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Preliminary Communications

Inhibition of Metastatic Spread by I.C.R.F. 159: Selective Deletion of a Malignant Characteristic

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Summary: Treatment with I.C.R.F. 159 completely inhibited metastasis formation in mice implanted with Lewis lung carcinoma at doses having little influence on the rate of growth of the primary implant. This inhibition was due to the effect of I.C.R.F. 159 on the development of blood vessels of the invading margins of the primary tumour. So far as is known, this is the first time a drug has induced a specific loss of the malignant characteristic of blood-borne tumour cell dissemination.

INTRODUCTION

Few attempts have been made to find new substances which prevent tumour cell dissemination (Handler, Sarris, and Wills, 1964; Donelli, Rosso, and Garattini, 1969; Rosso, Donelli, Franchi, and Garattini, 1969). This may largely be due to the difficulty of finding suitable experimental models, and most other workers have therefore bypassed the problem by injecting tumour cells intravenously. This expedient is not entirely satisfactory, since important early stages in the process of metastatic spread are thereby also bypassed.

We have used the Lewis lung carcinoma (3LL) in $C_{57}Bl$ mice as our test system because it has the important property, when implanted in the flank, of metastasizing spontaneously to the lungs (Ketcham, Wexler, and Minton, 1966; Wexler, Ryan, and Ketcham, 1969) and in our experience consistently, regularly, and predictably in all inoculated animals. Thus a precise experimental baseline is established of the organ invaded (the lungs) and of the time of invasion (nine days, microscopically). With this system a new cytostatic agent, (±)-1,2-bis(3,5-dioxopiperazin-1-yl) propane, I.C.R.F. 159 (Creighton, Hellmann, and Whitecross, 1969; Hellmann, Newton, Whitmore, Hanham, and Bond, 1969; Hellmann and Field, 1970; Sharpe, Field, and Hellmann, 1970), has controlled metastasis formation at doses having no overt influence on the growth of the primary tumour (Hellmann and Burrage, 1969). The mechanism of this inhibition has now been examined more closely, a preliminary account of the work being given elsewhere (Burrage, Hellmann, and Salsbury, 1970).

Essentially the experiments were designed to compare the microscopical changes in blood, lungs, and primary tumour after 3LL implantation in control and I.C.R.F. 159-treated animals to see if it was possible to discover morphological reasons for the inhibition of metastatic spread produced by I.C.R.F. 159. Cyclophosphamide was used for comparison.

MATERIALS AND METHODS

Effect on Primary Tumour Growth.—All mice used were $C_{57}Bl$ females of about 20 g. weight. All tumour inoculations were made subcutaneously in the flank. Methods of transplanting the tumour were those used routinely in the department of cancer chemotherapy of the I.C.R.F. (Hellmann, Marshall, and Stayt, 1967). Test mice received I.C.R.F. 159, 30 mg./kg., suspended in carboxymethyl cellulose solution intraperitoneally and the control mice received carboxymethyl cellulose alone intraperitoneally. Tumours were removed and examined macroscopically, and the test/control value was obtained by dividing the mean weight of test tumours by the mean weight of control tumours. The schedule of injections and the results are given in Table I.

TABLE I.—*Effect of I.C.R.F. 159 on Primary 3LL Tumour Growth*

Days After Implantation on which Injections Given	Days After Implantation on which Primary Tumour Removed	Mean Weight (g.) Test/Control (5 mice in each group)	Test/Control Value
1, 2, 3	4	0·056/0·075	0·73
1, 2, 3, 4	7	0·160/0·256	0·62
1, 2, 3, 4, 7, 8, 9	10	0·377/0·625	0·60
1, 2, 3, 4, 7, 8, 9, 10, 11	14	1·120/1·714	0·65
1, 2, 3, 4, 7, 8, 9, 10, 11, 14, 15	16	1·411/1·780	0·80
1, 2, 3, 4, 7, 8, 9, 10, 11, 14, 15, 16	19	1·780/2·343	0·76

Lungs and Blood in Mice Implanted with Tumour.—Tumour implantation and treatment were as described above, and are summarized in Table II. The lungs of these mice were removed, fixed, and individual lobes separated. After dehydration and embedding, a section (8μm.) was cut across the centre of each lobe. Pooled blood from the mice was collected into sodium edetate (Sequestrene) and averaged 4 ml. from each group of six. A nucleated cell concentrate was obtained by double centrifugation. Films were made of the whole of the nucleated cell layer, fixed in methyl alcohol, and stained with May-Grünwald-Giemsa.

TABLE II.—*Experimental Protocol to Show Effect of I.C.R.F. 159 on Pulmonary Metastases and Circulating 3LL Cells in Mice Implanted With 3LL Tumour. Six Mice in Each Group*

Mice Implanted with 3LL Tumour	
Days After Implantation on which I.C.R.F. 159 Given	Days After Implantation on which Lungs and Blood Removed
None	Each day from 1 to 14
Each day from 1 to 14	Each day from 1 to 14
None from 1 to 6. Each day from 7 to 14	Each day from 1 to 14*

*Blood only removed. 6 mice per group

Histological Examination.—Details of the experiment are given in Table III. Tumour implantation and treatment were

as described above. The dose of cyclophosphamide was 20 mg./kg. in carboxymethyl cellulose intraperitoneally. In each case the primary tumour was removed from the flank of the animal by wide excision and semiserial sections were cut across the whole of the specimen.

TABLE III.—*Experimental Protocol to Show Effect of I.C.R.F. 159 or Cyclophosphamide on the Histology of Primary 3LL Tumours*

No. of Mice in Each Group	Days After Implantation on Which Treatment Given	Days After Implantation when Sacrificed
4	C.M.C. .. 1, 2, 3, 4, 5,	7
4	C.M.C. .. 1, 2, 3, 4, 5	8
5	C.M.C. .. 1, 2, 3, 4, 5 7, 8, 9, 10, 11	14
4	I.C.R.F. 159 1, 2, 3, 4, 5	7
4	I.C.R.F. 159 1, 2, 3, 4, 5	8
5	I.C.R.F. 159 1, 2, 3, 4, 5 7, 8, 9, 10, 11	14
4	Cyclophosphamide 1, 2, 3, 4, 5	7
9	Cyclophosphamide 1, 2, 3, 4, 5 7, 8, 9, 10, 11	14

CMC= Carboxymethyl cellulose.

RESULTS

All treated and control mice remained in apparently good health as indicated by weight gain.

Effect on Lung Metastases.—In control mice implanted with 3LL tumour the first pulmonary malignant deposit was seen on Day 9. Thereafter the deposits rapidly increased in size and number. By Day 14 a total of 28 malignant deposits were seen in the six pairs of lungs examined, with three or more metastases in every specimen. Their sizes ranged from those of a few cells to 1 mm. in diameter. A few metastases were subpleural, and in one instance tumour was seen growing along the lumen of a pulmonary vein. The development of metastases was accompanied by a cellular reaction in the lungs. This took the form of perivascular cuffing by lymphocytes and plasma cells and actual infiltration of pulmonary tissue by lymphoid cells. The lungs from mice treated with I.C.R.F. 159 showed no evidence of metastatic growth at any time, nor were individual malignant cells seen, though cells of a comparable size (megakaryocytes) were readily detected in pulmonary capillaries. In the first seven days some areas of bronchopneumonia were seen, but in the second week these had largely resolved.

Effect on Circulating Malignant Cells.—In control mice malignant cells were first identified on Day 10, and rose to a maximum of 36 on Day 11. Fewer malignant cells were invariably present from Day 12 onwards. Malignant cells were never seen in blood samples from mice treated with I.C.R.F. 159, either from Day 1 or from Day 7 onwards. An increase in atypical mononuclear cells, probably immunoblasts, in the blood paralleled the inflammatory changes in the lungs of control mice but was small and transient in treated animals.

Effect on Primary 3LL Tumour.—Excised tumours from all control mice were very hyperaemic. On microscopical examination the congestion was confined to the margins of the tumour, particularly its deep aspect. In this area strands of tumour cells were separated by a dense network of vascular channels, and in many places malignant cells appeared to be in direct contact with blood cells (Fig. 1). There were also several areas of frank haemorrhage. The centre of the tumour was much less vascular, with some areas of necrosis and well-delineated blood vessels possessing a lining of endothelium.

All the 3LL tumours from mice treated with I.C.R.F. 159 were slightly smaller than the control tumours (see Table I). Their pale appearance was in distinct contrast to the congested haemorrhagic surface of control tumours. This effect tended to diminish with increasing age of the tumour at removal. The pallor was reflected microscopically in an absence of marginal congestion (Fig. 2) though invasion of surrounding tissue was just as pronounced as in control

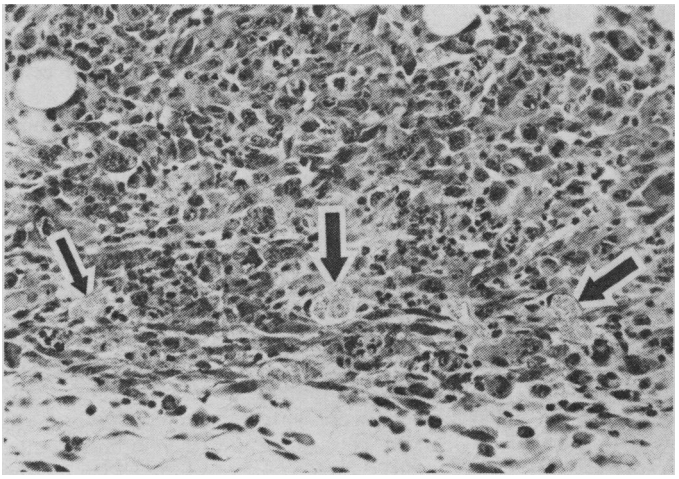

FIG. 2.—Section of a primary 3LL tumour seven days after implantation and treated with I.C.R.F. 159 (30 mg./kg. intraperitoneally. Days 1-5). Note blood vessels (arrowed). No red cells to be seen in the tumour. (H. & E. × 225.)

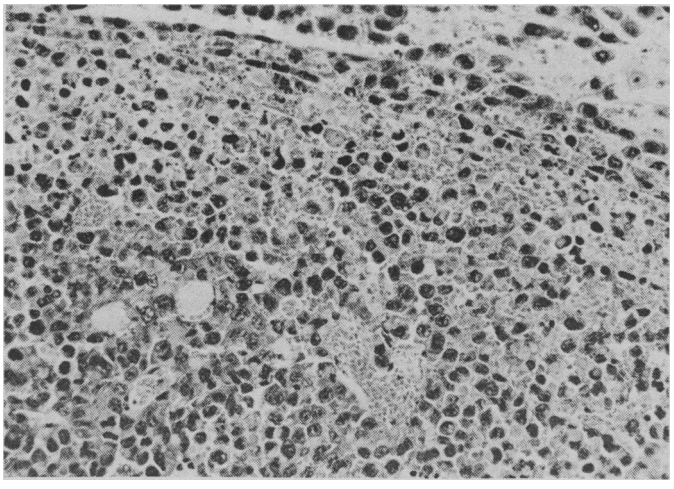

FIG. 1.—Section of a primary 3LL tumour seven days after implantation and treated with carboxymethyl cellulose only. Red blood cells can be seen to be streaming between strands of malignant cells. (Haematoxylin and eosin. × 225.)

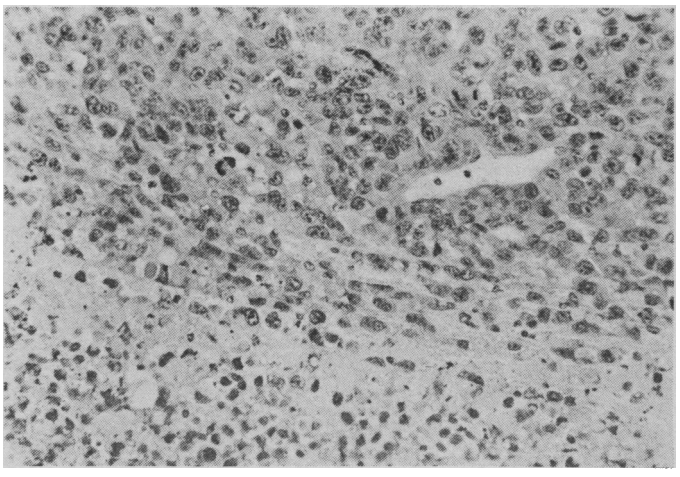

FIG. 3.—Section of primary 3LL tumour seven days after implantation and treated with cyclophosphamide (20 mg./kg. intraperitoneally. Days 1-5). Red blood cells streaming between tumour cells as in tumour treated with carboxymethyl cellulose (Fig. 1). (H. & E. × 225.)

tumours. Instead of the network of poorly defined vascular channels, only a few discrete blood vessels were present. Tumour cells were separated from blood cells by a layer of endothelium. Large haemorrhages were not seen at the margin but were present in the central necrotic zone, which was very similar in appearance to the centre of control tumours.

Effect of Cyclophosphamide.—All tumours treated with cyclophosphamide (20 mg./kg. intraperitoneally) and removed seven days after implantation were smaller than control tumours but similar in size to those treated with I.C.R.F. 159 for the same length of time. Vascularity around the tumour was about the same as in control tumours, partly in the form of discrete capillaries, but largely as areas of dilated vascular channels, with blood cells in apparent contact with malignant cells (Fig. 3). Most treated tumours removed 14 days after implantation were a little smaller than those from control animals, but their microscopical appearance was identical. Lungs from mice treated with cyclophosphamide and killed at 14 days showed metastatic infiltration in five out of nine mice. Metastases, however, were smaller and more scanty than in the controls.

Discussion

The absence of metastases in the lungs of mice treated with I.C.R.F. 159 could have resulted from several different mechanisms. Possibly the 3LL cells failed to implant in the lungs or they lodged there but did not develop. Possibly also they were liberated into the blood stream and killed in it by the drug; the least likely explanation seemed to be that cells were not liberated from the primary tumour.

The consistent presence from Day 10 onwards of circulating malignant cells in control animals and the complete absence of these cells in treated animals seem to rule out the first two possibilities, even though the techniques of collection and concentration may not have been sensitive enough to pick up all circulating 3LL cells. The latter is indicated by finding malignant cells in the blood of controls only from a day after the first pulmonary deposit was observed. Nevertheless, it was unlikely that any malignant cells had reached the lungs, since even individual cells were never seen there. On the other hand, individual non-malignant cells of a comparable size (megakaryocytes) were easily detected. Failure of implantation of metastatic cells, or their development into secondary tumours, could therefore be excluded (Hellmann and Burrage, 1969). The malignant cells were probably not killed in the blood stream, since none of them—not even necrotic forms—were seen in blood concentrates. It also seems unlikely that every 3LL cell would undergo mitosis while in the blood, and I.C.R.F. 159 appears to kill cells only during the late premitotic (G_2) or early mitotic phase of the cell generation cycle (Sharpe *et al.*, 1970).

Thus the antimetastatic effect of I.C.R.F. 159 on the 3LL tumour may have been due to a direct action on the primary tumour or a host vascular reaction to it. Though little inhibition in tumour growth and local invasiveness was noted in treated mice, the vascularity of the tumour margin was greatly reduced. Probably, therefore, the presence in treated animals of a well-formed vascular barrier between tumour cells and blood cells was the main factor in preventing escape of malignant cells into the blood stream. It was not, however, clear at first whether the difference in the pattern of tumour blood vessels as between mice treated with I.C.R.F. 159 and controls was due to an effect of I.C.R.F. 159 on the fundamental growth pattern of the tumour, possibly as a result of metabolic disturbances, or secondary to an effect on tumour growth giving more time and better circumstances for blood vessels to mature.

Mice treated with a dose of cyclophosphamide (20 mg./kg. intraperitoneally) having an inhibitory effect similar to 30 mg. of I.C.R.F. 159 per kg. on the growth of the primary tumour, as judged by weight, however, showed no change in their tumour vasculature. This was in keeping with previous findings (Hellmann and Burrage, unpublished observations) that about 50% of mice treated with cyclophosphamide (20 mg./kg. intraperitoneally) still develop some pulmonary metastases. Thus retardation of growth of the primary tumour cannot alone account for the inhibition of metastasis formation by I.C.R.F. 159. Probably therefore the mechanism by which I.C.R.F. 159 prevents 3LL tumour cell dissemination is through a direct effect on the growth pattern of the primary tumour.

Attempts to prevent metastases and increase the survival time of cancer patients by adjuvant cytotoxic chemotherapy following surgery have been disappointing (Higgins and White, 1968), but there is no evidence that the cytotoxic agents used had any specific inhibitory action on any phase of the development of metastasis. It remains to be seen whether the vascular changes produced by I.C.R.F. 159 in the Lewis lung carcinoma have a parallel in other experimental tumours and human malignancies. The therapeutic value of I.C.R.F. 159 in this context is being investigated.

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