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Therapy of Spontaneously Metastatic HSV-2 Induced Hamster Tumours with Cortisone Acetate Administered with or without Heparin

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Abstract—*Subcutaneous injection of cortisone acetate administered with or without oral heparin retarded the growth of two HSV-2 induced hamster fibrosarcomas. Histological sections showed no obvious difference between the vasculature of treated and untreated tumours although there were fewer infiltrating lymphocytes in treated tumours. Treatment was, however, found to be ineffective against metastatic development following resection of primary tumours. Natural killer cell activity was found to be greatly reduced in animals receiving heparin and/or cortisone acetate treatment and this may influence the effectiveness of treatment on the metastatic process. We conclude that treatment of tumours with cortisone plus heparin is no different from the response to cortisone used alone.*

INTRODUCTION

THE INITIAL growth phase of embolic tumour deposits is an avascular process relying on diffusion to supply nutrients and remove metabolic wastes [1]. For development to continue, however, the tumour mass must establish its own blood supply [1–2] and recruitment of host endothelial cells to form an intratumoural capillary network has been shown to be augmented by the secretion of a soluble factor from the tumour, termed Tumour Angiogenic Factor (TAF) [3]. TAF production has been demonstrated in several *in vitro* and *in vivo* tumours [2–5] and the importance of this substance on tumour expansion has been suggested as a possible method of tumour chemotherapy [6]. Falkman *et al.* [7] found that cortisone acetate administered with heparin inhibits vascularization on the chick chorioallantoic membrane. This group [7] also reported that treatment *in vivo* caused the partial, and in some cases complete, regression of some established mouse tumours, although certain chemically-induced mouse neoplasms have proved resistant to this treatment protocol [7]. Inhibition of tumour angiogenesis was described as the mechanism of reduced or regressing tumour development. In other experiments hydrocortisone alone was reported to cause tumour regression or a reduction in tumour growth rate, in both spontaneous and chemically-induced murine sarcomas [8] and was attributed to a reduction in the number of tumour infiltrating macrophages. In contrast, Folkman *et al.* [7] found no antitumour activity using cortisone alone. In the present communication we report on the effects of cortisone acetate administered with or without heparin on the primary growth and metastatic capacity of a herpes-induced tumour of hamsters [9, 10, 11, 12, 13]. The most effective type of heparin in Folkman's studies (Panheparin, Abbot) is no longer commercially available and in the present investigation sodium heparin from Weddel Pharmaceuticals was employed.

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MATERIALS AND METHODS

Animals

Male Syrian golden hamsters aged 6–10 weeks and weighing 60–90 g were used in all experiments. The animals were obtained from a closed randomly-bred colony at the University of Sheffield, and have previously been shown to be syngeneic by skin-grafting experiments (Potter and Jennings, unpublished findings) and *in vitro* mixed lymphocyte reactions (Teale, unpublished findings). All animals were fed sterile food and given

sterile water. Hamsters consumed an average of 7 ml drinking water/animal/day. In all experiments 10 hamsters were used per group unless otherwise stated.

Tumours

The HSV-2-333-2-26 cell line used in the present study was originally derived by *in vitro* transformation of hamster embryo fibroblasts with inactivated HSV-2: this cell line was kindly provided by Dr. F. Rapp, Department of Microbiology, Pennsylvania State University, Hershey, Pennsylvania, U.S.A. and is referred to as the Parent cell/tumour. This cell line has been shown to be weakly metastatic *in vivo* [9, 10]. Following resection of parent tumours growing s.c. a lung foci was excised from a single animal showing macroscopic metastases, and maintained by s.c. transplantation. This tumour was designated Met B [9, 10]. Cell suspensions of both Parent and Met B subcutaneous tumours were prepared by removing necrotic tissue from excised tumours of 1.0–1.5 cm dia., and digesting the tumour fragments with 0.25% trypsin (Difco 1 : 250) containing DNase at a concentration of 0.02% (w/w) to prevent cell clumping. Cell suspensions were washed three times in Hanks Balanced Salt solution (Hanks BSS), and resuspended in Hanks BSS to the required concentration. Only cell preparations of greater than 90% viability, as assessed by trypan blue exclusion, were used for animal inoculation.

Hamsters were inoculated s.c. with 1×10^3 cells in an 0.1 ml vol. unless otherwise specified. Tumour growth was assessed daily by measuring tumour diameters, or by weighing tumours following their excision. To evaluate metastatic potential, subcutaneous tumours of 1.0–1.5 cm dia. were resected, and the animals observed for signs of illness or respiratory distress [9] at which time the animals were killed and the extent of metastatic spread assessed by post-mortem examination.

Chemicals

Cortisone acetate injections BP (25 mg/ml), without preservative, was obtained' from Boots Drug Company PLC, Nottingham, England: injections were given s.c. into the right flank. Sodium heparin injection B.P. (25,000 U/ml), with 0.15% chlorocresol, was obtained from Weddel Pharmaceuticals, Ltd., Wrexham, Clwyd, U.K. and administered in the drinking water.

Natural killer (NK) cell cytotoxicity assay

Peripheral blood lymphocytes were recovered from normal and heparin and/or cortisone acetate-treated animals by cardiac puncture and separated on lymphocyte separation medium as described previously [14]. The lymphoid cells were removed, washed twice in buffer (RPMI–NBCS), and fractionated on nylon wool columns. Non-adherent cells were eluted from the column, washed and counted and the cytolytic activity measured against K562 in a 4 hr chromium-release assay the details of which, including target cell labelling, medium and incubation requirements, have been described previously [14, 15].

Statistics

Non-parametric $2 \times K$ contingency test or linear regression analysis carried out on the growth rates of the Parent and Met B cell lines has previously shown no significant difference between the growth rate of tumours within each experiment nor between experiments [9]. Statistical analysis of results presented here between groups of tumours within individual experiments were analysed by means of Students *t*-test.

RESULTS

Effect of cortisone acetate and heparin on subcutaneous tumour growth

To assess the activity of heparin and cortisone acetate on tumour growth, hamsters were inoculated s.c. with 1×10^3 live tumour cells, and palpated daily for the appearance of tumour nodules. After approx. 20 days when primary tumours had reached 0.2–0.5 cm dia., the animals were divided into groups for treatment. One group of animals (10 hamsters/group), received cortisone acetate at an initial dose of 250 mg/kg body weight daily for 6 days followed by 125 mg/kg for 1 day, 62.6 mg/kg for 1 day and then a maintenance dose of 31 mg/kg per day (the same dose schedule used in Folkman's study [7]); the second group received the same cortisone acetate treatment, but were also given 500 U/ml of heparin in the drinking water; and a third group of animals received no treatment. All animals were examined twice weekly for tumour growth, and the results are shown in Fig. 1.

The growth of Parent and Met B tumours in control animals increased rapidly from day 16, and reached 2.0 cm dia. by day 31; animals were killed when the primary tumours reached 2.5–3.0 cm dia. In contrast, tumours in animals given cortisone acetate or cortisone acetate and heparin grew less quickly, and at 34–36 days post-inoculation the mean diameter was 1.0 cm (Fig. 1). This was statistically significant ($P = <0.001$). In order to determine the optimal concentration of cortisone acetate and heparin required for treatment the amount of each drug administered was varied whilst keeping the other drug constant. The

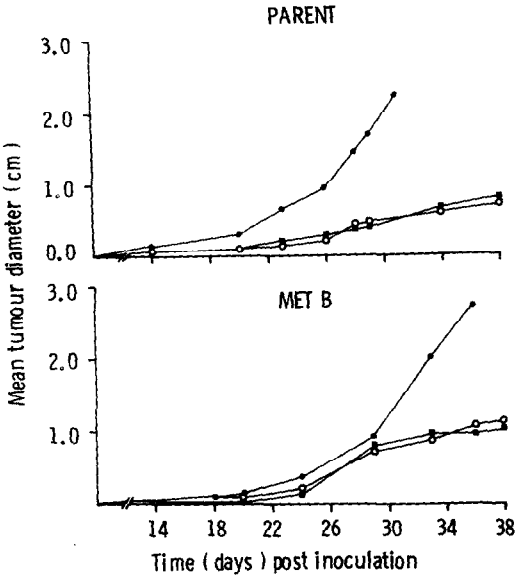

Fig. 1. The effect of cortisone acetate (C/A) administered with or without heparin on the growth of parent or Met B primary tumours. (○—○) 250 mg/kg C/A days 1–6; 125 mg/kg C/A day 7; 62 mg/kg C/A day 8; 31 mg/kg on day 9 and maintained on this dose: plus 500 U heparin/ml drinking water. (■—■) cortisone acetate given as above. (●—●) control animals. The difference between the control group and the test groups is statistically significant (P = <0.001).

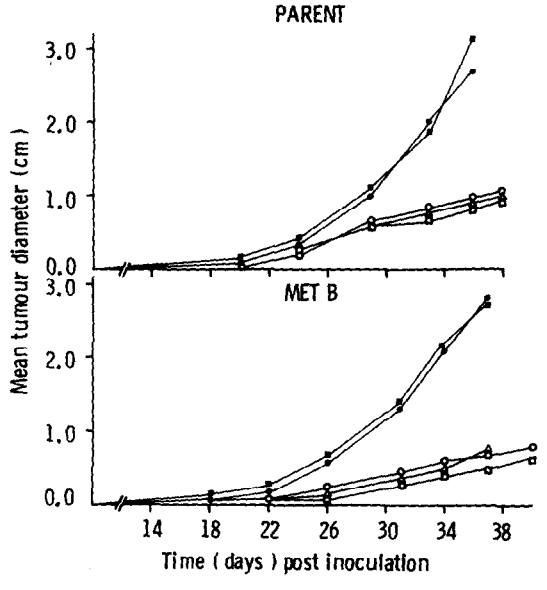

Fig. 2. Effect of varying concentrations of cortisone acetate (C/A) on parent or Met B primary tumour growth. C/A given as follows: X mg/kg days 1–6; $\frac{X}{2}$ mg/kg day 7; $\frac{X}{4}$ mg/kg day 8 and maintained. Starting concentrations: (○—○) 250 mg/kg C/A plus 500 U heparin/ml water; (□—□) 25 mg/kg C/A plus 500 U heparin/ml water; (■—■) 2.5 mg/kg C/A plus 500 U heparin/ml; (△—△) 25 mg/kg C/A only; (●—●) Control. There was a significant reduction (P = <0.001) *in tumour growth rate for animals inoculated with cortisone acetate compared with control animals. There was no significant difference between tumour growth rates for control animals (●—●) and animals given the lowest dose of cortisone acetate plus heparin (■—■).*

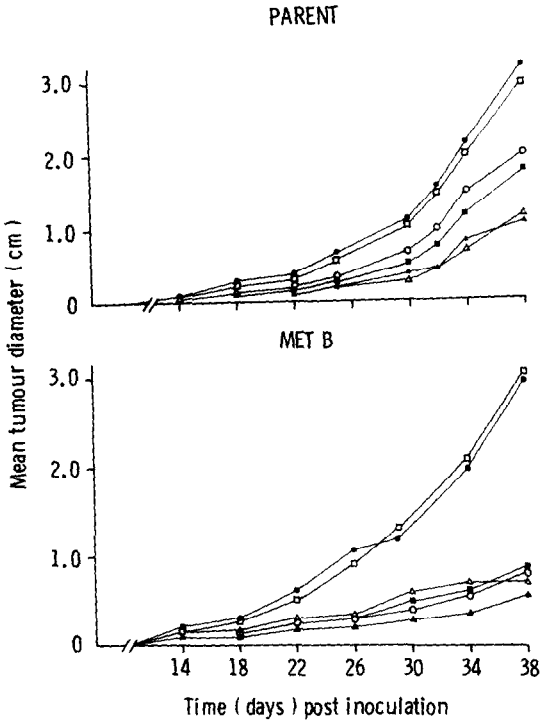

Fig. 3. Effect of varying concentrations of heparin on Met B primary tumour growth. Cortisone acetate given at 25 mg/kg days 1–6, 12.5 mg/kg day 7, 6.2 mg/kg day 8, 3.1 mg/kg day 9 and maintained. (△—△) 250 U heparin/ml drinking water plus C/A; (○—○) 500 U heparin/ml water plus C/A; (■—■) 1000 U heparin/ml water plus C/A; (□—□) 500 U heparin/ml water only; (▲—▲) cortisone acetate only; (●—●) control animals. There was a significant reduction (P = <0.001) *in the tumour growth rate for groups of animals inoculated with cortisone acetate (± heparin) compared to the control group. Animals given heparin alone had similar tumour growth rates to the control group* (P = >0.05). *Tumour growth rate for animals given 250 U heparin plus cortisone or cortisone alone were also reduced compared to animals given 500 U heparin plus cortisone* (P = <0.01). *(Parent tumour only.)*

treatment regime was as described above and the results are shown in Figs 2 and 3. Thus, cortisone acetate was found to be most effective at an initial starting concentration of 25 mg/kg body weight whilst the concentration of heparin was shown not to influence tumour growth. Cortisone acetate at 250 mg/kg was also effective although animals suffered excessive weight loss and died prematurely at this concentration. Post-mortem examination indicated monocytopenia and associated complications to be the cause of death.

Optimal time of treatment

Having determined the optimum concentration for tumour therapy it was necessary to establish the optimal time at which to commence treatment. Owing to the similarity of results obtained for the Parent and Met B tumour all subsequent experiments were confined to just one tumour; Met B. Thus, groups of hamsters were inoculated s.c.with 1×10^3 Met B tumour cells and 24 hr later were

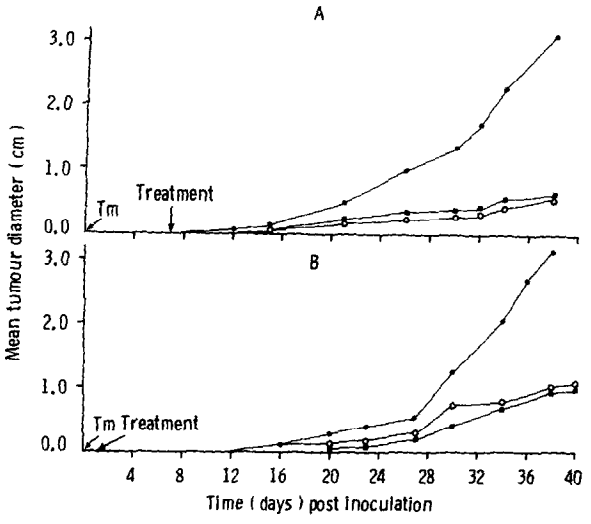

Fig. 4. Effect of early and delayed treatment on Met B primary tumour growth. (○—○) 25 mg C/A/kg days 1–6; 12.5 mg/kg day 7; 6.2 mg/kg day 8, 3.1 mg/kg day 9 and maintained plus 500 U heparin/ml drinking water; (■—■) cortisone acetate only (as above); (●—●) control animals. Tumours inoculated on day 0 and treatment commenced as indicated.

given 25 mg/kg cortisone acetate daily for 6 days followed by 12.5 mg/kg for 1 day, 6.2 mg/kg for 1 day and then a maintenance dose of 3.1 mg/kg per day; a second group of hamsters received the same cortisone treatment but were also given 500 units of heparin/ml drinking water; and control animals received no treatment.

A parallel group of animals was given tumour therapy starting 7 days after tumour cell inoculation. The results are shown in Fig. 4.

Tumours growing in untreated animals reached 2.0 cm dia. by day 34, post-inoculation. In contrast, tumours grew less quickly in cortisone acetate and cortisone acetate plus heparin treated animals given the drugs immediately following tumour cell inoculation; tumours in this group were approx. 1.0 cm dia. by day 40 (Fig. 4B). In experiments where treatment was delayed until 7 days after tumour cell inoculation control animal tumours developed much quicker than those in treated animals, reaching approx. 2.0 cm by 30 days, post-inoculation. In contrast, treated animals had developed tumours which were approx. 0.5 cm mean dia. by day 35, post-inoculation (Fig. 4A).

Effects of cortisone acetate/heparin treatment on tumour metastases

To investigate the effect of heparin and/or cortisone acetate on the growth of metastases, hamsters were inoculated s.c. with 1×10^4 Met B tumour cells and the resulting tumours resected approx. 3 weeks later when the mean tumour diameter was 10–15 mm. Following tumour resection the animals were divided into three groups. One group

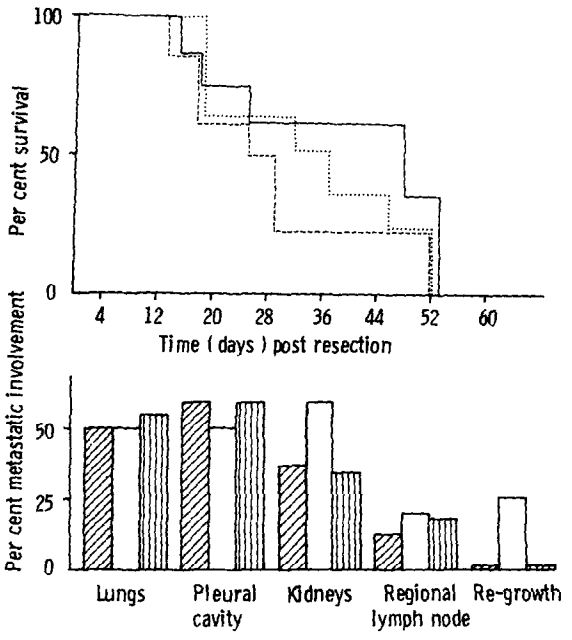

Fig. 5a. Survival of heparin and/or cortisone acetate treated animals following Met B tumour resection. Initial starting concentration of C/A was 25 mg/kg titrating to 3.1 mg/kg on day 9. Heparin was supplied at 500 U/ml drinking water.
(----) Cortisone acetate and heparin treated;
(. . . .) Cortisone acetate treated;
(——) Untreated control animals.
Differences between treated and control animals are not statistically significant by 2 × k contingency test (. . . .) $\chi^2 = 1.6$, P = 50%; (----) $\chi^2 = 1.31$, $P = >50\%$.

Fig. 5b. Incidence and organ distribution of metastatic deposits assessed up to 8 weeks following resection of Met B primary tumours. The number of animals with metastases in each organ was scored as a percentage. Metastatic propensity was essentially bulk involvement since animals were examined when moribund.

□ *Cortisone acetate plus heparin treated;*

□ *Cortisone acetate only;*

□ *Untreated control animals.*

received 25 mg cortisone acetate daily for six days, followed by 12.5 mg/kg on day 7, 6.2 mg/kg on day 8 and maintained with 3.1 mg/kg from day 9. A second group received the same cortisone acetate injection but were also given 500 U/heparin/ml drinking water. The third group were untreated. Animals showing signs of respiratory distress were killed and examined for metastatic spread. Those surviving 12 weeks after resection were killed and a post-mortem examination performed. All control animals had died or been sacrificed by 54 days post-resection and those receiving treatment, cortisone acetate with or without heparin, were dead by 52 days post-resection (Fig. 5A). This difference was not statistically significant ($P = >0.05$) On post-mortem examination the incidence of metastases was the same in all these groups, all animals showing extensive metastatic involvement in the

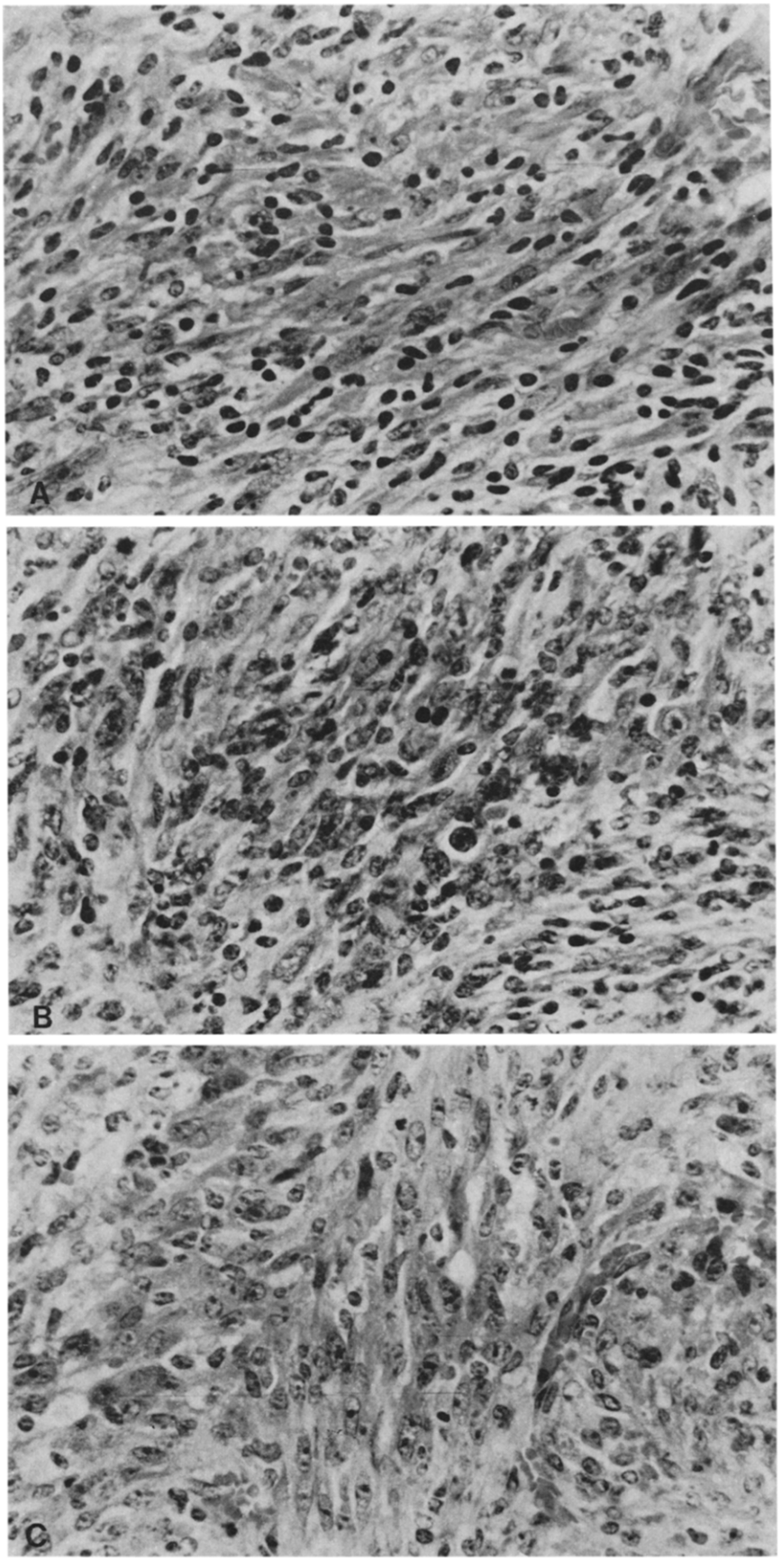

Fig. 7. Tumour histology: (*a*) *untreated tumour showing marked infiltration by lymphocytes and macrophages*; (*b*) *cortisone acetate and* (*c*) *cortisone acetate and heparin-treated tumours showing diminished infiltration. Haematoxylin and eosin* (× 520).

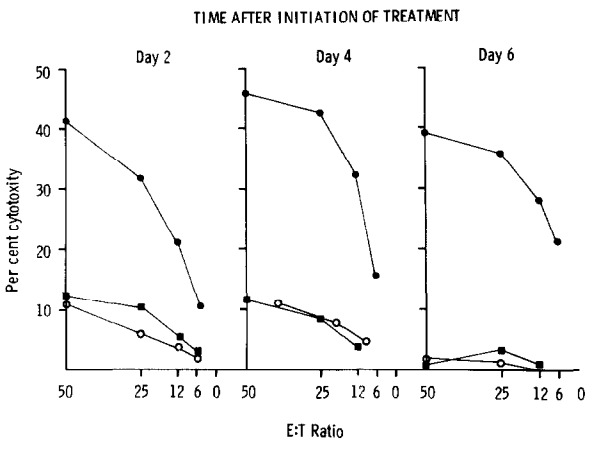

Fig. 6. Natural cytotoxicity, assessed in a 4-hr chromium release assay, of hamsters treated with cortisone acetate with or without heparin given in the drinking water. (■—■) 25 mg C/A kg given daily plus 500 U heparin/ml water; (○—○) 25 mg C/A/mg given daily; (●—●) control animals.

lungs, pleural cavity, kidneys and regional lymph nodes (see Fig. 5B).

Effect of cortisone acetate with or without heparin on natural killer (NK) cell activity

Natural killer cells are an important subset of lymphocytes which are postulated to have functional reactivity against tumour formation and metastatic spread [10, 15, 16]. The use of cortisone acetate in mice, however, reduces NK activity [17], induces monocytopenia [18] and inhibits T-cell dependent functions [19] and it was therefore of interest to investigate the NK status of hamsters undergoing cortisone acetate and heparin treatment. Three groups of animals were investigated; one group were inoculated s.c. with 25 mg cortisone acetate/kg for 6 days; a second group were given the same cortisone acetate treatment plus 500 U heparin/ml in drinking water and the third group received no treatment. The hamsters were bled on days 2, 4 and 6 following the start of treatment, and NK activity of PBL's assessed in a 4-hr chromium release assay. The results are shown in Fig. 6. Untreated hamsters showed relatively high levels of NK cytotoxicity on each of the three days tested (Fig. 6). In contrast, both groups of treated animals showed a significant reduction in NK activity ($P = <0.001$), which was completely abolished by day 6. The reduction was similar for cortisone acetate treatment with or without heparin treatment (Fig. 6).

Histological examination of tumours

Met B primary tumours of similar mean diameter were resected from untreated, cortisone acetate treated and cortisone acetate plus heparin treated animals used in the experiments shown in Fig.4; these were examined histologically. Tumours excised from control, cortisone acetate treated or cortisone acetate plus heparin treated animals showed no histologically evident differences in tumour architecture or vascularity. However, both groups of treated animals showed a marked reduction in the number of tumour-infiltrating lymphocytes; this difference in shown in Fig. 7.

DISCUSSION

The present investigation was initiated to confirm and extend the observations that treatment with heparin in the presence of cortisone limited tumour growth in mice [7]. Thus, cortisone acetate together with heparin, or a non-coagulent fraction of heparin, prevented angiogenesis of the chorio-allantoic membrane of a hen's egg, and caused regression of the B16 melanoma, a reticulum cell sarcoma, the Lewis lung carcinoma and a bladder carcinoma of mice (MB49)[7]. The same study failed to show similar results for four transplantable methylcholanthrene-induced tumours. We report here the effect of cortisone acetate, administered with or without heparin, on the growh of two tumour lines of an HSV-2 induced hamster fibrosarcoma [11, 12]. The results showed that cortisone acetate plus heparin decreased the rate of tumour growth, although cortisone acetate alone showed the same property. Although these results are in contrast to those of Folkman's who showed no antitumour activity of cortisone alone [7], they are in agreement with Penhaligon and Camplejohn [21] who have recently described the ineffectiveness of heparin and cortisone on RIF-1 tumours and mouse mammary tumours of C3H/He mice. In addition to this cortisone treatment proved extremely toxic in our hamster model and in the C3H/He mouse model [21], a phenomenon not observed by Folkman in his original communication [7]. Histological examination of the tumours from treated and untreated animals indicated that these treatments had no obvious effect upon tumour vascularization; but tumours from treated animals had fewer infiltrating lymphocytes. Thus, the apparent reduction in tumour size may not be due to anti-tumour activity but to fewer total cell numbers brought about by restriction of host cell infiltration. This restriction did not apply to host endothelial cells initiating tumour angiogenesis, since the extent of vascularization in tumours from both treated and control animals was similar.

Studies on the effect of hydrocortisone on the growth of the MFS6 and MN/MCA1 sarcomas, 3LL carcinoma and the M109 carcinoma of mice has shown that this compound inhibited growth of all these tumours *in vivo* but augmented metastases following primary tumour resection or *in vivo* inoculation [8]. Again the results for primary tumours

are similar to those of the present study, and reduced tumour growth in these models was effected by a restriction of infiltrating host cells, since hydrocortisone has been reported to induce monocytopenia which could result in a reduction of phagocytic macrophages/monocytes migrating to and infiltrating the primary tumour [18, 8]. The present studies did not confirm the augmentation of metastases in treated animals [8], and although treated hamsters became ill more quickly than control animals, the incidence of metastases was similar in the two groups. However, since treated animals beame ill more quickly, they were examined earlier for metastases and the experimental procedure may have led to an underestimation of the extent of metastases in treated animals. In addition, treatment may have an augmenting effect on metastases since the extent of metastases can be associated with NK cell activity of this model [10], and cortisone acetate significantly depressed NK cell activity. Retardation of tumour growth, in this system, therefore, seemed to be due to cortisone alone. The activity of the heparin used or the ability of the hamster gut to degrade heparin into an active fragment is, however, an open question.

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