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Comparison of excision versus cryosurgery of an HSV-2-induced fibrosarcoma

I. Survival, extent of metastatic disease and host immunocompetence following surgery

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Summary. Cryosurgery and excision were used to treat primary tumours of HSV-2-transformed hamster tumour sublines, and post-operative survival and the extent of metastatic disease were compared in the two groups. An inferior prognosis was observed following cryosurgery although the extent of metastatic disease was similar in both groups. Using this model it would appear that cryosurgery enhances the development of micrometastases rather than affecting the number of cells shed from the primary tumour during surgery. To investigate the underlying causes of the decrease in survival following cryosurgery, in vitro assays were used to monitor host immunocompetence following surgery. The results showed that whilst natural killer cell cytotoxicity was only marginally depressed, mitogen responsiveness and lymphocyte participation in a mixed lymphocyte reaction were severely reduced 3–7 days post-cryosurgery. In parallel with immunosuppression, extensive cell proliferation in the spleen of cryosurgically treated tumour-bearing animals was observed. Histological examination of the spleen demonstrated the presence of large numbers of transformed cells which correlated with the loss of mitogen responsiveness and the ability to participate in a mixed lymphocyte reaction. Further studies (manuscript submitted for publication) have demonstrated that spleen cells from animals whose tumour is treated by cryosurgery are capable of suppressing immunocompetence in vitro, implying they have a role in the uncontrolled growth of micrometastases in vivo.

Introduction

Cryosurgery is a recognised method by which the controlled cryogenic destruction of benign and malignant tissue may be achieved in situ [26]. Freezing a primary tumour in situ has been reported to induce a specific anti-tumour immune response [27] which it has been claimed destroys tumour cell distant from the primary freezing site [29, 2, 13]. The development of a systemic immune response distant from the primary tumour is of potential significance in the treatment of malignant disease, since radical excision of malignant tumours is only successful if surgery is performed prior to the establishment of micrometastasis, the latter being responsible for many deaths after apparently curative surgery.

Most of the experimental studies used to investigate the potential of cryosurgery in the reduction of metastasis used highly immunogenic, chemically induced tumour models, contrary to current knowledge of human tumour immunogenicity [7, 12]. It was therefore felt relevant to re-examine the effect of cryosurgery on a primary tumour in a less immunogenic model. The *Herpesvirus hominis*-induced tumour in Syrian golden hamsters offers such features. Different sublines are available which display a range of metastatic profiles which correlate with their immunogenicity and in vitro killing by the immune system [30].

This study reports on the effect of cryosurgery on a primary tumour compared to conventional excision, in relation to survival, metastatic disease and host immunocompetence. The study was undertaken using a metastatic tumour line of low immunogenicity (Met B) and a related line which is weakly metastatic and immunogenic (Met G).

Materials and methods

Animals. Male Syrian golden hamsters 10 to 12 weeks old were obtained from a closed, randomly bred colony at the University of Sheffield. Skin grafting experiments between the hamsters resulted in 100% take (Potter and Jennings, unpublished observations). Male MB strain Syrian golden hamsters 10 to 12 weeks old were obtained from an outbred colony (Shamrock Farms, Henfield, West Sussex).

Tumours. The parent tumour line, HSV-2-333-2-26, was derived by in vitro transformation of hamster embryo fibroblasts with UV-inactivated herpes simplex virus type 2. This cell line was provided by Dr. F. Rapp (Department of Microbiology, Pennsylvania State University, Hershey, Pa., USA). Following inoculation of these cells into hamsters, a transplantable fibrosarcoma was established. This cell line has been shown to be weakly metastatic in vivo [25].

Inoculation of 1×10^4 parent tumour cells s.c. into the hamster resulted in the growth of a primary tumour which in 1 out of 20 animals spontaneously metastasised to the lungs and kidney. Individual lung metastasis from such animals were implanted s.c. into fresh animals and Met G and Met B were derived from the resultant tumours [33]. On s.c. injection the clones produced fibrosarcomas which displayed different levels of metastatic disease, Met G was

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weakly metastatic in contrast to Met B which was metastatic [30]. The tumours were maintained in vivo by trocar transplantation.

Tumour cell suspensions were prepared by digesting non-necrotic tumour tissue in a mixture of 0.25% trypsin (Difco 1:250, East Molesey, Surrey) and 0.02% w/v DNase (Sigma Chemical, London) for 20 min at 37° C. The digest was filtered through sterile gauze, the cells washed X3 in phosphate-buffered saline (PBS) and resuspended in PBS at the required cell concentration.

Tumour-bearing (T.B.) animals were prepared by injecting 1×10^4 viable tumour cell s.c. into the flank of the hamster.

Whilst HSV-2 virus was used initially to transform the hamster embryo fibroblasts, viral antigens have not been detected on any of the tumour cell lines using monoclonal antibodies against HSV glycoproteins (Abdulwahab SA, PhD Thesis, The University of Sheffield).

Surgical procedure. Animals were anaesthetised with ether prior to tumour excision or cryosurgery. The excision group had their tumours excised together with overlying skin under aseptic conditions. Cryosurgery was performed with a CS-76 TM liquid nitrogen cryosurgical system (Downs Surgical PLc, Mitchan, Surrey). Pressurised liquid nitrogen was applied to the tumour using a probe tip temperature of −196° C. Peripheral tumour temperature was recorded using a thermocouple located within the tumour to ensure that the whole of the tumour was exposed to a temperature of −40° C or lower. Two freeze-thaw cycles were employed to ensure maximum tumour cell death.

Following surgery on the primary tumour, the animals were observed, and those showing signs of respiratory distress were killed and examined for metastatic disease. The experiment was terminated 100 days after surgery, any surviving animals killed and a post-mortem examination performed.

The effect of surgery on the immune response was also investigated following cryosurgery or excision of animals with and without tumours. Cryosurgery and excision were performed as above on T.B. animals. Tumour-free animals either received two freeze-thaw cycles of cryosurgery on the flank or an incision comparable in size and position to the lesions produced in the T.B. animals which underwent surgery. Additional controls comprised T.B. and tumour-free animals which did not receive surgery.

Preparation of spleen cell suspensions. At various times post-surgery, spleens were aseptically removed and weighed. Single cell suspensions were prepared using sterile steel gauze. The cells were washed in PBS and resuspended in HB103 medium (DuPont Ltd, Stevenage, Herts.) for the blastogenesis assays or in RPMI + 10% fetal calf serum (FCS) for the natural killer (NK) assays. HB103 had previously been shown to reduce the control background variability caused by the mitogenic properties of some batches of bovine serum.

Histology. A piece of spleen and some vertibrae from control and experimental animals were fixed in buffered formal saline, processed routinely into paraffin wax, sectioned and stained with H&E. The vertibrae were decalcified for 72 h with 12% w/v EDTA pH7 prior to processing.

NK cell cytotoxicity assay. The spleen was removed and a single cell suspension made as before, 4 h, 1, 2, 5, 7, 10 and 21 days following surgery. Red blood cells and neutrophils were removed from the suspension by centrifugation over Lymphoprep (Nyeggaard (UK) LTD, Birmingham.) for 20 min at 600 *g*. Cells from the interface were collected, washed in PBS and resuspended in RPMI +10% FCS. The cells were then incubated for 1 h on a nylon wool column (Travenol Ltd, Norfolk.) at 37° C. After incubation, non-adherent cells were eluted from the column with 30 ml of pre-warmed RPMI +10% FCS. The cells were washed and resuspended in RPMI +10% FCS at the required density. The resultant cell suspension has previously been shown to be enriched for hamster NK activity [31].

The hamster NK susceptible targets Raji, a Burkitt's lymphoma cell line and an in vitro culture of Met G were used. Although K562 and YAC, the human and murine NK indicator targets were susceptible to hamster NK, Raji was a more sensitive target for the assessment of hamster NK cytotoxicity [31]. All cell lines were routinely screened for mycoplasma and only mycoplasma-free lines were used as targets. NK cytotoxicity was measured using a 4-h chromium release assay, as previously described [31].

Blastogenesis assays. Concanavalin A (Con A) (Sigma) was used at a final concentration of 10 μg/ml and lipopolysaccharide (LPS) at 25 μg/ml (Sigma). Spleen cells were added to round-bottomed microtitre plates (Gibco, Paisley, Scotland.) in 0.1 ml of HB103 medium at a final concentration of $1.25-2.5 \times 10^6$ cells/ml. Mitogens were added in a further 0.1 ml and the plates incubated at 37° C for 48 h, and pulsed with 0.5 μCi of ^{3}H-thymidine, (20 Ci/mmol) (NEN, Stevenage, Herts.), in 10 μl of PBS for the last 4 h of the assay. At the end of the incubation period the cells were harvested on glass filter paper using a cell harvester and ^{3}H-thymidine uptake measured by scintillation counting.

Mixed lymphocyte reaction (MLR). Single cell suspensions of stimulator lymphocytes were prepared from the spleens of MB hamsters and incubated for 30 min at 37° C with 20 μg/ml mytomycin C (Sigma). They were then washed 3 times in PBS and resuspended in HB103 at 2×10^6/ml.

Responder lymphocytes were suspended in HB103 at 5×10^6/ml. Then 0.1 ml of responder and stimulator spleen cells were incubated at 37° C in round-bottomed microtitre plates for 5 days. At 18 h prior to the end of the assay 0.5 μCi of ^{3}H-thymidine (20 Ci/mmol) was added to each well in 10 μl PBS. The cells were harvested on glass fibre filters and ^{3}H-thymidine uptake determined as before.

Ex-blast assay. It became apparent that the spleens of T.B. animals which had received cryosurgery became grossly enlarged due to an influx of cells which were characterised by their large blast-like appearance and constituted up to 50% of the spleen cell population. To control for possible dilution effects of these cells on the mitogen response of other lymphocyte populations, assays were performed where cultures contained 2.5×10^5 cells excluding blast cells. Since this increased the density of the cultures by up to 50%, additional wells were included with control spleen cells at a similar density.

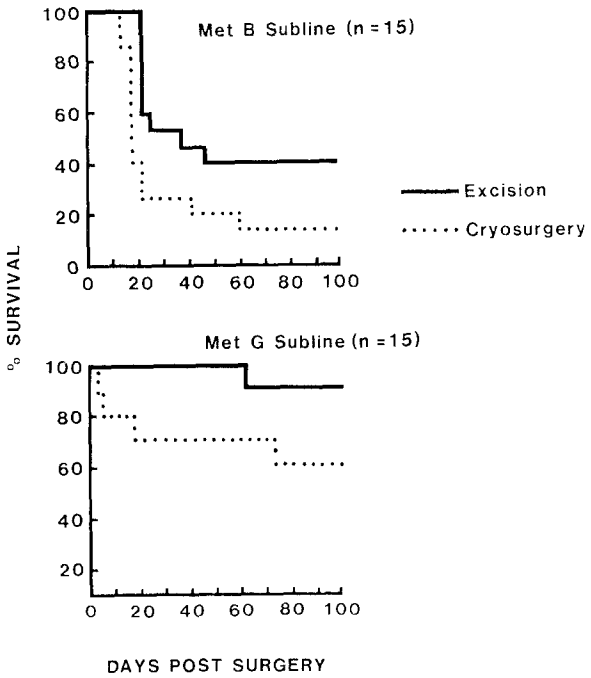

Fig. 1. Percent survival of hamsters following s.c. inoculation of 1×10^4 Met G or Met B, followed by either tumour excision or cryosurgery of the primary tumour at 15 mm diameter

Results

Metastasis of Met G, Met B and S7A following surgery

Tumour tissue was digested, resuspended in PBS and 1×10^4 tumour cells injected s.c. into hamsters. When the tumour reached an average diameter of 15 mm, animals either received excision or cryosurgery. Figure 1 shows representative survival curves following cryosurgery or excision of the two tumour lines.

Cryosurgery of the non-immunogenic metastatic Met B tumour resulted in a significant reduction in survival compared with excision ($P < 0.01$). It was subsequently shown that cryosurgery of the immunogenic weakly metastatic Met G tumour, resulted in the development of metastatic disease in a small number of animals. Despite the consistency of this finding, there was no significant difference in survival following either excision or cryosurgery.

Post-mortem examination of the regional lymph nodes, kidneys, lungs and pleural cavity revealed no significant difference in the distribution or number of metastases in the animals from either group which exhibited metastatic disease following primary Met B surgery (Table 1).

The remaining data was derived from animals which underwent surgery of a primary Met G tumour, however, it was subsequently demonstrated that identical results were achieved when animals with Met B primary tumours were used.

Spleen weight and total white cell number following surgery

Spleen weight and total white cell counts were measured 0.2, 1, 3, 5, 7 and 10 days post-surgery (Fig. 2) Student's *t*-test of the sham and control groups showed there was no significant difference between the two groups ($P > 0.05$) at any time point, or from day to day, and so this data was pooled and expressed as the mean ± 2 X standard error of the mean (± 2 SE). At all the time points spleens from the T.B. and cryosurgery groups were significantly heavier ($P < 0.001$), and contained more cells than the pooled controls ($P < 0.001$). In addition, at day 3, both the spleen weight and cell number in the cryosurgery group was significantly higher than the T.B. controls ($P < 0.001$). Until day 5 there was no significant difference between the excision and T.B. controls. After day 5 both spleen weight and cell number fell in the excision group to within the control range.

Table 1. Percent incidence and organ distribution of metastatic disease following excision or cryosurgery of Met B tumour-bearing animals

Organ	Excision (18/29)[a]	Cryosurgery (25/29)
Regional lymph node	49.5± 7.8%[b]	58.3± 4.6%
Pleural cavity	88.5± 1.0% (11–20)[c]	74.8± 8.5% (11–20)
Lungs	100.0± 0.0% (11–20)	94.0±18.7% (11–20)
Kidney	55.6±15.8% (1–10)	59.0±36.3% (1–10)

[a] Number of animals with metastatic disease/number of animals operated
[b] Percent incidence of metastatic disease ± SD
[c] Number of metastases per organ

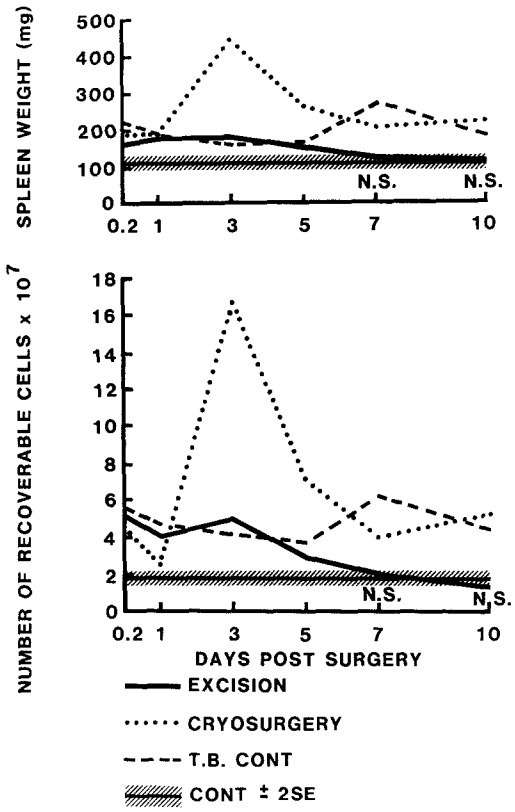

Fig. 2. Spleen weight and the total white cell number were estimated at 0.2, 1, 3, 5, 7 and 10 days post-surgery. Between 3 and 4 spleens were measured per time point for each group. The significance of increase in weight and cells recovered was determined using Student's *t*-test. There was no significant difference ($P > 0.05$) between the sham surgery and the control groups at any time point, or from day to day, and so a mean was estimated to produce a pooled control value ± 2 SE. The cryosurgery, excision and the tumour bearer control groups were significantly different from the pooled control ($P < 0.05$) unless indicated

Histology

Control hamster spleens showed well delineated aggregates of lymphoid tissue around the major arterial branches, this was the white pulp. It was organised zonally into an area adjacent to the artery populated by T-cells, and a more peripheral area of B-cells, the latter may be characterised by a germinal centre. The intervening part of the spleen was composed of a network of sinusoids which usually contained erythrocytes and were designated the red pulp. Beneath the splenic capsule, adjacent to small fibrous septae, and sometimes adjacent to lymphoid aggregates there were small collections of cells with a small dense nucleus; these seemed to be late erythroblasts. Some collections of the latter were associated with larger cells with a more open nucleus and no definite cytoplasmic differentiation: these blast cells could not identified definitively but their anatomical distribution suggested they were also erythroid precursors.

Animals receiving sham excision showed splenic histology identical to controls throughout days 0 to 10. Those animals receiving sham cryosurgery had a transient minor increase of blast cells in the sinusoids on days 3 to 5. The T.B. control animals showed normal appearances in the white pulp but a slightly variable mild to moderate increase in the number and size of erythroblast islands with associated primitive blast cells; there was no change in these features throughout the period of observation.

Animals that had their tumour surgically excised showed a transient though fairly substantial increase in the number of blast cells in splenic sinusoids. This appeared to be maximal at day 1 and by day 5 this sinusoidal infiltration and paratrabecular accumulation of cells had mostly resolved and returned to resemble the appearances in the untreated T.B. group.

In those animals which had their tumour treated by cryosurgery, on day 0 the appearances were similar to those seen in the untreated T.B. animals (Fig. 3). By day 1 primitive blast cells were proliferating in many sites though relatively few late erythroblasts were present. On day 3 the red pulp was filled with enormous numbers of erythroblasts and other primitive blast cells far in excess of the numbers of cells seen in any other situation (Fig. 4). Days 5 to 7 were characterised by falling numbers of primitive cells and a higher proportion of late erythroblasts; this adds further to the contention that the undifferentiated cells were erythroid precursors. By day 10 the cellular proliferation had regressed to levels seen in the other tumour animal groups at the same time (Table 2).

Examination of vertebral bone marrow in control and T.B. animals treated by cryosurgery revealed a mild but just detectable increase in number and size of erythrons (groups of erythropoietic cells) on day 1. On days 3 to 5 erythropoietic activity was increased such that adjacent erythrons virtually ran into one another. By day 7 the appearance had returned to that seen before treatment. Other bone marrow cells appeared unaltered throughout.

NK cell cytotoxicity

The effect of surgery on NK cytotoxicity was investigated using two NK susceptible targets, Raji and Met G (Fig. 5).

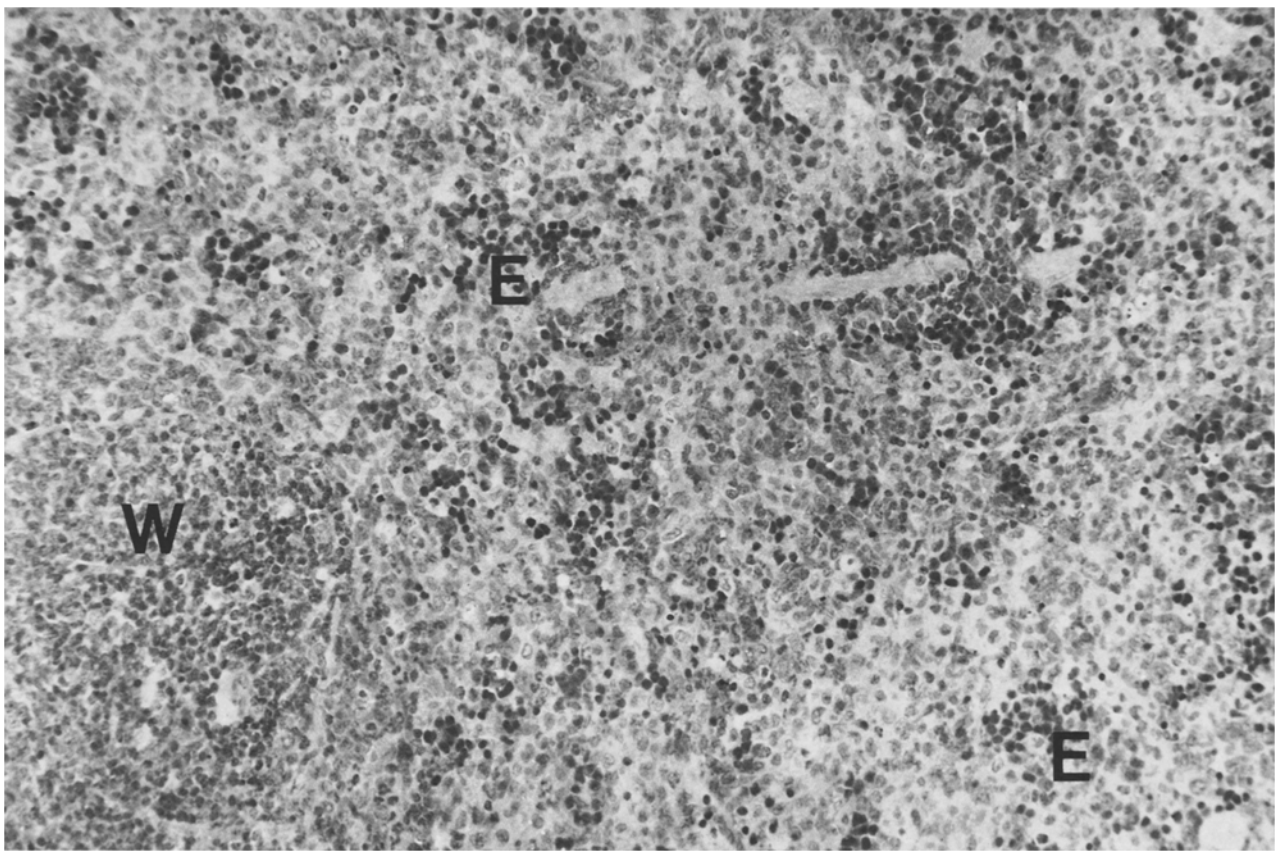

Fig. 3. Hamster spleen, tumour treated by cryosurgery, day 0. White pulp (W) and many groups of erythroblasts (E) in the red pulp. (×280)

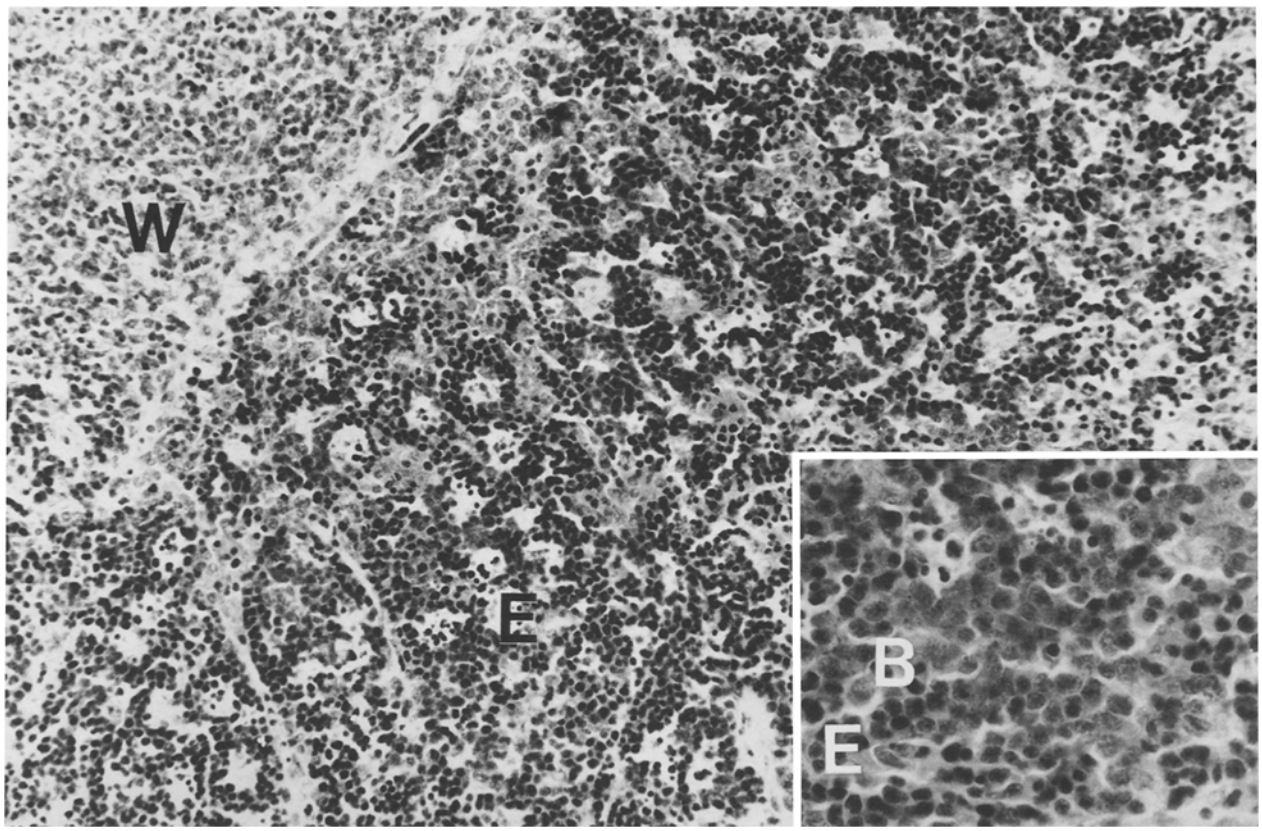

Fig. 4. Hamster spleen, tumour treated by cryosurgery, day 3. White pulp (W); sinusoids are filled with primitive blast cells and erythroblasts (E). The inset shows the blast cells (B) and erythroblasts (E). (× 280, inset × 560)

Table 2. Erythroblasts in hamster spleen

Treatment	Days post-surgery					
	0	1	3	5	7	10
Control	+	+	+	+	+	+
Sham excision	NT	+	+	+	+	NT
Sham cryosurgery	+	+	+/++	+/++	+	+
Tumour-bearing control	+/++	+/++	+/++	+/++	+/++	+/++
Excision	NT	++/+++B	++	+/++	+/++	NT
Cryosurgery	+/++	++B	+++B	++	+/++	+/++

+ occasional to +++ numerous
B = large numbers of primitive blast cells
NT = not tested

As there was no significant difference in NK activity between the sham surgery and the control groups, this data was pooled and adjusted to a value of 100%. The excision and cryosurgery results were expressed as a percentage of the control value. The results indicated, with the exception of cryosurgery on day 1, that neither cryosurgery nor tumour excision significantly effected NK cytotoxicity. The difference observed on day 1 in the cryosurgery group, although statistically significant, was of short duration and unlikely to be of biological significance.

Blastogenesis assays and MLR

To investigate the effect of surgery on lymphocyte transformation Con A and LPS were used (Fig. 6). With the exception of T.B. animals treated by cryosurgery, the different surgical procedures did not affect the host's ability to respond to both mitogens or stimulator MB strain spleen cells. The suppression in the T.B. cryosurgery group was first evident on day 3, and by day 7, the mitogen responsiveness, but not MLR activity had returned to a level comparable to the other surgical groups. The MLR activity was not completely restored until day 14.

The degree of response in the surgically treated animals to mitogenic stimulation and MLR participation was lower than that of the controls, but this response was variable and although not statistically significant ($P > 0.05$), may reflect immunosuppression due to surgical stress. The immunosuppression observed in T.B. animals treated by cryosurgery was significantly different from the response of the other surgery groups and the control animals ($P < 0.02$).

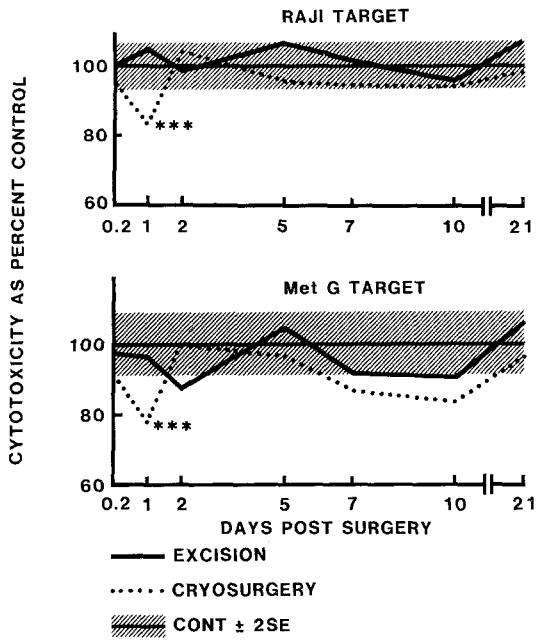

Fig. 5. Natural killer cell cytotoxicity was measured in a 4-h ^{51}Cr release assay against Raji and Met G targets at a 50:1, effector:target ratio following surgery. There was no significant difference using Student's *t*-test between the cytotoxicity of the control group and the two sham surgery groups, and so these values were pooled and adjusted to 100% ±2 SE. The excision and cryosurgery results are expressed as a percentage of this control value and the significance determined by Student's *t*-test. (*** $P < 0.001$). Unless indicated the results were not significantly different to control values

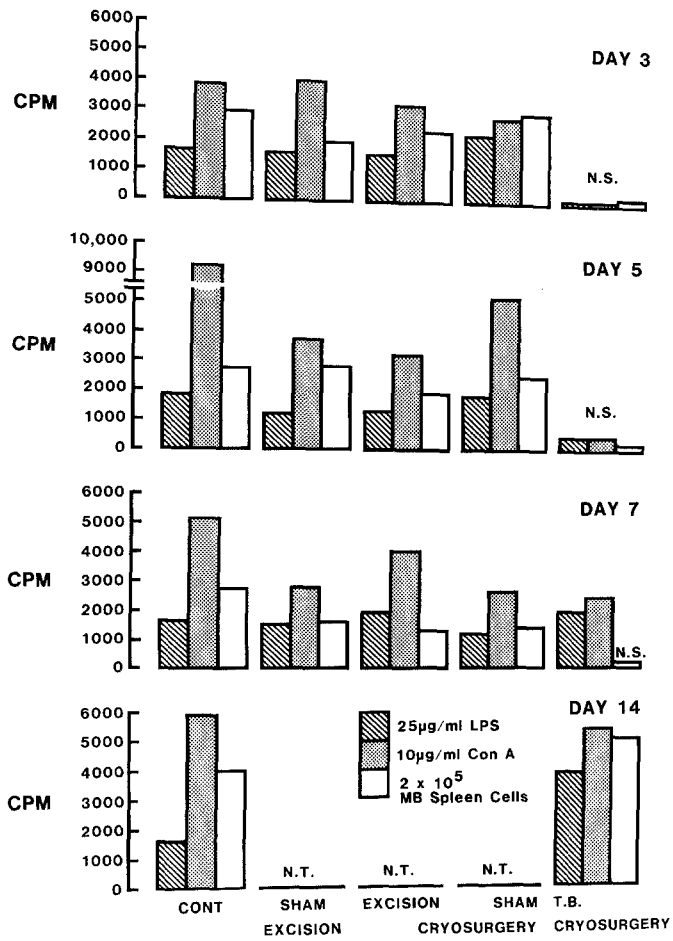

Fig. 6. The effect of surgery on lymphocyte blastogenesis. Mitogenic response and their ability to participate in an allogeneic mixed lymphocyte reaction was measured 3, 5, 7, and 14 days following surgery. The level of significance was determined using Student's *t*-test. Unless indicated there was a significant difference ($P < 0.05$) between ^3H-thymidine uptake of cells with or without mitogen or stimulator spleen cells. (N.T. not tested)

Ex-blast assay

To ensure that the observed immunosuppression in T.B. cryosurgery-treated hamsters was not due to the excessive number of blasts in the cultures so reducing the number of cells capable of responding to Con A to below a threshold density, wells were set up containing 2.5×10^5 spleen cells including or excluding blasts in the cell counts (Fig. 7). The results showed that at both time points tested, failure to respond to Con A could not be associated with the presence of blast-like cells. As an appropriate control, wells were set up with control spleen cells at an equivalent density. The results showed that at both densities, control cells were able to respond to Con A, and this was significantly higher than that of the spleen cells from T.B. cryosurgery animals cultured at the same density.

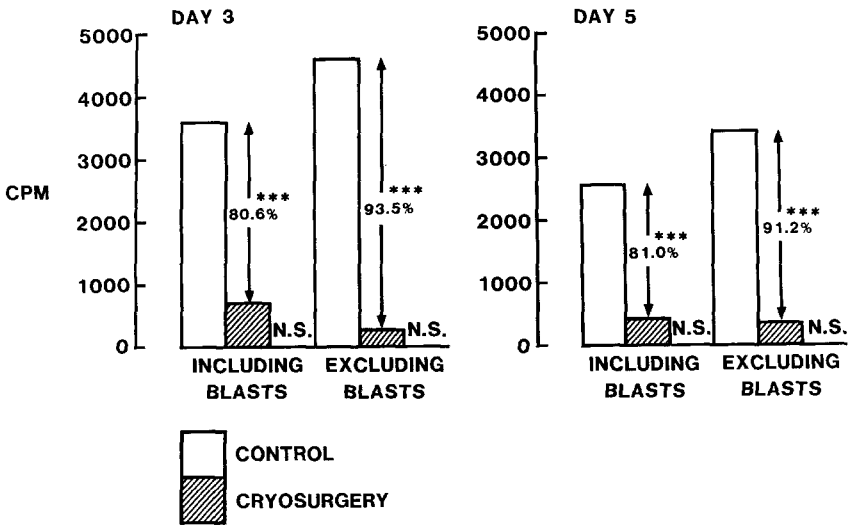

Fig. 7. Effect of adjusting spleen cell densities to include or exclude the presence of blasts, on their ability to respond to concanavalin A, measured at 3 and 5 days post-surgery. Spleen cells from animals whose tumour had been cryosurgically treated were adjusted to 2.5×10^5 cells/wells in HB103 including or excluding blasts (*shaded bars*). Control spleen cells at the same cell density were included (*open bars*). Concanavalin A was added at 10 μg/ml and the plates incubated for 48 h. The wells were set up in triplicate and the results of three experiments pooled. There was a significant difference ($P < 0.02$) between wells with and without mitogen unless indicated (N.S.) using Student's *t*-test. The percent reduction in the cryosurgery response compared to the appropriate controls is indicated and the significance determined by Student's *t*-test (*** $P < 0.001$)

Discussion

Previous studies have shown enhanced tumour-specific transplantation immunity to tumour re-challenge following cryosurgery as compared to excision [19, 20] and this correlated with increased spleen weight and heightened lymphocyte cytotoxicity from cryosurgically treated animals up to 19 days post-surgery. Similar results could be achieved by excision of the primary tumour, followed by injection of frozen tumour antigen [8]. This led to the hypothesis that cryosurgery of a primary tumour induced a systemic anti-tumour immune response, possibly through the continued release of tumour antigen. It was further suggested that such a response might account for the reported regression of lung metastasis following cryosurgery of carcinoma of the prostate [2].

In the present study, cryosurgery of animals with the non-immunogenic metastatic Met B tumour resulted in a significant decrease in survival compared with excision. Since the incidence of metastatic disease remained similar in both treatments, but the cryosurgically treated animals displayed earlier evidence of metastatic disease, we conclude that cryosurgery influenced the rate at which pre-existing micrometastases developed, rather than altered the number of cells shed from the primary tumour during surgery.

Gross splenomegaly was observed following cryosurgery of the primary tumour. Histological examination demonstrated that this was due to erythroblast proliferation, mainly in the sinusoids of the red pulp. Proliferation was evident on days 1–3 as determined by the number of primitive blast cells present. The number of cells in the red pulp continued to remain elevated up to day 5, due to the presence of the erythroblasts. Only limited erythroblast proliferation was seen on days 3–5 in the sham cryosurgery groups, and in the T.B. controls.

The results from this study concerning the in vitro measurement of NK cytotoxicity were inconsistent with the extensive literature which shows cryosurgery to enhance lymphocyte cytotoxicity [19, 20, 8]. In addition, enhanced NK activity has been reported during anaesthesia and surgery in patients with benign disease and cancer [10, 11]. The explanation for the enhanced NK cytotoxicity was demonstrated to be an increase in the number of phenotypically circulating NK cells, which fell to pre-operative levels by 48 h post-operation. A recent study showed that amputation of the hind limb in mice led to a reduction in NK cell cytotoxicity following surgery [23, 24]. The immunosuppression was caused by the presence of suppressor cells in the spleen of the surgically treated animals and required cell-cell contact with the NK cells for the effect to be apparent.

The findings of the present study failed to show a correlation between NK cell status and in vivo tumour metastasis, suggesting that variations in NK cytotoxicity do not account for the in vivo observation that cryosurgery results in a poorer prognosis compared to excision of a primary tumour.

Stress-induced lymphocyte immunosuppression has been demonstrated in vitro as an inability to respond to mitogens [4, 5], or to act as responders or stimulators in MLR [28] and in vivo studies have also demonstrated lymphocyte defects in delayed-type hypersensitivity reactions [28]. The cause of these defects are probably multifactorial, and may include the induction of corticosteroids and catecholamine, as well as decreased interleukin-2 production [9, 6, 34, 1].

With the exception of the T.B. cryosurgery group, the observed immunosuppresion is likely to be due to one or more of the classical stress-induced mechanisms discussed. The absolute immunosuppression as measured by mitogen response and MLR participation in the cryosurgery group appeared to be induced by an additional mechanism, and the results suggest that the immunosuppression is due to the inability of the cryosurgery lymphocytes themselves to respond to mitogens and participate in a MLR.

Attempts to separate lymphocytes from blast cells using density gradients or flow cytometry were unsuccessful, but using counterflow centrifugal elutriation greater than 98% pure lymphocyte preparations could be obtained. Despite this enrichment, the lymphocytes failed to respond to mitogens. (Wing et al. manuscript submitted for publication).

The cell types present in the spleen capable of suppressing lymphocyte blastogenesis include NK cells [32, 16], macrophages [21], T-lymphocytes [15] and erythroblasts [24]. Other studies are currently in progress to identify the cell types responsible for suppressing lymphocyte blastogenesis. Whilst it appears that erythroblast proliferation is responsible for the observed increase in spleen weight and cell number, this cell has yet to be conclusively proven to be responsible for suppressing lymphocyte blastogenesis. The possibility that cryosurgery could induce a suppressor cell population has been previously reported [35], and may explain the enhanced metastasis observed in this group. Tumour cell growth has been demonstrated to be enhanced in rats immunized with soluble tumour extracts [18] and such material would be expected to be presented systemically in the host following cryosurgery, and may be important in the induction of anergy and suppressor cells [22, 14, 17, 3].

A recent study demonstrated increased spleen weight following hind limb amputation in mice, and generation of a cell population which suppressed NK cell function [24]. Although in our study depressed NK activity following cryosurgery was not demonstrated, both studies show that the surgical procedures used result in the proliferation of spleen cells capable of suppressing host immune responses. Identification of the cell type(s) responsible for inducing immunosuppression have shown it to be non-adherent (Wing et al. manuscript submitted for publication), and development of a protocol which either inhibits their growth or action following cryosurgery is essential if cryosurgery is to be used as a means of treating patients with micrometastases.

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