. injection of alginate-encapsulated hybridoma cells on tumour growth. SP2/0 cells were cultured in RPMI 1640 containing 10% FCS. 1C5F5 (IgG2a), a hybridoma cell line of BALB/c origin [11], producing MoAbs directed against viral 50- and 29-kD (glyco)proteins present on the surface of RMB-1 cells, was used as a positive control to evaluate our method of immunotherapy using alginate-encapsulated hybridoma cells.

Mice and tumour

Male and female BALB/c mice (H-2^d) were bred and kept in our own colony and used at 12–14 weeks of age. The microbial status of the mice fulfilled the standard of 'specific pathogen free V' according to the criteria of the Dutch Veterinary Inspection, as described in the law on animal experiments.

In all tumour experiments, mice were injected intravenously with 1×10^7 RMB-1 tumour cells, causing the rapid growth of disseminated tumours in liver and haematopoietic organs. This tumour kills the mice in 10–15 days, with a mean survival time (MST) of 12 days.

Flow cytometry

Flow cytometry was performed to determine the reactivity of the MoAbs with retroviral antigens on the surface of RMB-1 cells and to determine the presence of specific MoAbs in mouse serum after alginate injection.

RMB-1 cells were washed twice with cold serum-free medium and were incubated with culture supernatant of hybridoma cell lines or with diluted mouse serum (1:100), both containing 20 mM NaN_3, for 10 min at room temperature. After washing the cells three times with cold buffer (PBS, 0·5% bovine serum albumin (BSA), NaN_3) a FITC-labelled secondary antibody (rabbit anti-mouse-FITC; Dako, Glostrup, Denmark) was allowed to incubate for 10 min at room temperature. Finally, the cells were washed and fluorescence was recorded using a Becton Dickinson FACScan. Antibody binding to cell surfaces was analysed and mean fluorescence units were used as a measure of antibody binding. Normal mouse serum or antibody MPC11 (IgG2b; ATCC, Rockville, MD) were used as negative control antibodies.

To determine the number of RMB-1 tumour cells in mice at several time points after tumour inoculation, spleen cell populations were analysed. To obtain spleen cell preparations, mice were killed by CO_2 exposure and spleens were removed. Cell suspensions were prepared and cells were washed three times with cold medium containing 5% serum and counted using a Coulter counter. Two-colour analysis had to be performed to be able to distinguish 1C5F5-positive RMB-1 cells from mouse B cells, which were also stained positive by the secondary antibody rabbit anti-mouse-FITC. In these experiments we used rat anti-B-220 MoAb, directed against mouse B cells (clone RA3 6B2 [15]), followed by incubation with a PE-labelled secondary antibody (goat anti-rat-PE; Caltag, S. San Francisco, CA) and 1C5F5, followed by rabbit anti-mouse-FITC. In this way FITC-labelled tumour cells could be distinguished from FITC and PE double-labelled murine B cells.

Gel electrophoresis and immunoblotting

Gel electrophoresis and immunoblotting were performed to detect retroviral p15E-reactive antibodies in mouse serum after i.p. injection of alginate-encapsulated hybridoma cells. Experiments were carried out as described earlier [12] with slight modifications. Lysed MuLV particles (150 μg) were electrophoresed using a preparative comb with one reference well in the mini-PROTEAN II slab cell system (BioRad, Hercules, CA). The nitrocellulose sheet was incubated overnight at 4°C with purified control MoAb (19F8 0·1 μg/ml) or with diluted mouse serum (1:100) using a Miniblotter system (Immunetics, Cambridge, MA).

Alginate encapsulation of hybridoma cells

Cultured hybridoma cells were washed four times in cold sterile saline and mixed with two parts of a sterile 1·2% alginate solution at room temperature (FMC, Vallensbaek Strand, Denmark). The mixture was transferred into a syringe equipped with a 25 G needle and squirted into a fresh prepared solution of 80 mM $CaCl_2$ (in water) at room temperature under continuous gentle mixing. Newly formed alginate capsules were washed four times with cold saline and mice were injected intraperitoneally with a volume of 1 ml, containing 2×10^6 hybridoma cells, using a 22 G needle. A few alginate capsules containing hybridoma cells were cultured for several days in medium to serve as a control for sterility and antibody production.

In all our experiments, not one of the mice developed peritoneal tumours accompanied by the formation of ascites

fluid, caused by outgrowth of hybridoma cells, due to breaking of the alginate capsules as was described earlier [16].

Therapy experiments

The ability of the MoAbs 19F8, ER-IS5 and 1C5F5 to delay tumour growth, or cure tumour-bearing animals, was evaluated in several therapy experiments.

The delay in tumour cell growth in the spleen of treated *versus* control mice was determined using two-colour FACScan analysis of spleen cell populations as described above. Mice were injected with encapsulated hybridoma cells 2 days before tumour inoculation. They were killed and spleens were taken out and analysed at 5 or 10 days after tumour inoculation.

For survival experiments, groups of seven or eight mice were inoculated with RMB-1 cells. In these experiments, antibody treatment with alginate-encapsulated hybridoma cells was started at different time points, either 2 days before tumour inoculation, or 2 days after tumour inoculation. Also, the effect of two injections with alginate-encapsulated hybridoma cells, 3 days before and 4 days after tumour inoculation, on survival was determined.

In each experiment, non-tumour-bearing control mice were injected with alginate-encapsulated hybridoma cells in order to monitor the antibody titres present in the serum at consecutive days.

19F8 antibody titres were measured with Western blot, using its specificity for MuLV-p15E. 1C5F5 antibody titres in mouse serum were measured with FACScan analysis, using its strong reactivity for RMB-1 cells.

In therapy experiments, animals were monitored daily to score their mortality. The results were expressed graphically as survivors against time and the MST was scored. Survival curves, as observed at day 60 after inoculation of tumour cells, obtained from different groups were tested for similarity using the Mann–Whitney test. Values of $P < 0{\cdot}05$ were considered significant.

RESULTS

The main goal of our experiments was to investigate whether neutralization of immunosuppressive p15E could be a successful approach in immunotherapy in cancer. To achieve this, p15E-neutralizing MoAbs were used which did (19F8) or did not (ER-IS5) recognize cell surface p15E on RMB-1 tumour cells. Both antibodies allowed us to study the immunotherapeutic potential of anti-p15E antibodies by neutralization and cytotoxicity.

In our experiments we applied the MoAb 1C5F5 as a control antibody to evaluate the efficacy of the method of alginate encapsulation of hybridoma cells in immunotherapy against cancer in mice. Purified 1C5F5 has successfully been used before in immunotherapy of mice bearing disseminated RMB-1 tumours [17]. 1C5F5 has a very strong reactivity for retroviral antigens at the surface of RMB-1 cells, as shown in Fig. 1.

Reactivity of 19F8 and ER-IS5 with RMB-1 tumour target cells

To determine the reactivity of the anti-p15E MoAbs with surface proteins on the tumour target cell line RMB-1, indirect immunofluorescence using FACScan analysis was performed. Culture supernatant of 19F8 reacted with surface p15E on viable RMB-1 cells (Fig. 1). This observation indicates that RMB-1 cells are in principle a suitable target for antibody-mediated cytotoxicity using 19F8. ER-IS5 showed no reactivity for surface proteins on the RMB-1 cells beyond the negative control MPC11 (IgG2b) (Fig. 1). Because ER-IS5 does neutralize p15E in serum (like 19F8 [12]), this antibody was included in the immunotherapy studies, to evaluate the effect of serum-p15E neutralization on tumour growth and survival.

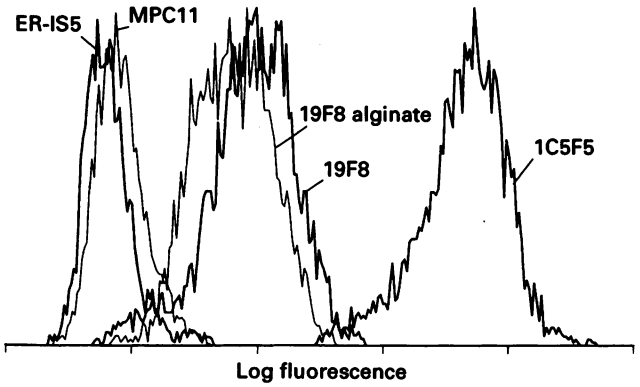

Fig. 1. Cell surface expression of 19F8, ER-IS5 and 1C5F5 antigens on RMB-1 cells. Cells were labelled with 19F8 cell culture supernatant (25 μg/ml) or with culture supernatant of 19F8 encapsulated in alginate, with ER-IS5 cell culture supernatant (20 μg/ml) or with 1C5F5 cell culture supernatant (12·5 μg/ml). Fluorescence was determined using a FACScan. Thin line represents isotype control value of MPC11 supernatant.

Technical aspects of hybridoma encapsulation for immunotherapy

Encapsulation of hybridomas in alginate was performed according to the method described by Savelkoul *et al.* and Hashimoto & Shirai [16,18]. Encapsulated hybridoma cells produced MoAbs *in vitro* as expected: supernatant derived from cultures of encapsulated hybridoma cells showed the same reactivity for RMB-1 cells as did supernatant from hybridoma tissue cultures. This is shown for 19F8 alginate and tissue culture supernatants in Fig. 1.

In the present study we applied alginate encapsulation to distribute MoAbs for immunotherapy in our mouse tumour model. Alginate-encapsulated antibody-producing cells caused high *in vivo* antibody titres which remained constant over a period of several weeks.

To determine the rate at which specific antibodies appeared in the serum after i.p. injection of encapsulated hybridoma cells, kinetic experiments were performed.

Mouse serum, taken before 19F8 alginate injection at day 0, showed no reactivity for retroviral proteins on Western blots, but already 2 days after alginate inoculation, 19F8 antibodies which detected p15E (19 kD) were present in the serum of alginate-injected mice (Fig. 2a). The level of MoAb in the serum peaked between days 5 and 19 and then slowly declined, disappearing after 3–4 weeks. The concentration of 19F8 antibody in mouse serum was determined by comparison with the reactivity of purified MoAb in a titration series. The intensity of staining of diluted (1:100) mouse serum taken 2 weeks after alginate injection was comparable to the intensity of 1 μg/ml of the purified antibody, indicating that we were able to reach serum concentrations for 19F8 of at least 100 μg/ml.

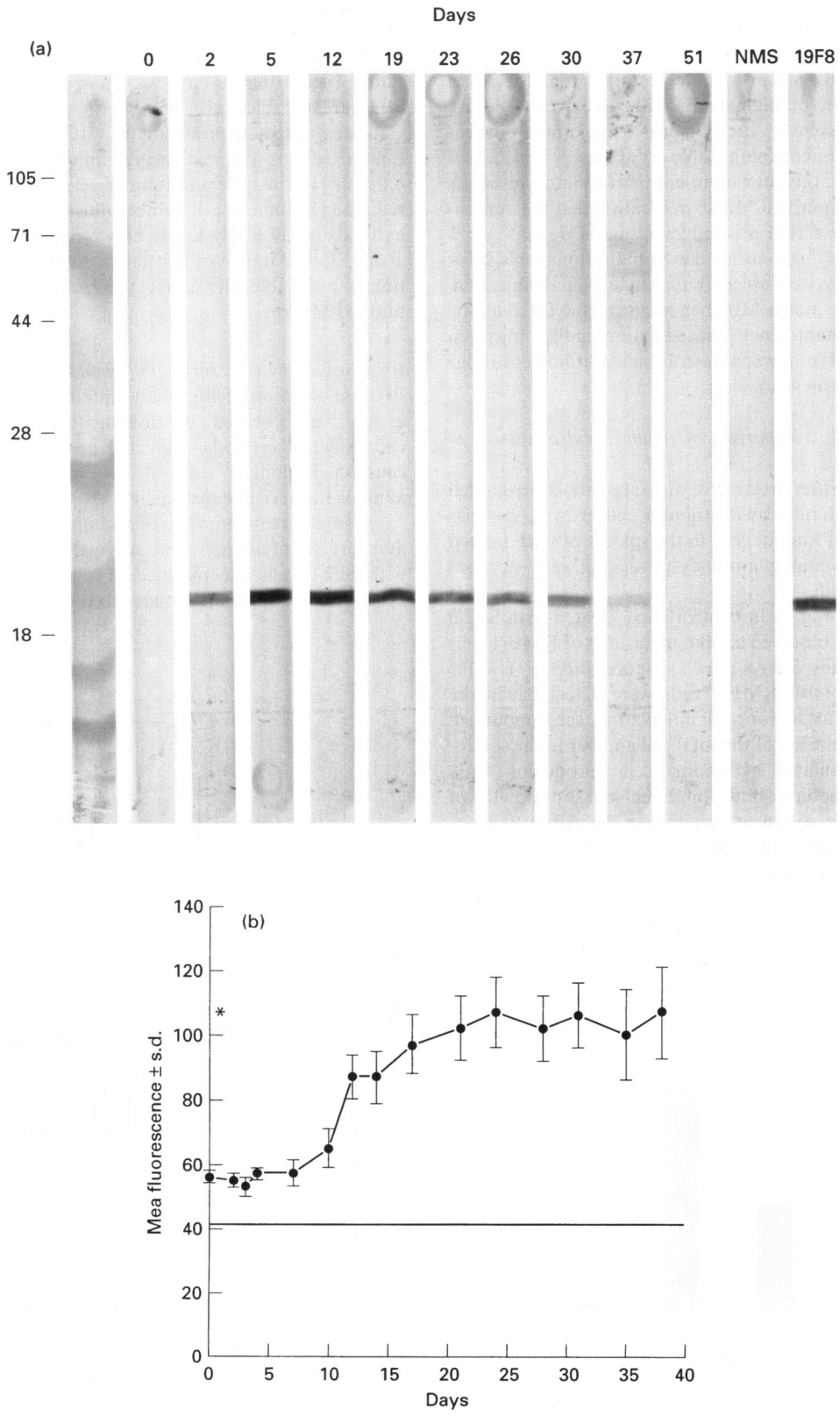

Fig. 2. Kinetics of 19F8 and 1C5F5 appearance in mouse serum after i.p. injection of 2×10^6 hybridoma cells encapsulated in alginate. (a) P15E-specific 19F8 in mouse serum was detected on MuLV blot. Normal mouse serum (NMS) was used as negative control, purified 19F8 (0·1 μg/ml) was used as positive control. Results are from one representative mouse out of four. (b) Levels of 1C5F5 were expressed as mean fluorescence of RMB-1 cells incubated with diluted mouse serum (1:100). Each point represents the mean of four mice ± s.d. The horizontal line represents background fluorescence. The mean fluorescence of purified 1C5F5 (0·5 μg/ml) is also depicted (*).

Serum samples from mice injected with 1C5F5 cells encapsulated in alginate were taken and analysed for their reactivity with RMB-1 cells using flow cytometry. Control serum, taken at day 0, showed only background fluorescence (Fig. 2b). Seven days after injection of alginate, 1C5F5 titres raised above background fluorescence. After 2 weeks a maximum concentration was reached, remaining stable for at least 5 weeks. As is depicted in Fig. 2b, this maximum concentration corresponds to 0·5 μg/ml of the purified MoAb, indicating that the concentration of 1C5F5 in serum reached values of 50 μg/ml.

Although the alginate-encapsulated hybridoma cells show different kinetics, we were able, after a single alginate injection, to detect the specific mouse MoAb in mouse serum for at least 3 weeks, indicating that alginate encapsulation and i.p. injection is a suitable method for the administration of antibodies for our immunotherapy studies in mice.

Immunotherapy (1): comparison of tumour load in spleens of alginate-treated mice

To investigate whether treatment with alginate-encapsulated hybridoma cells interfered with tumour cell growth, we analysed the number of tumour cells in the spleens of mice treated with alginates containing antibody-producing cells or non-producing SP2/0 cells.

As is shown in Fig. 3, in control mice, treated with SP2/0 cells in alginate, we observed a rapid metastasis of RMB-1 cells to the spleen: already 5 days after i.v. inoculation of 1×10^7 RMB-1 cells, $6{\cdot}5 \times 10^7$ RMB-1 cells were detectable in the spleen. Ten days after tumour cell inoculation, the tumour cell load comprised over 65% of the total cell number in the spleen.

Alginate-encapsulated hybridoma cells, producing 19F8 and ER-IS5 antibodies, both inhibited the outgrowth of tumour cells in the spleen at day 5 (Fig. 3). After 10 days, however, a difference in the effectiveness of the therapy with different hybridomas was observed. In mice treated with 19F8 we still observed a decrease in cell number compared with control SP2/0-treated mice, but the efficacy of the therapy varied between individual animals. Whereas in some animals this therapy caused a reduction in tumour cell number, 19F8 treatment was not sufficient in others to prevent the tumour from outgrowth. In ER-IS5-treated mice, the number of tumour cells after 10 days was comparable to that of control SP2/0-treated mice, over 65%.

Using alginate-encapsulated 1C5F5 hybridoma cells, tumour cell growth was strongly inhibited, and after 10 days only very low numbers of tumour cells could be detected. This reduction in tumour cell number illustrated the capacity of this antibody to kill RMB-1 cells, as was already shown by Berends *et al.* [17], and it proved that the method of alginate encapsulation is a reliable alternative to repeated i.p. injections with purified MoAbs.

Immunotherapy (2): survival of alginate-treated mice

Because a tumour cell growth-inhibiting effect of the MoAbs *in vivo* was observed, we investigated whether the alginate-encapsulated antibodies directed against p15E were able to cause a prolonged survival or even cure tumour-bearing animals in survival experiments.

Alginate treatment in itself had no effect on tumour growth and survival of animals. The survival curve of animals treated with SP2/0 cells encapsulated in alginate was similar to the survival curve of animals that received no treatment, with all

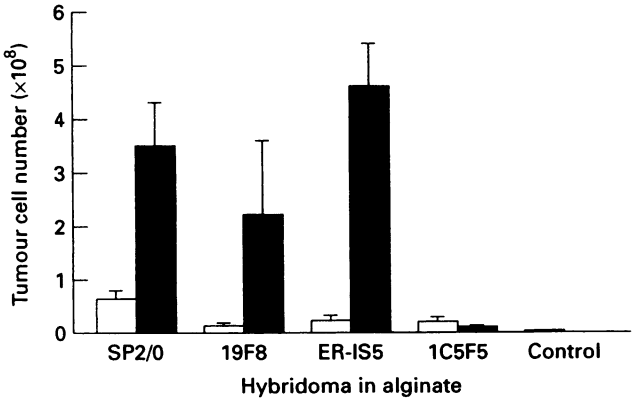

Fig. 3. RMB-1 tumour cell load in the spleen of tumour-bearing mice 5 (□) and 10 days (■) after tumour cell inoculation. Mice were injected with alginate-encapsulated hybridoma cells 2 days before tumour cell inoculation. Spleen cell suspensions were incubated with 1C5F5 and the number of tumour cells was determined using FACScan analysis. A non-tumour-bearing control mouse was used to obtain background levels. Each bar represents the mean of four mice ± s.d.

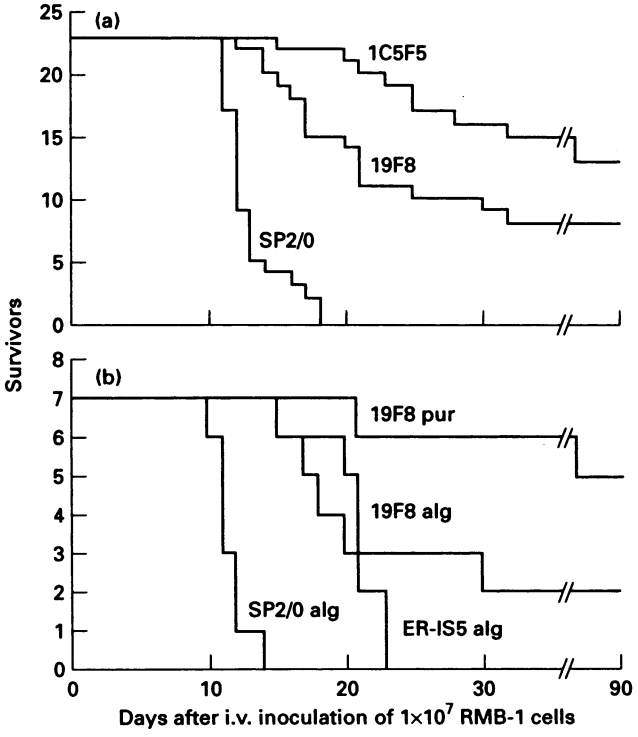

Fig. 4. Survival of RMB-1 tumour-bearing mice after immunotherapy with alginate-encapsulated hybridoma cells. (a) Comparison between treatment with control alginate containing SP2/0 cells and alginate containing 19F8 or 1C5F5 hybridoma cells. Data are from three experiments. (b) Comparison between control (SP2/0) alginate treatment and anti-p15E treatment with alginate containing 19F8 or ER-IS5 hybridoma cells and treatment with purified 19F8 antibodies (four injections with 200 μg/ml on days 1, 4, 8 and 11 after tumour cell inoculation).

Table 1. Effect of antibody treatment on host survival

Hybridoma	Form	Survivors	MST, days	Average survival time ± s.d.	*P*, treated *versus* control
SP2/0	Alginate	0/7	12·5	12·6 ± 1·4	—
19F8	Alginate	2/7	21·0	32·4 ± 19·3	0·018
19F8	Purified	5/7	60·0	54·4 ± 14·7	0·007
ER-IS5	Alginate	0/7	20·0	19·6 ± 3·0	0·026
1C5F5	Alginate	6/7	60·0	53·6 ± 17·0	0·007

Groups of seven mice were inoculated with RMB-1 tumour cells (at day 0) and the immunotherapeutic effect of hybridoma cells encapsulated in alginate (injected at day −3 and 4) and of purified 19F8 (injected at days 1, 4, 8 and 11) was determined. Median and average survival times were scored after 60 days.

mice dying within 10–15 days (Fig. 4), with a MST of 12·5 days (Table 1).

Treatment with encapsulated 19F8 hybridoma cells showed a significant prolonged survival of tumour-bearing mice, with a MST of 21 days (Table 1). In three combined experiments this anti-p15E MoAb was able to cure eight out of 23 animals, with an average survival time of 30·1 ± 18·7 days ($P < 0·001$) (Fig. 4a). With the p15E-neutralizing antibody ER-IS5, we detected a significantly prolonged survival, with a MST of 20 days (Table 1). Therapy with this MoAb alone, however, was not enough to cure any of the seven treated animals (Fig. 4b).

Treatment with a single injection of encapsulated 1C5F5-producing cells showed significantly better survival ($P < 0·001$) (Fig. 4a), with a MST of 60 days (Table 1). Out of 23 animals, 13 were cured. These results are comparable to those obtained by Berends *et al.* after multiple i.p. injections of 1C5F5 ascites [17].

Immunotherapy (3): comparison of alginate and purified antibody treatment

In order to compare the efficacy of alginate treatment with multiple injections of purified antibody in immunotherapy directed against p15E, we treated a group of seven mice with 19F8 hybridoma in alginate and a group of seven mice and two control mice with purified 19F8 antibody. The mice received i.p. injections with 0·2 mg of protein A purified MoAb on days 1, 4, 8 and 11 after i.v. tumour inoculation. This treatment resulted in immediate high serum titres of 19F8 in the non-tumour-bearing control mice, from day 1 until day 17, thereafter declining. This kinetics is comparable to the kinetics we found after one single injection with alginate-encapsulated hybridoma cells.

Repeated injections of purified MoAb until day 11 after tumour inoculation cured five out of seven mice, with a MST of 60 days (Fig. 4b). In the alginate-treated group in the same experiment, two out of seven mice were cured. This difference was not significant ($P > 0·05$).

Immunotherapy (4): effect of timing of alginate injection and survival

Since encapsulated antibody-producing cells require several days in order to produce detectable and effective serum levels of antibody (see also Fig. 2), we investigated the effect of the timing of alginate injection in tumour-bearing mice. To this purpose, groups of mice were treated with alginate containing either 19F8 or ER-IS5 2 days before or 2 days after tumour inoculation. No significant differences were found ($P > 0·1$). We also found no significant improvement in the effectiveness of the therapy when we injected alginate twice, i.e. 3 days before and 4 days after tumour cell inoculation.

In contrast to anti-p15E antibodies, the variation in time of administration of 1C5F5 resulted in different MST per group. We observed that when treatment with 1C5F5 was started 2 days after tumour cell inoculation the MST of the tumour-bearing animals was only 30 days, compared with the MST of 60 days when we started alginate treatment 2 days before tumour inoculation. This could be attributed to the fact that the encapsulated 1C5F5 hybridoma cells needed a period of at least 1 week to give rise to detectable serum levels of antibody (see also Fig. 2).

Together, our data indicate that anti-p15E antibodies can be applied for immunotherapy in tumour-bearing mice when injected in the form of alginate-encapsulated hybridoma cells. However, permanent cure was only observed in mice treated with anti-p15E antibodies which detected p15E both at the tumour cell surface and in the serum.

DISCUSSION

Tumours are often accompanied by immunosuppression [19]. One of the tumour-associated immunosuppressive factors is a retroviral p15E-related protein [4]. As early as 1979, Mathes *et al.* reported a role for p15E in feline leukaemia virus-induced immunosuppression [3]. Nelson *et al.* (1985) were the first to apply antibodies specific for p15E in immunotherapy studies. They were able to restore the depressed DTH in mice after injection with immunosuppressive products from bovine ocular squamous cell carcinoma (BOSCC) by using MoAbs against p15E [20]. In 1987, Thiel *et al.* demonstrated that the incidence of retrovirally induced AKR leukaemia in mice could be reduced by treatment of mice with a combination of MoAbs directed to the viral proteins gp70 and p15E. Where anti-gp70 was virus-neutralizing, anti-p15E antibodies were required to inactivate the immunosuppressive peptide [21]. Also, the growth of a p15E-expressing rat yolk sac tumour could be inhibited after therapy with the purified MoAb 19F8, directed against p15E [22].

In the present study we investigated whether antibodies directed against the immunosuppressive domain of p15E and neutralizing serum p15E, could be applied in immunotherapy

in a syngeneic mouse model. Intraperitoneally injected alginate-encapsulated hybridoma cells were used as the source of *in vivo* antibody production. The retrovirus-transformed mouse myeloid cell line RMB-1 was injected in mice to induce a fast growing (MST 12 days), p15E-positive tumour in mice.

Our data indicate that MoAbs directed against the immunosuppressive protein p15E (19F8 and ER-IS5) inhibit tumour cell growth, and that a single injection of alginate-encapsulated hybridoma cells is an efficient method to study the immunotherapeutic potential of MoAbs in our mouse model.

The method of alginate encapsulation of secretory cells has recently been applied succesfully for long-term administration of rat MoAbs which neutralize IL-6 in mice [23] or for the administration of cytokines produced by cytokine-transfected cells which were encapsulated in alginate and injected in mice [24,25].

Our results confirm the notion that alginate encapsulation of hybridoma cells is a powerful technique to maintain constant high antibody titres over a period of several weeks. After i.p. injection of our different hybridoma cells encapsulated in alginate, antibody concentrations of 50–100 μg/ml could be detected in mouse serum. 19F8 antibodies appeared in mouse serum already 2 days after alginate injection, therefore immunotherapy with this antibody could be started either before or after tumour cell inoculation. We found no beneficial effect of a second injection with hybridoma cells in alginate, indicating that antibody titres after a single injection are optimal for tumour immunotherapy in our model.

We also found no statistical difference between treatment with 19F8 in alginate or with purified antibodies. However, the survival curves are not identical (see Fig. 4b). We feel that the lack of significance could be a consequence of the rather small groups (n = 7). The immediate high serum titres at day 1 after injection of purified 19F8 antibodies caused an optimal immunotherapeutic effect. After injection of 19F8 in alginates, high serum titres were only obtained after 2 days. At this time point, the tumour load is already larger, and therefore therapy was not successful in all treated mice.

The alginate-encapsulated hybridoma 1C5F5 needed at least a week to raise detectable antibody titres in mouse serum. For the most optimal therapy results, 1C5F5 in alginate had to be injected in mice before tumour cell inoculation.

Serum levels of ER-IS5 could not be monitored, because this antibody did not react with antigens on RMB-1 cells, and it has only a weak reactivity for retroviral p15E on Western blot. However, we know from *in vitro* alginate cultures that the antibody production of encapsulated hybridoma cells is comparable to production in conventional hybridoma cultures (20 μg/ml).

Treatment of tumour-bearing mice with a single injection of alginate-encapsulated hybridoma cells directed against retroviral p15E (19F8) or against the immunosuppressive domain of p15E (anti-CKS-17, ER-IS5), caused a significant delay in the outgrowth of tumour cells in the spleen of tumour-bearing mice. RMB-1 cells express retroviral p15E antigens on their surface, indicating that 19F8 could exert its therapeutic effect through antibody-mediated cytotoxicity. In this respect, Lindvall & Sjögren, using 19F8 (IgG2b), showed that ADCC was the effector mechanism in their rat yolk sac tumour model [22].

In survival experiments encapsulated 19F8 hybridoma cells cured eight of 23 tumour-bearing animals (survival over 90 days). Immunotherapy with 19F8 did not result in cure of all tumour-bearing mice. This could be explained by the observation that the number of 19F8 antigens expressed at the cell surface of RMB-1 cells is low compared with the expression of 1C5F5 antigens, and that some cells are even p15E-negative (see also Fig. 1). Since 19F8 also binds circulating p15E, the amount of free antibody required for tumour cell binding and cytotoxicity may be reduced, resulting in inefficient cellular cytotoxicity.

ER-IS5 does not recognize p15E at the surface of RMB-1 tumour cells. Still, treatment with ER-IS5 caused a significant delay in the onset of disease. This effect is probably caused by the ability of ER-IS5 to neutralize p15E-related immunosuppressive factors in the circulation, thereby improving the immune system of the treated mice.

In this respect, recently performed experiments showed that neutralization of p15E in tumour-bearing mice caused an improvement of the humoral IgE immune response of these tumour-bearing mice against TNP-KLH: the suppressed secondary immune response against TNP-KLH in tumour-bearing mice was improved after treatment with anti-p15E antibodies (Lang & Savelkoul, manuscript in preparation).

The importance of CKS-17 as target epitope for immunotherapy, directed against the immunosuppressive protein p15E, is based on the following observations. First, Nelson *et al.* described that the CKS-17 epitope could be held responsible for the immunosuppressive effects of p15E-related tumour products [26]. Second, in a study from our group we have shown that 19F8 is directed against a linear epitope within the immunosuppressive CKS-17 domain [27]. Our results using CKS-17 specific and neutralizing antibodies provide more evidence for the importance of CKS-17 as a functional epitope in tumour-associated immunosuppression.

We feel that immunotherapy directed against the immunosuppressive domain of p15E could be used in addition to conventional therapy, like surgery or radiotherapy, in the therapy of early detected, small recurrent tumours, or to improve the results of immunotherapy protocols, which until now have had limited success, possibly because of the immunosuppressed state of the patient.

A combination of MoAbs blocking the immunosuppressive epitope of proteins like p15E and recognizing tumour cell surface antigens should be used in order to obtain maximal immunotherapy. In this respect, squamous cell carcinoma of the head and neck offers an interesting field of research, because these tumours are associated with p15E-mediated immunosuppression [5,9]. Besides, for this group of patients, several functional MoAbs directed against tumour-associated surface antigens have already been developed [12,28,29]. Studies in relevant animal models (nude, SCID or RAG -\-) using a combination of neutralizing and tumour cell-specific antibodies together with relevant human tumour cell lines, could provide more insight into more successful immunotherapy protocols for patients with (head and neck) cancer.

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