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# Effect of monoclonal antibodies to early pregnancy factor (EPF) on the in vivo growth of transplantable murine tumours

**K. A. Quinn* and H. Morton**

University of Queensland Department of Surgery, Royal Brisbane Hospital, Queensland 4029, Australia

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**Summary.** Neutralisation studies with monoclonal antibodies (mAbs) specific for early pregnancy factor (EPF) have shown it to be essential for the continuation of pregnancy in mice and the growth of some tumour cells in vitro. These studies report that the mAbs are also able to limit the growth of two murine tumour lines transplanted s.c. The development of MCA-2 tumours in CBA mice was unaffected by the injection of 1 mg anti-EPF IgM at the time of tumour cell inoculation. However, four doses of 500 μg anti-EPF, injected one dose per day for 4 days after tumour cell inoculation, significantly retarded tumour development such that no tumours were palpable on day 13. A similar dose regimen of control IgM had no effect on tumour size. Dose/response studies revealed that lower doses of anti-EPF administered after tumour cell inoculation were effective in retarding the growth of the MCA-2 tumours. The effect of anti-EPF mAb administration on the growth rate of palpable B16 tumours established s.c. in C57BL/6 mice was also determined. Tumours injected with 6 mg anti-EPF 5/341 or anti-EPF 5/333 mAbs showed significant decrease in the uptake of [$^3$H]thymidine into tumour tissue, measured 16 h after injection. Furthermore, titration of sera for active EPF showed that a significant reduction in the EPF titre was associated with a significant inhibition of tumour DNA synthesis. Thus it appears that neutralisation of EPF retards tumour growth both in vitro and in vivo. In vitro the effects must be due to anti-EPF mAb interfering with a direct mechanism that contributes to the maintenance of cells in the active growing phase. However, in vivo host immunological mechanism that are modified to allow tumour survival may also be affected. The presence of EPF-induced suppressor factors curculating in the serum of tumour-bearing mice has been confirmed and the contribution of such factors to tumour progression must now be investigated.

**Present address:* NCl-Navy Medical Oncology Branch, Bldg 8, Room 5101, Naval Hospital Bethesda, Bethesda, MD 20814 USA

*Offprint requests to:* H. Morton

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## Introduction

Early pregnancy factor (EPF) is a polypeptide hormone that is present in serum of a wide range of mammalian species within hours of fertilisation [12]. EPF has been shown to be immunosuppressive and its production may prevent adverse maternal responses against the antigenically foreign fetus [14]. To define the role of EPF more precisely, monoclonal antibodies (mAbs) to EPF have been produced, providing a source of specific reagents for use in neutralisation studies [1, 17]. These have shown that, as well as being a monitor of the viable embryo, EPF, either directly or indirectly, is necessary to maintain this viability [1, 2]; neutralization of EPF in pregnant mice with specific monoclonal antibodies resulted in embryonic loss [1, 2].

The production of EPF is not confined to pregnancy. EPF has been detected in the serum of patients bearing a diversity of tumours [17, 19] and continued production by cultured tumour and transformed cell lines has suggested that the source is the tumour cell itself [17]. EPF production by cultured tumour cells is associated with growth; production peaks whilst cells are in the logarithmic phase of growth and ceases after the induction of differentiation or growth arrest [17].

These in vitro characteristics were suggestive of EPF playing a role that is not concerned with immunomodulation. Confirmation of this has been achieved by utilising the anti-EPF mAbs to study the effect of EPF neutralisation on the in vitro growth pattern of tumour cells. Culture of murine tumour cell lines B16 [8] and MCA-2 [11], in the presence of two different anti-EPF mAbs, caused a non-complement-dependent, dose-dependent decrease in the rate of DNA synthesis by the cells. These results suggested EPF may be necessary for the optimal growth of tumour

cells in culture and be functioning via an autocrine mechanism.

Having established that both MCA-2 and B16 tumour cells show decreased growth in vitro when exposed to anti-EPF mAbs, the following studies were undertaken to investigate the EPF dependence of these cells in vivo. Both types of tumour cells grow rapidly when transplanted into syngeneic mice and the time course of the appearance of EPF in serum after inoculation of cells has been established. Experiments have been carried out, using two different protocols, to test the effect of neutralization of EPF in vivo on the growth of tumour cells. First, anti-EPF mAbs were administered to CBA mice at the time of s.c. inoculation of MCA-2 tumour cells; the growth of the tumour cells 9–13 days later was recorded. Secondly, antibodies were administered to C57BL/6 mice with palpable B16 tumours and the effect on the level of circulatory EPF and rate of DNA synthesis of the tumour cells determined.

## Materials and methods

*Mice.* Male C57BL/6 and CBA mice (aged 5–7 weeks) and outbred male Quackenbush mice (aged 8–12 weeks) were obtained from the Central Animal Breeding House, University of Queensland, and housed in a light-controlled (12 h light/12 h dark) and temperature-controled (22–26° C) room with free access to water and mouse pellets. Animal care followed the guidelines of the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (1990) and ethical clearance for the experiments was obtained from the University of Queensland Animal Experimentation Ethics Committee.

*Assay for EPF – the rosette inhibition test.* To date, the rosette inhibition test remains the only means of detecting EPF in complex biological mixtures. This assay is dependent on the original finding of Bach and coworkers [3] that an immunosuppressive anti-lymphocyte serum can inhibit spontaneous rosette formation in vitro between lymphocytes and heterologous red blood cells. The highest dilution of an anti-lymphocyte serum that will inhibit the number of rosettes formed to 75% or less of the number formed without serum was termed the rosette inhibition titre [3, 13]. A modification of the assay was introduced to detect EPF after it was demonstrated that lymphocytes, preincubated in EPF, give a significantly higher titre with an anti-lymphocyte serum than do lymphocytes from the same donor without EPF [12]. The assay described in this study was performed with spleen cells from outbred, male Quackenbush mice, rabbit anti-mouse lymphocyte serum and human red blood cells; one batch of anti-lymphocyte serum was used throughout the experiments [13, 20]. For each test, $1.8 \times 10^7$ freshly isolated spleen cells were incubated at 37° C for 0.5 h with 0.2 ml test sample, diluted in Hanks' balanced salt solution without $Ca^{2+}$ or $Mg^{2+}$ (HBSS) containing 0.01% w/v bovine serum albumin (BSA). After incubation, the cells were washed twice in HBSS, reconstituted to 1.0 ml in HBSS and used to estimate the rosette inhibition titre of an antilymphocyte serum. A positive (purified mEPF, 5 ng/ml in HBSS/BSA [5]) and a negative (HBSS/BSA) control were included with each set of tests. With each test the titre was expressed as $\log_2$ (reciprocal dilution of anti-lymphocyte serum $\times 10^{-3}$); a titre $\geq 16$ was positive for EPF and titre $<16$ was negative for EPF [13].

In the present study, tenfold dilutions of each sample were prepared in HBSS/BSA and each dilution tested in the rosette inhibition test. The results were recorded as an EPF titre, that is, the reciprocal of the highest dilution of a sample to test positive for EPF.

*Anti-EPF monoclonal antibodies.* The preparation and characteristics of anti-EPF IgM mAbs 7/342 and 5/341 and control IgM mAb 7/331 have been described previously [1, 17]. A third clone producing anti-EPF IgM, 5/333 (see hybridoma supernatant 1, Fig. 2 [17]) has been characterised subsequently and used as an additional source of specific mAb. As discussed previously [1, 17], binding of anti-EPF IgM mAbs to EPF in a solid-phase immunoassay is not highly significant; at best, levels of 2.5 times background are observed with purified antigen. With EPF in complex biological mixtures such as serum, significant binding is not demonstrated. However, these antibodies will neutralise the activity of EPF in either a purified or crude form in the liquid phase, as demonstrated in the rosette inhibition test [1]. Furthermore the monoclonal antibodies, bound to CNBr-Sepharose 4B (Pharmacia-LKB, Uppsala, Sweden), will remove EPF from solution [16] (Cavanagh et al. in preparation). These characteristics are in keeping with those generally found for IgM antibodies, which tend to be of low affinity but, because of their high valency, bind with quite respectable avidity to antigens [18 a].

Hybridoma 7/342 was grown as ascites tumours in mice, while 5/341, 5/333 and 7/331 were grown in vitro in spinner culture [16]. IgM was isolated from ascites and conditioned medium by ion-exchange chromatography followed by euglobulin precipitation [1]. The mAbs were stored in 0.05 M sodium phosphate buffer pH 7.4 containing 0.15 M sodium chloride/0.01% w/v sodium azide and, before injection into mice, exchanged into HBSS by dialysis.

*Tumour cell lines.* Two murine tumour cell lines, a fibrosarcoma (MCA-2; 11) and the B16 melanoma [8] were studied. Cells were retrieved from liquid $N_2$ storage and cultured under standard conditions in Dulbecco's modification of Eagle's medium (DMEM) +10% fetal calf serum (Flow Laboratories, Irvine, UK) for the minimum time necessary for population expansion (generally 1–2 weeks). Before inoculation into mice, monolayers were washed in serum-free medium, then dissociated by a short exposure to trypsin/versene solution (CSL, Melbourne, Australia). After three further washes in DMEM, $10^6$ viable tumour cells in 0.2 ml HBSS containing 1% mouse serum, were injected s. c. into the abdominal flanks of mice. With both tumour cell lines, palpable tumours were present in 90% of mice within 7 days of tumour cell inoculation.

*EPF in serum after tumour cell inoculation.* C57BL/6 mice ($n = 2$) were bled, via the retrobulbar sinus under light halothane (Fluothane; ICI Australia, Melbourne, Australia) anaesthesia, every 48 h after B16 tumour cell inoculation. CBA mice ($n = 3$) were bled 7 and 10 days after MCA-2 tumour cell inoculation. Tenfold dilutions of serum were prepared in HBSS/BSA and tested in the rosette inhibition test to determine EPF titre.

*Capacity of mAbs to neutralize EPF activity in serum.* The capacity of anti-EPF mAbs to neutralise EPF activity in serum from mice bearing B16 tumours s.c. was compared with their capacity to neutralise EPF activity in medium conditioned by B16 tumour cells in vitro ($10^5$–$10^6$ cells/ml) and in serum from mice collected on day 10 of pregnancy. Dilutions of mAbs, in the range 1 mg/ml–1 pg/ml, were prepared in HBSS/BSA; 0.1 ml of each was incubated for 0.5 h at 37° C with an equal volume of undiluted conditioned medium or serum then the mixtures were tested for EPF in the rosette inhibition test. The lowest concentration of each mAb that completely neutralised the EPF activity was determined to gauge the relative affinity of each mAb for the different preparations of EPF.

*Effect of anti-EPF mAb administration on tumour establishment.* Preliminary experiments were performed to determine an effective mAb dose regimen for limiting the development of tumours. Groups of mice ($n = 4$) were inoculated at $t = 0$ with $10^6$ MCA-2 cells and treated in the following ways: (a) no treatment; (b) $4 \times 0.5$ ml 0.9% w/v sodium chloride (saline) at 0, 24, 48 and 96 h; (c) $4 \times 500$ µg control mAb 7/331 in 0.2 ml HBSS at times indicated in (b); (d) 1 mg anti-EPF 7/342 in 0.2 ml HBSS at the time of tumour cell inoculation; (e) $4 \times 500$ µg anti-EPF 7/342 in 0.2 ml HBSS at times indicated in (b). Injections were directly into the site of tumour cell inoculation. Using the same protocol, tumour cells were pre-incubated in 1 mg anti-EPF 7/342 in DMEM +10% fetal calf serum (not inactivated) for 0.5 h at 37° C, and washed three times in HBSS/1% mouse serum before inoculation. On day 13, the diameter of the tumours was measured with vernier callipers.

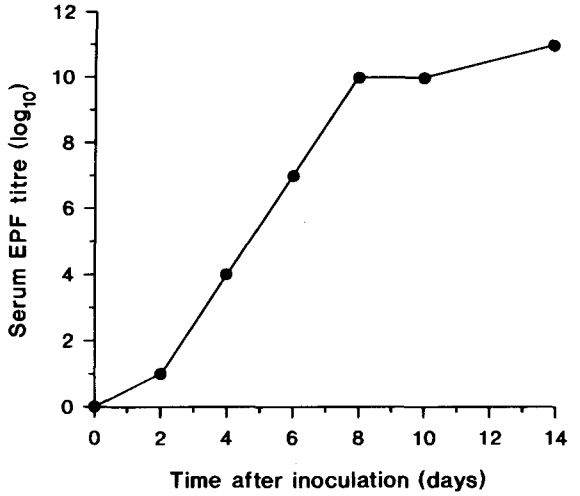


**Fig. 1.** Time course of the appearance of early pregnancy factor (*EPF*) in serum of C57BL/6 mice after inoculation s.c. with $10^6$ viable B16 tumour cells. Serum was titrated and each dilution tested in the rosette inhibition test. The appearance of EPF is expressed as $\log_{10}$ (reciporcal of the highest dilution of serum positive in the rosette inhibition test [Serum EPF titre ($\log_{10}$)]. The results shown are means of tests done on two mice

**Table 1.** The lowest concentration of anti-EPF mAbs to neutralise all EPF activity in various samples

| | anti-EPF[a] mAb 5/341 | mAb 5/333 | (ng/ml) 7/342 |
|---|---|---|---|
| Mouse pregnancy serum[b] (EPF titre $10^5$)[e] | 0.1 | 1.0 | 1000 |
| B16 cell complete medium[c] (EPF titre $10^8$)[e] | 0.01 | 0.1 | 100 |
| In vivo B16 mouse serum[d] (EPF titre $10^{11}$)[e] | 0.01 | 0.1 | 100 |

[a] The lowest concentration of anti-EPF mAb to neutralise all EPF activity in a sample

[b] Serum from mouse taken on day 10 of pregnancy

[c] Supernatant from confluent culture of B16 tumour cells ($10^5 - 10^6$ cells/ml) in Dulbecco's modified Eagle's medium +10% fetal calf serum

[d] Serum from C57BL/6 mice, 14 days after inoculation of B16 tumour cells

[e] EPF titre is the reciprocal of the highest dilution of the preparation positive in the rosette inhibition test

In the following experiments, the growth of the tumours was assessed by estimation of tumour weights [9, 15]. First, a normal growth curve of MCA-2 tumour cells inoculated s.c. into mice was established. A group of mice ($n = 4$ ) was inoculated on day 0 with $10^6$ cells s.c. and, on days 9, 13 and 15, the length and width of the tumours were measured using vernier callipers; measurements were adjusted for skin thickness, which was measured on the flank opposite that with the tumour, and subtracted from the tumour measurements. Estimations of the tumour weight ($m$) were then made from the adjusted linear measurements using the formula $m$ (mg) = $(a^2 \times b)/2$, where $a$ represents the width and $b$ the length, both in millimeters [9, 15].

Based on the findings in the preliminary experiment that $4 \times 24$ h doses of 500 µg anti-EPF 7/342 mAb after tumour cell inoculation was most effective in limiting the establishment of the tumours (Fig. 2), this procedure was repeated with decreasing amounts of mAb to establish a dose/response curve. Doses of 50, 100, 250 or 350 µg anti-EPF 7/342 mAb were given to mice ($n = 3$) in 0.2 ml HBSS at 0 h, 24 h, 48 h and 72 h after MCA-2 cell inoculation. As it had been established that four doses of 500 µg control mAb 7/331 had no effect on the development of the tumours (Fig. 2), the control group ($n = 3$) in this experiment received 0.2 ml HBSS alone. On day 9, the weight of each tumour was determined, as previously described.

*Administration of anti-EPF into palpable B16 tumours.* Mice with palpable tumours were selected 7 days after tumour cell inoculation. Two groups ($n = 6$) received injections of either 0.5 ml DMEM (control group) or 500 µg anti-EPF 5/341 in 0.5 ml DMEM, directly into the tumour tissue on days 7, 8, 9 and 10 after inoculation. Each mouse received a total of 2 mg antibody. On day 13 the size of the tumours was measured and the weight calculated, as described above.

*Effect of anti-EPF on the rate DNA synthesis in established tumours.* Mice with palpable B16 tumours on day 7 after inoculation were injected at 5 p.m. on day 7 with 0.2 ml DMEM or with 0.2 ml DMEM containing 6 mg anti-EPF 5/341 or 5/333 or control 7/331 s.c. directly around the tumour site. Next morning mice received 10 µCi [*methyl*]-[$^3$H]thymidine 5′-triphosphate ([$^3$H]thymidine; Amersham, UK) in 0.1 ml saline by cardiac puncture, under light halothane anaesthesia. Mice were exsanguinated 2 h later and tumour tissue excised. Serum was harvested from the blood and 100-fold dilutions, prepared in HBSS/BSA, were tested in the rosette inhibition test to determine the relative amount of active EPF remaining in the circulation; the negative controls were serum samples from untreated mice and positive controls were from tumour-bearing mice receiving no antibody. Excised tumour tissue was tested for [$^3$H]thymidine uptake and DNA concentration as follows.

*Determination of [$^3$H]thymidine uptake.* Tumour tissue was homogenised in five volumes of ice-cold deionised water. A sample of homogenate (2 ml) was mixed with an equal volume of 10% w/v trichloroacetic acid and precipitated on ice for 1 h. The precipitate was centrifuged (400 *g*, 10 min, 4° C) and washed three times with 5% trichloroacetic acid. DNA was extracted from the precipitate by heating (0.5 h, 70° C) in 1 ml 5% trichloroacetic acid. After centrifugation, the supernatant was removed and the precipitate washed with a further 0.5 ml 5% trichloroacetic acid; the supernatant and final wash were pooled. A 1-ml sample of extract was mixed with 10 ml scintillation cocktail (Optifluor, Packard Instrument Company, Downer's Grove, Ill., USA) and β emissions counted. The remaining extract was used for estimation of DNA content. The uptake of [$^3$H]thymidine in the tissue was expressed as cpm incorporated/µg DNA.

*Estimation of DNA concentration.* The DNA content of extracts was estimated using the method of Burton [4]. Phenylamine reagent [0.2 g phenylamine (Sigma) dissolved in 20 ml glacial acetic acid/0.55 ml concentrated sulphuric acid] was freshly prepared for each estimation. The unknown samples (0.2 ml) were mixed with 0.4 ml phenylamine reagent, boiled for 10 min, and the absorbance of each sample was read at 600 nm. A standard curve was constructed with known concentrations of calf thymus DNA (Sigma) diluted in 5% w/v trichloroacetic acid from a stock solution containing 1 mg/ml in 10 mM Tris/HCl/1 mM EDTA, pH 7.5.

## Results

### *Time course of serum EPF after tumour cell inoculation*

After inoculation s.c. of B16 tumour cells into C57BL/6 mice, EPF appeared in serum within 48 h. The time course of EPF production is shown in Fig. 1. Similar results were obtained when MCA-2 tumours were palpable in CBA mice ($n = 2$); on day 7 the EPF titre of sera from both mice was $10^8$ and by day 10 it was increased to $10^{10}$.

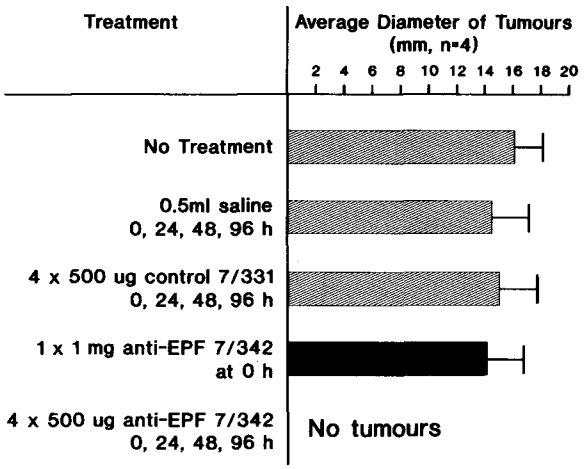


**Fig. 2.** Preliminary experiments investigating the effect of EPF neutralisation on MCA-2 tumour development. Doses of anti-EPF mAb 7/342 or control mAb 7/331 were administered to CBA mice (four mice per group) at the site of MCA-2 tumour cell inoculation at the times indicated. On day 13, the diameter of the tumours was measured with vernier callipers. The results shown are the mean (+ SD) of these values in each group

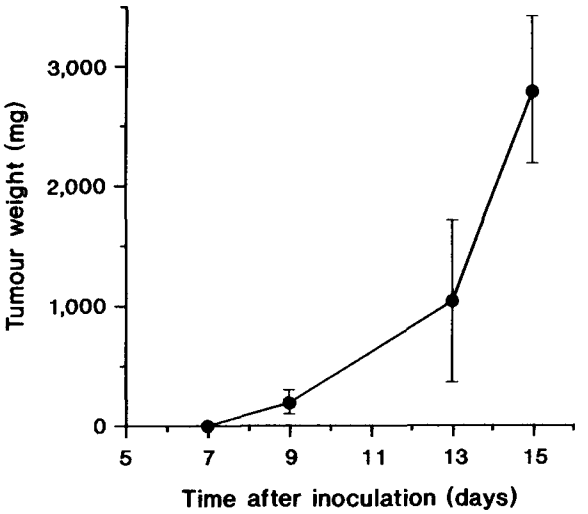


**Fig. 3.** Normal growth characteristics of MCA-2 tumour cells determined as tumour weight. Palpable tumours were evident in all mice ($n = 4$) 7 days after inoculation of $10^6$ viable MCA-2 tumour cells s.c. into CBA mice. The width and length of the tumours were measured on days 9, 13 and 15 using vernier callipers. After adjustment for skin thickness, the tumour weight ($m$) was estimated using the formula $m$ (mg) = $(a^2 \times b)/2$, where $a$ represents the width and $b$ the legnth (both in mm). The results are illustrated as means (± SD) of each group

*Neutralisation of EPF by anti-EPF mAbs*

The titration of purified anti-EPF mAbs against the activity of EPF in the rosette inhibition test revealed a difference in the reactivity of the three anti-EPF mAbs against tumour and pregnancy-derived material (Table 1). A comparison of the EPF titre of medium conditioned by B16 tumour cells in vitro and serum obtained from a mouse bearing a B16 tumour s.c. suggested that there was 1000-fold more EPF in serum. In concordance, 1000-fold more anti-EPF was reqired to neutralise the activity of serum. In contrast,

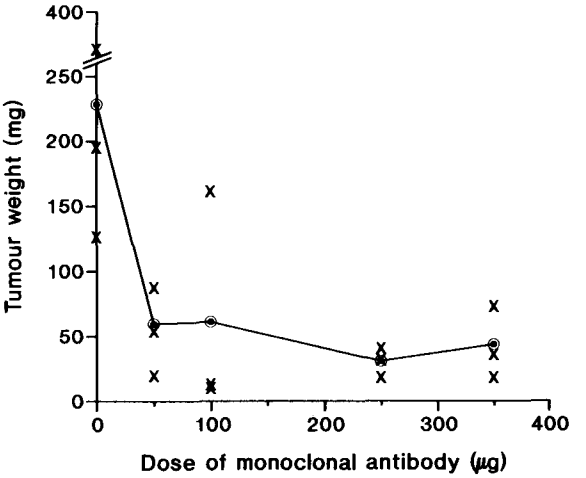


**Fig. 4.** Dose-dependent effect of anti-EPF administration on the establishment of MCA-2 tumours. Doses of anti-EPF mAb 7/342 (50, 100, 250 or 350 µg) were administered at the site of tumour cell inoculation to CBA mice (three mice per group) 0, 24, 48 and 72 h after inoculation of $10^6$ MCA-2 tumour cells s.c. The width and length of the tumours were measured on day 9 using vernier callipers. After adjustment for skin thickness, the weight of each tumour (×) was estimated using the formula $m$ (mg) = $(a^2 \times b)/2$, where $a$ represents the width and $b$ the length (both in mm); the mean tumour weight (⊙) is indicated. Administration of control mAb 7/331 did not affect the tumour growth (see Fig. 2)

the EPF titre of mouse pregnancy serum was 1000-fold less than that of B16 complete medium, but only 10-fold less mAb was needed to neutralise its activity, indicating qualitative differences between the pregnancy- and tumour-derived materials.

*Anti-EPF mAb 7/342 inhibits tumour establishment*

The results of preliminary experiments to determine an effective dose regimen for limiting the establishment of MCA-2 tumours s.c. are shown in Fig. 2. The prolonged exposure of the tumour to anti-EPF mAb (four doses over 4 days) was the most effective dose regimen, with these mice having no palpable tumours by day 13, when all mice in the other groups bore sizeable tumours. On day 21, two out of four mice in the affected group had very small palpable tumours, demonstrating that some viable tumour elements survived the antibody treatment.

That the anti-EPF mAb 7/342 was not retarding the tumours by antibody-dependent cell-mediated cytotoxicity, due to the tumour-cell-bound IgM [18b], is indicated by the normal development of tumours after the cells were preincubated for 0.5 h in anti-EPF mAb and washed before inoculation. The average tumour diameter in this group ($n = 3$) on day 13 was 14.5 ± 5.00 mm, which was not significantly different from that of untreated tumours (Fig. 2).

In the next series of experiments, the size of the tumours was estimated as tumour weight. The normal growth characteristics of the MCA-2 tumour, inoculated s.c. into CBA mice, are illustrated in Fig. 3. As the preliminary study demonstrated that 4 × 24-h doses of 500 µg anti-EPF 7/342 mAb after tumour cell inoculation were most effective in limiting the establishment of tumour (Fig. 2), this

**Table 2.** Tumour DNA synthesis is suppressed after administration of anti-EPF mAbs to C57BL/6 mice with palpable tumours

| Treatment[a] | DNA synthesis[b] | | | Serum EPF titre[c] | | |
|---|---|---|---|---|---|---|
| | *n* | cpm/μg DNA (mean ± SD) | *P*[d] | *n* | $\log_{10}$ EPF titre (mean ± SD) | *P*[d] |
| Saline | 8 | 56.65 ± 12.63 | – | 7 | 8.0 ± 1.2 | – |
| 6 mg anti-EPF 5/341 | 4 | 37.33 ± 14.7 | <0.05 | 4 | 5.0 ± 2.0 | <0.02 |
| 6 mg anti-EPF 5/333 | 5 | 24.21 ± 6.3 | <0.001 | 5 | 4.0 ± 1.6 | <0.001 |
| 6 mg control 7/331 | 5 | 60.90 ± 13.2 | NS | 4 | 8.5 ± 1.0 | NS |

[a] Preparations administered to mice s. c. into the tumour site

[b] [$^3$H]Thymidine injected into mice by cardiac puncture, 16 h after antibody administration and 2 h prior to tumour excision, followed by determination of cpm/μg DNA. *n*, number of mice tested

[c] Serum EPF titre of sample (see Table 1) taken at time of tumour excision. *n*, number of mice tested

[d] Student's *t*-test; NS, not significant

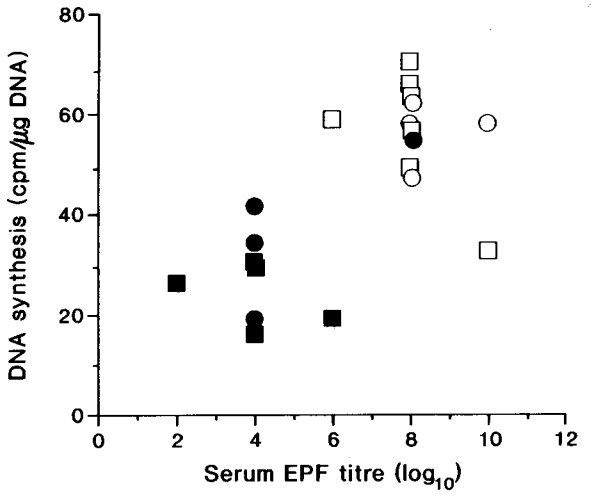


**Fig. 5.** Decreased tumour DNA synthesis, after anti-EPF administration, is associated with decreased serum EPF. C57BL/6 mice were inoculated with $10^6$ viable B16 tumour cells. After 7 days, anti-EPF 5/341 or 5/333 (6 mg), control mAb 7/331 (6 mg) or saline was administered into the established tumours, followed 16 h later by 10 μCi [$^3$H]thymidine in 0.1 ml saline by cardiac puncture. The mice were exsanguinated and tumour tissue excised 2 h later. Serum was harvested from the blood and EPF titre determined. DNA was extracted from tumour tissue and the uptake of [$^3$H]thymidine in tissue expressed as cpm/μg DNA. ●, Anti-EPF mAb 5/341; ■, anti-EPF mAb 5/333; ○, control mAb 7/331; □, saline

procedure was repeated with decreasing amounts of mAb to establish a dose/response curve. The results showed that, when assessed 9 days after cell inoculation, administration of considerably lower doses of anti-EPF mAb were still effective in retarding growth of the MCA-2 tumour (Fig. 4).

*Anti-EPF mAb inhibitis the growth of established tumours*

Four doses of anti-EPF 5/341, administered daily into palpable B16 tumours 7 days after tumour cell inoculation, affected the increase in tumour size such that, when measured 3 days after the last injection, the estimated weight of the anti-EPF-treated tumours [468.6 (±99.0) mg, $n = 6$] was less than half that of DMEM-treated tumours [1109 (±616.3) mg, $n = 6$]. However, as the DMEM-treated tumours showed such a wide variation in size, the statistical significance of these results was questionable.

Determination of the effect of anti-EPF IgM mAbs on the rate of DNA synthesis by the tumour yielded more conclusive results. A single injection of 6 mg anti-EPF 5/341 or 5/333 into mice at the tumour site on day 7 after inoculation was sufficient to inhibit the uptake of [$^3$H]thymidine into the tumour significantly, when measured 18 h later (Table 2). Although the dose of antibody was large, a similar amount of control IgM injected into the tumour had no effect on the rate of tumour DNA synthesis. Furthermore, the specificity of the mAb treatment was confirmed by determining the serum EPF titre after antibody treatment. Only mice with significant reductions in serum EPF titre showed significant decreases in tumour DNA synthesis (Table 2, Fig. 5).

## Discussion

To date, a number of EPF-related factors have been identified from various sources. Their relationship to each other was indicated, first, by their shared capacity to induce from lymphocytes the genetically restricted suppressor factors, which mediate the activity of EPF in the bioassay – the rosette inhibition test [21, 22], and, secondly, by serological cross-reactivity assessed with polyclonal antisera raised against purified preparations of pregnancy EPF [17]. More recently, close similarities in their biochemical characteristics have been demonstrated (Cavanagh et al., manuscript in preparation). Definitive characterisation of these factors and their relationship to each other is still underway.

The availability of mono-specific anti-EPF mAbs has enabled studies that confirm the presence of shared epitopes within the pregnancy- and tumour-derived substances and assess the biological requirement for these active substances. The neutralisation studies reported here have identified qualitative differences in the capacity of the anti-EPF mAbs to neutralise the activity of tumour- and pregnancy-derived material in the bioassay. There may be two explanations for the appparent difference. It may be a reflection of differences in the structure of the serologically cross-reactive EPF species. The EPF, used for immunisa-

tions in the production of the mAbs, was purified from human choriocarcinoma cell conditioned medium and it is, therefore, likely that the mAbs would show a greater affinity for tumour-derived EPF. Alternatively, a difference in the specific activity of the pregnancy serum may be responsible for the apparent decreased affinity of the anti-EPF mAbs. Cavanagh et al. [6] have demonstrated that the specific activity of EPF in different samples is not constant and can be altered by other factors in the sample. One inhibitor of EPF has been identified in mouse serum during oestrus and the pre-implantation period [6]. Similar factors may be circulating in later pregnancy, which affect specific activity such that a greater amount of EPF is required for response in the bioassay. As a result, a greater amount of antibody would be required for neutralisation.

EPF appears rapidly in the serum of mice inoculated s.c. with B16 or MCA-2 tumour cells and these studies have demonstrated that anti-EPF mAbs are able to limit the growth of both these tumours in vivo. The transplantable tumours studied here have been well established as tumour lines for some time and grow rapidly when transplanted in vivo. In the first set of studies, it was established that anti-EPF administration, over a 4-day period after tumour cell inoculation, was effective in limiting the size of the tumours when measured on days 9 and 13. In the second set of studies, the effect of anti-EPF administration on the growth of established tumours was investigated. This was a much more stringent test of the anti-proliferative capacity of the mAbs, as, at this stage, the tumour burden is increased substantially and the tumour has established advantageous interactions with the host, such as extensive vascularisation. Intitial studies, which monitored the effect of anti-EPF administration on the size of the tumours, suggested that anti-EPF was affecting the growth of the tumours. However, the relevance of tumour size to the actual growth rate of the tumour is debatable as other factors such as degrees of fibrosis, fat or water content could affect the result obtained, particularly when various solutions have recently been injected directly into the tumour tissue.

Determination of the rate of DNA synthesis by the tumours provided conclusive evidence that the anti-EPF mAbs were inhibiting the growth of established tumours. That a decrease in the serum EPF titre was associated with inhibition of tumour DNA synthesis confirms that EPF is required by tumour cells to maintain optimal levels of cell division. In these studies the antiproliferative effect of EPF neutralisation was determined on highly anaplastic, transplantable murine tumours. The relevance of EPF production to the progression of more differentiated, primary human tumours must now be investigated.

The limitation of tumour cell growth in vitro [17] is apparently due to anti-EPF mAbs interfering with a mechanism that acts directly to maintain the tumour cell in the active phases of growth. In vivo, there may be additional host mechanisms that also act to limit the tumour growth after anti-EPF administration. The capacity of EPF to initiate a suppressor cell cascade, the soluble products of which are active in the EPF bioassay, has been established [21, 22]. Preliminary studies have confirmed the presence of the EPF-induced suppressor factors in the serum of CBA mice bearing MCA-2 tumours s.c. (B. E. Rolfe, personal communication). Whether the inhibition of in vivo tumour growth was due in some part to the abrogation of the immunosuppressive properties of EPF is unclear at this point. A number of studies, analysing the reaction of the host's immune system against syngeneic transplantable tumours, have described the induction of suppressor cells. Haubeck and Kolsch [10] demonstrated that activation of specific suppressor cells appears to be the first reaction of the host's immune system during the initial phases of tumorogenesis. The consequence of this induction was the suppression of a T cell cytotoxic response against a transplantable plasmacytoma, syngeneic in BALB/c mice. The relevance of the EPF-induced suppressor factors to survival of the tumour in vivo must be investigated further.

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