Histological Analysis of the Antimetastatic Effect of 
\((\pm)-1,2\text{-Bis}(3,5\text{-dioxopiperazin-1-yl})\text{propane}\)

A. J. Salsbury, Karen Burrage, and K. Hellmann

Haematology Department, Brompton Hospital, Fulham Road, London, S.W. 3 [A. J. S.], and Cancer Chemotherapy Department, Imperial Cancer Research Fund, Lincoln's Inn Fields, London, WC2A 3PX [K. B., K. H.], England

SUMMARY

The antimetastatic effect of 
\((\pm)-1,2\text{-bis}(3,5\text{-dioxopiperazine-1-yl})\text{propane}\) (ICRF 159) on the Lewis lung carcinoma has been examined at tissue level. The effect has been compared with that of cyclophosphamide on the same tumor and with ICRF 159 on other tumors. Histological changes induced by ICRF 159 in the Lewis lung carcinoma tumor have been followed over a period of time. They lead to the conclusion that the antimetastatic activity of ICRF 159 is due to the striking changes that it induces in the morphology and physiology of the developing tumor vascular structure with the result that their appearance and behavior resemble that of normal blood vessels. Malignant cells no longer line the tumor vascular channels and are also probably unable to enter the normalized tumor vessels. Blood-borne tumor dissemination is thereby prevented, but the propensity of the treated tumors for uncontrolled proliferation and invasion of adjacent tissues remains unimpaired.

INTRODUCTION

It has long been recognized that treatment of cancer would be considerably improved if metastases could be prevented. With this in mind, nonspecific cytotoxic agents have been used in numerous attempts to influence the course of clinical cancer, but most of these attempts have been disappointing (5, 13) or, at best, marginally better than conventional treatment.

Efforts to discover substances specifically able to inhibit the formation of metastases have been hampered by a lack of suitable experimental systems. Hellmann and Burrage (10), however, used 3LL, in which the malignant cells escape spontaneously from the primary tumor, implant in the same distant organ, and develop there into visible metastases at about the same time in all the experimental animals. To set criteria such as these for an experimental tumor was previously thought to be unrealistic.

Examination in this 3LL system of the antimetastatic effect of some well-known anticancer agents and of a new class of antitumor agents, the bisdioxopiperazines (3), showed that some agents like 5-fluorouracil and ICRF 159 completely prevented the appearance of 3LL secondaries, without overt influence on the growth rate of the primary implant (12), while others like cyclophosphamide reduced the number of secondaries in direct proportion to their effect of the primary tumor (12).

This paper deals with changes seen at tissue level during the production of the antimetastatic effect by ICRF 159 and compares it with changes in other tumors that do not normally produce metastases and with the effects of cyclophosphamide on the 3LL carcinoma.

MATERIALS AND METHODS

Implantation and Treatment of Primary Tumors. We implanted 315 female C57/BL mice with 3LL, 184 male Schneider mice with Sarcoma 180, and 16 male C57/BL mice with Ca755. All inoculations were made s.c. into the flanks of mice weighing about 20 g. Routine methods of tumor implantation were used (11).

Mice treated with ICRF 159 received 30 mg/kg/day suspended in 0.5% CMC in 0.9% NaCl solution i.p. This dose has previously been shown to prevent metastases, without much inhibition of the primary tumor (10, 15). Mice implanted with Sarcoma 180 received only 3 mg ICRF 159 per kg per day since doses above this inhibit the growth of this tumor. Mice treated with cyclophosphamide received 20 mg/kg/day in CMC i.p. Control mice received CMC alone.

Examination of Lungs. Groups of 6 mice were implanted with 3LL tumor and received ICRF 159 or CMC daily. Each day from 1 to 14, 1 test and 1 control group were sacrificed and the lungs were removed and placed in 4% neutral buffered formalin. Following fixation, each lung was dissected and individual lobes were separated, dehydrated, and embedded in paraffin wax. A section was cut across the center of each lobe and stained with hematoxylin and eosin. Since Sarcoma 180 and Ca755 do not metastasize spontaneously, lungs from animals implanted with these tumors were not examined.

Examination of Blood from Mice Given Implants of 3LL and Sarcoma 180 Tumors. Mice given implants of 3LL or Sarcoma 180 tumors were treated and killed as shown in Table 1. Blood from each group of mice was obtained by cutting the subclavian vein and transferring the blood into se-
A. J. Salsbury, Karen Burrage, and K. Hellmann

questrene. The blood from each group of 6 mice was pooled, yielding an average of 4 ml. A nucleated cell concentrate of the pooled blood was made by double centrifugation. The blood was first centrifuged in a centrifuge tube for 20 min at 2000 rpm, and the buffy coat was removed. This was again centrifuged for 20 min at 2000 rpm in a hematocrit tube. Films were made of the whole of the nucleated cell layer, fixed in methyl alcohol and stained with May-Grunwald-Giemsa stain.

Examination of Primary Tumors. Mice given implants of 3LL, Sarcoma 180, or Ca755 tumors were treated as shown in Table 2. In each case, after the animal was killed, the primary tumor was removed from its flank together with a wide margin of macroscopically normal skin and s.c. tissue. The tumors were examined macroscopically and fixed in 4% neutral buffered formalin, semiserial sections were cut of the whole specimen, and the preparations were stained with hematoxylin and eosin.

All slides were coded and examined "blind."

RESULTS

Lung Appearances

Control Mice Receiving CMC. The results are shown in Chart 1. There was a considerable degree of endarteritis obliterans on the 1st day. This gradually diminished to vanish altogether by the 6th day after implantation but reappeared again on Day 13. On Days 13 and 14, some small blood vessels were obstructed by thrombus.

A considerable cellular reaction was observed. This initially took the form of an increase in perivascular cuffing by lymphocytes, reaching a maximum between Days 4 and 6. The cuffing diminished thereafter but again became prominent from Day 12 onward. Plasma cells were present among the lymphocytes from Day 3 onward and formed an appreciable part of the cuffing from Day 4 onward.

In addition to the perivascular cuffing, actual infiltration of pulmonary tissue by lymphoid cells was observed from Day 3 onward and tended to increase subsequently. At first, the infiltration took the form of discrete nodules in relatively close relation to bronchioles or blood vessels, but later it became more diffuse and extended onto the pleural surfaces of the lungs. However, even when infiltration was widespread, some relation to blood vessels was still preserved.

The 1st pulmonary deposit of 3LL tumor was seen on Day 9. It consisted of 5 or 6 malignant cells in close relation to a blood vessel (Fig. 1). Thereafter, the deposits rapidly increased, both in size and in number. By Day 14, some 28 deposits were seen in the 6 pairs of lungs examined, with 3 or more in every specimen. Their size varied from deposits composed of a few cells to tumors 1.0 mm in diameter. A few metastases were subpleural and, in 1 instance, tumor was seen growing on the intimal aspect of a pulmonary vein.

Mice Treated with ICRF 159. The results are shown in Chart 1. The sections of lung showed no evidence of metastatic growth at any time. There was a slight initial increase in perivascular lymphocyte and plasma cell cuffing, but this was transitory and had virtually returned to normal by Day 7. Lymphoid infiltration of lung parenchyma was never seen.

In the 1st 7 days, some areas of bronchopneumonia were seen. During the 2nd 7 days, most of these areas resolved and the inflammatory cell exudate was largely replaced by phagocytic cells.

In the lungs taken on Day 12, an apparent early malignant deposit in 1 lung was subsequently identified as 2 megakaryocytes lying in pulmonary capillaries (Fig. 2).

Findings in Blood Concentrates

Control Mice Given Implants of 3LL Tumor and Receiving CMC. Malignant cells were first identified in blood concentrates on Day 10 (Chart 2). Their numbers rose to a maximum of 36 on Day 11, clusters of up to 5 cells being noted (Fig. 3). From Day 12 onward, smaller numbers of malignant cells were present. The cells found in blood concentrates bore a close resemblance to a smear of cells from the primary tumor that had been processed in a similar fashion.

Atypical mononuclear cells (immunoblasts) were also seen in blood concentrates. The number of atypical mononuclears rose rapidly to a maximum of 596 cells on Day 4 and then fell equally rapidly but remained at a level of around 30 to 40 cells for the remainder of the 14 days.

Mice Given Implants of 3LL and Treated with ICRF 159. Malignant cells were never seen in mice re-
**Antimetastatic Effect of ICRF 159**

**Table 2**

*Experimental details of mice sacrificed for examination of primary tumor*

C57/BL female mice, approximately 20 g, were used for implantation of 3LL, and Swiss Schneider male mice, approximately 20 g, were used for Sarcoma 180. C57/BL male mice, approximately 20 g, were used for Ca755.

<table>
<thead>
<tr>
<th>Tumor implanted</th>
<th>Treatment and days after implantation on which it was given</th>
<th>Days after implantation on which tumor removed</th>
<th>No. of mice in group</th>
</tr>
</thead>
<tbody>
<tr>
<td>3LL</td>
<td>CMC daily from 1 to 5</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>3LL</td>
<td>CMC daily from 1 to 5</td>
<td>8</td>
<td>4</td>
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<tr>
<td>3LL</td>
<td>CMC daily from 1 to 5 and 7 to 11</td>
<td>14</td>
<td>5</td>
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<tr>
<td>3LL</td>
<td>ICRF 159 daily from 1 to 5</td>
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<td>ICRF 159 daily from 1 to 5 and 7 to 11</td>
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<td>5</td>
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<tr>
<td>3LL</td>
<td>Cyclophosphamide daily from 1 to 5</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>3LL</td>
<td>Cyclophosphamide daily from 1 to 5 and 7 to 11</td>
<td>14</td>
<td>9</td>
</tr>
<tr>
<td>Sarcoma 180</td>
<td>CMC daily from 1 to 5</td>
<td>7</td>
<td>4</td>
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<td>Sarcoma 180</td>
<td>CMC daily from 1 to 5 and 7 to 11</td>
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<td>Sarcoma 180</td>
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<td>Ca755</td>
<td>ICRF 159 daily from 1 to 5 and 7 to 11</td>
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Receiving ICRF 159 either daily for 14 days or only from Day 7 onwards (Chart 2).

In mice treated with ICRF 159 daily for the duration of the experiment, an earlier and less marked rise in atypical mononuclears occurred than in control mice. Such cells were virtually absent from the blood from Day 5 onwards. In mice treated with ICRF 159 daily from Day 7 only, the initial rise in atypical mononuclears paralleled that of untreated mice but the cells rapidly disappeared from the blood after that.

**Control Mice Given Implants of Sarcoma 180 and Receiving CMC.** Malignant cells were identified, in small numbers and sporadically, from Day 3 onward (Chart 3). The number of atypical mononuclear cells is shown in Chart 4.

**Mice Given Implants of Sarcoma 180 and Treated with ICRF 159.** No specimen examined contained any malignant cells. A very slight and transient increase in mononuclear cells was noted (Chart 4).

**Appearance of Primary Tumors**

**Control Mice Given Implants of 3LL and Receiving CMC.** The appearances, apart from size, were essentially similar in 7-, 8-, and 14-day tumors; the following description applies to all specimens.

Excised tumors were markedly hyperemic to the naked eye (Fig. 4). On microscopy, the congestion was found to be confined to the margins of the tumor on its deep aspect. In this area, strands of tumor cells were separated by a network of poorly formed vascular channels (Fig. 5). Malignant cells appeared to be in direct contact with blood cells (Fig. 6). There were also several areas of frank hemorrhage. The central vessels possessed a lining of endothelium. Some areas of necrosis were present toward the center of the tumor.

**Mice Given Implants of 3LL and Treated with Cyclophosphamide.** Tumors removed at 7 days were smaller...
Tumors at 7 days were moderately vascular. Large blood vessels lined with endothelium ran throughout the tumor. There were some areas of hemorrhage. Many dilated vascular channels were present at the periphery of the tumors, but the majority were lined by endothelium and blood was not in direct contact with malignant cells. By 14 days, the tumors were similar in structure but were larger and more vascular, areas of necrosis and hemorrhage being more prominent. Malignant cells were in apparent contact with blood cells in a few places at the margins of the tumors, but the blood was mainly confined to dilated, endothelial-lined vessels.

Mice Given Implants of Ca755 Tumor and Receiving ICRF 159. At 7 days, tumors were of approximately the smaller than control tumors. The center of each tumor was necrotic, with a margin of more or less intact cells. Many of these cells, particularly on the central aspect, showed nuclear and cytoplasmic degenerative changes and were often widely separated, one from another. Vascularity around the tumor was approximately the same as in control tumors and was partly in the form of discrete capillaries. However, in addition, many areas of dilated vascular channels were seen, with blood cells in apparent contact with malignant cells.

The majority of tumors removed at 14 days were a little smaller than control tumors and their microscopic appearance was identical, with marked congestion and malignant cells in direct contact with wide, poorly formed vascular channels. One tumor was slightly less vascular than the controls, but the actual vascular pattern was similar.

Control Mice Given Implants of Sarcoma 180 Tumor and Receiving CMC. Tumors at 7 days were relatively avascular, with only a few discrete capillaries around their periphery. Three tumors showed small areas of poorly formed vascular channels in close relation to malignant cells. By Day 14, vascularity had increased, but to a slightly lesser extent than in the control 3LL tumors. Many areas of malignant cells in close contact with blood cells in vascular spaces were noted, but a higher proportion of the peripheral congestion could be ascribed to the presence of capillaries than in the 3LL tumor.

Mice Given Implants of Sarcoma 180 and Treated with ICRF 159. Sarcoma 180 tumors removed at 7 days were approximately the same size as control tumors but contained somewhat larger necrotic areas at their center. Around the periphery of the tumors, vascularity and vascular pattern were approximately the same as in control tumors. In contrast, at 14 days the tumors were roughly the same size as control tumors, but vascularity was much less and blood cells were confined almost entirely to discrete capillaries and were well separated from malignant cells.

Control Mice Given Implants of Ca755 and Receiving ICRF 159. At 7 days, tumors were of approximately the same size as control tumors but contained somewhat larger necrotic areas at their center. Around the periphery of the tumors, vascularity and vascular pattern were approximately the same as in control tumors. In contrast, at 14 days the tumors were roughly the same size as control tumors, but vascularity was much less and blood cells were confined almost entirely to discrete capillaries and were well separated from malignant cells.
same size and vascularity as control tumors. No hemorrhages were present. A large number of capillaries lined by endothelium ran around the periphery of the tumors. After 14 days, the tumors were slightly smaller and slightly less vascular than control tumors, but with much more necrotic centers. However, the microscopic appearance of the blood vessels at their periphery was essentially the same as control tumors.

DISCUSSION

Prevention of dissemination by inhibiting the release of malignant cells from the primary growth transforms a malignant to a quasibenign tumor. There appear to be a number of substances that when given systemically can produce such an effect, and the activity of one of them, ICRF 159, has now been studied at tissue level.

Detailed histological examination has revealed only 1 major difference between ICRF 159-treated and control 3LL primary tumors, the normalization of the vascular system at the periphery of the tumor. This difference could well account for the antimetastatic effect (4, 14).

Two major questions arise: Is 3LL alone in its response to ICRF 159 and is ICRF 159 unique in producing this response?

It is clear from the changes induced in the Sarcoma 180 that 3LL is by no means alone in its response to ICRF 159. Sarcoma 180 showed changes in its vasculature very like those seen in 3LL after ICRF 159 with the similar result that the tumor cells could not then be found in the circulation. The vasculature of the Ca755 carcinoma, however, is, even in control animals, well endothelialized and no change on treatment with ICRF 159 could be detected. The integrity of the vasculature is undoubtedly an important factor in the ability of any tumor to disseminate.

It is unlikely that ICRF 159 is unique in its effect on tumor blood vessels. Other antitumor bisdioxopiperazines also inhibit 3LL metastases (those that have no antitumor action have no antimetastatic action), but their effect on tumor blood vessels has not as yet been investigated. 5-Fluorouracil also has a similar antimetastatic activity (12), but its effect on tumor blood vessels has not yet been investigated either. Gitterman and Luell (7), on the other hand, have described a new agent with profound antimetastatic activity and an effect on the tumor vasculature similar to that seen with ICRF 159.

The pattern of growth of the 3LL and the Sarcoma 180 is rather more like that of a human sarcoma, with blood vessels lying in close relation to tumor cells, than that of a carcinoma, where vessels and tumor cells are generally separated by a fibrous tissue stroma. It is possible, therefore, that the most promising results with ICRF 159 may occur in such cancers as osteogenic sarcoma, in which a poor prognosis is linked to the frequent development of blood borne metastases, or in such poorly differentiated carcinomas as oat cell carcinoma of the bronchus.

Nevertheless, venous invasion occurs in a surprisingly high proportion of cases of human carcinoma, e.g., rectal carcinoma (1). Areas of hemorrhage are also not uncommon in and around malignant tumors. Venous invasion has been shown to bear a definite relationship to the degree of differentiation of a carcinoma (2, 8) and to poor prognosis with the occurrence of distant blood-borne metastases (6, 9, 16). It may well be that the disturbance of carcinoma growth pattern produced by ICRF 159 might lead to a retardation in the development of venous invasion and a reduction in its incidence, with a consequently improved prognosis. Clearly, however, the influence of any drug that prevents metastases is limited by the number of patients who present at a time when their tumor has already disseminated.

REFERENCES

Fig. 1. Small deposit of 3LL in lung of mouse receiving CMC on 9th day after implantation. H & E, × 500.
Fig. 2. Two megakaryocytes in lung of mouse treated with ICRF 159 on 12th day after implantation. H & E, × 500.
Fig. 3. Cluster of malignant cells from blood concentrate (mouse receiving CMC 11 days after implantation). May-Grünwald-Giesma, × 2000.
Fig. 4. Primary tumors removed on Day 7 after implantation. Control mice below, mice receiving ICRF 159 on Days 1 to 4 above. × 3.
Fig. 5. Margin of untreated tumor to show poorly formed vascular channels. H & E, x 250.

Fig. 6. A higher-powered view of Fig. 5 to show close contact between malignant cells and blood cells. H & E, x 500.

Fig. 7. Margin of tumor from mouse treated with ICRF 159 to show well-formed capillaries. H & E, x 250.
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