Successful Immunotherapy of Murine Experimental Hepatic Metastases with Lymphokine-activated Killer Cells and Recombinant Interleukin 2

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ABSTRACT

Lymphokine-activated killer (LAK) cells are generated in vitro by the incubation of normal murine splenocytes in interleukin 2. We have shown previously that the systemic injection of LAK cells in conjunction with recombinant interleukin 2 can reduce the number of established pulmonary metastases in mice. In an attempt to study this approach in the treatment of hepatic metastases, we developed a technique for the induction of hepatic metastases in mice based on the intrasplenic injection of tumor cells and have tested the effects of LAK cells and recombinant interleukin 2 produced in Escherichia coli (RIL-2) therapy on these metastases. Treatment with LAK cells alone in 14 consecutive experiments rarely produced significant reduction in metastases over control (mean percentage reduction, 12%). Therapy with RIL-2 alone produced a dose-dependent reduction in the number of liver metastases. In 20 consecutive experiments when RIL-2 was administered i.p. three times a day at doses varying from 1,000 to 5,000, 10,000 to 15,000, and 25,000 units, a statistically significant (P < 0.05) reduction in liver metastases was seen in 2 of 12, 2 of 4, and 8 of 12 determinations, respectively (percentage reduction, 0 to 97; mean, 42%). At doses greater than 25,000 units, the reduction in metastases was highly reproducible (percentage reduction, 66 to 95; mean, 83%) and was statistically significant in 14 of 14 determinations. When LAK cells were given i.v. in addition to RIL-2 administration in 16 consecutive experiments, the percentage reduction in liver metastases was markedly increased over that seen with RIL-2 alone (mean percentage reduction, 77% at doses of 5,000 to 25,000 units of RIL-2 and mean reduction, 97% for doses greater than 25,000 units of RIL-2). At doses of 5,000, 10,000, 25,000, and greater than 25,000 units of RIL-2 plus LAK cells, significant reduction of liver metastases (P < 0.05) was achieved in 3 of 7, 2 of 2, 8 of 8, and 6 of 6 determinations, respectively. When animals were given fresh splenocytes or splenocytes cultured in complete medium without RIL-2 instead of LAK cells, no reduction in liver metastases was seen except for that attributable to the administration of RIL-2 alone. Sublethal total body irradiation of the mice prior to therapy abrogated the therapeutic effects of RIL-2, but the effects of treatment with LAK cells plus RIL-2 were maintained. Thus, treatment with RIL-2 alone or in combination with LAK cells is effective in reducing the number of established hepatic micrometastases in a murine model. These studies are in accord with our previous observations concerning the effective therapy of established pulmonary metastases with RIL-2 plus LAK cells and provide a rationale for the extension of these observations to the treatment of metastatic cancer in humans.

INTRODUCTION

Passive immunotherapy involves the transfer to the tumor-bearing host of previously sensitized immunological reagents, such as cells or antibodies that have the ability to mediate antitumor responses. The term adoptive immunotherapy is usually used to connote passive immunotherapy with cells such as lymphocytes or macrophages. In general, the use of cells obtained from highly immunized animals has been essential to the success of adoptive immunotherapy in animal tumor models (1, 2).

We have recently described a simplified method for generating lymphoid cells with selective antitumor reactivity that can be used in the adoptive immunotherapy of tumors (3–6). The incubation of normal murine or human splenocytes in IL-2, a lymphokine produced by lectin or antigen-activated T-cells (7, 8), gives rise to lymphoid cells that are specifically cytotoxic to fresh, non-cultured, autologous, syngeneic, and allogeneic primary and metastatic tumor cells regardless of their natural killer susceptibility but are not toxic to normal cells. We have called these cytotoxic cells LAK cells. They bear the Thy-1+, Lyt-1−2+ cell surface phenotype in the mouse (5) and are OKT-3−4+8+ in the human (3).

The recent availability of large amounts of RIL-2 with known biological activity (9) has made it possible to evaluate the antitumor effectiveness of this lymphokine in vivo either alone or in conjunction with LAK cells. In a recent report, Mule et al. (10) documented the effectiveness of transfused LAK cells plus repetitive injections of RIL-2 in the treatment of established pulmonary micrometastases from multiple sarcomas. The effects of low doses of RIL-2 or of LAK cells alone were not significant; however, concomitant administration of both caused a significant reduction in the number of metastatic foci in the lungs. In later studies, we showed that administration of RIL-2 alone was effective in reducing the number of metastases but only when given in very high doses (11).

In a new experimental model designed to produce metastases solely in the liver, we undertook studies to assess the effectiveness of adoptive therapy with RIL-2 alone and LAK cells plus RIL-2 on metastatic foci in this location. This model also tested the ability of i.v. injected LAK cells to mediate antitumor effects in an organ that did not represent the first capillary bed (i.e., the lung) encountered by LAK cells after i.v. injection. In this paper, we have demonstrated that treatment with high doses of RIL-2 alone can mediate the successful therapy of hepatic micrometastases. The therapeutic effect is enhanced by the concomitant i.v. injections of LAK cells. The factors responsible for successful therapy in this model have been analyzed.
MATERIALS AND METHODS

Animals. C57BL/6 mice were obtained from the Animal Production Colonies of the National Institutes of Health, Bethesda, MD. Mice were fed standard mice chow and water ad libitum and were used in experiments when 12 weeks or older.

Splenocytes. Splenocytes from C57BL/6 female mice were harvested aseptically and studied with 70% ethanol and washed with sterile HBSS. The cell suspension and spleen fragments were transferred to a single layer of 100-gauge nylon mesh (nitex; Lawshe Industrial Co., Bethesda, MD) and the erythrocytes were lysed osmotically with 0.1% buffered ammonium chloride solution (NIH Media Unit) at room temperature for 2 min. The cells were then centrifuged and washed 3 times with HBSS.

RIL-2. The gene for IL-2 (12), isolated from a high-producer Jurkat cell line, was expressed in high levels in Escherichia coli and purified to apparent homogeneity as recently described (9). This human RIL-2 was kindly supplied by the Cetus Corp. (Emeryville, CA). Purified RIL-2 had a specific activity of 3 to 4 x 10^6 units/ml. The endotoxin level in the purified preparation was less than 0.1 ng/10^6 units of RIL-2 as measured in a standard limulus assay. RIL-2 was lyophilized and reconstituted with distilled water. The preparation contained 5% mannitol and sodium dodecyl sulfate, 131 µg/ml RIL-2 as a vehicle.

Tumor. MCA-105, a sarcoma syngeneic in C57BL/6 mice was used in these experiments. This tumor was induced in our laboratory by the injection of 0.1 ml of 1% 3-methylcholanthrene in sesame oil as described previously (13). A large number of vials of MCA-105 from the first passage generation were cryopreserved. After thawing from storage at -70°C, tumor was injected s.c. in C57BL/6 mice for serial passage and was always used within the first 7 transplant generations, at which time a new vial was thawed and used. Single-cell suspensions of tumor were prepared as described previously (10). Briefly, fresh sarcoma tumors were excised, minced with scissors, and stirred in a triple enzyme solution of deoxyribonuclease, hyaluronidase, and collagenase (Sigma Chemical Co., St. Louis, MO) for 3 h at room temperature, filtered through 100-gauge nylon mesh (Nitex), washed 3 times in HBSS without calcium or magnesium, and resuspended at the appropriate cell concentration for injection in HBSS without calcium or magnesium.

Generation of LAK Cells. LAK cells were generated by placing 5 x 10^6 fresh splenocytes (prepared as described above) into 175-sq cm (750 ml) flasks (Falcon Labware, Becton, Dickinson and Co., Oxnard, CA) containing 175 ml of complete medium, which consisted of RPMI 1640 (Biofluids) with 10% fetal calf serum (Biofluids), 0.03% fresh glutamine, streptomycin, penicillin (all from the NIH Media Unit), 0.1 mm nonessential amino acids, 0.1 µm sodium pyruvate (all from Microbiological Associates, Walkersville, MD), 5 x 10^-5 M of 2-mercaptoethanol (Aldrich Chemical Co., Milwaukee, WI), gentamicin (50 µg/ml) (Shearing, Kenilworth, NJ), and fungizone (0.5 M9/ml) (Flow Laboratories, McLean, VA). RIL-2 (175,000 units) was added to each flask. The flasks were incubated supine at 37°C in a moist atmosphere for 5% CO2 for 72 h. The cells (LAK) were then harvested into sterile 250-ml centrifuge tubes (Corning No. 25350; Corning Glass Works, Corning, NY) passed over Ficol (Lymphocyte-M; Cedarlane Laboratories Ltd., Hornby, Ontario, Canada) to remove dead cells, and washed 3 times with HBSS before resuspending in HBSS for i.v. injection. Aliquots of LAK cells were tested for cytotoxicity in vitro at a standard of 5 x 10^5 MCA-105 cells in HBSS prepared as described above was then injected into the upper pole of the exposed extraabdominal spleen using a 27-gauge needle (American Hospital Supply, McGaw Park, IL). During the injection, the spleen swelled slightly, and then the injected suspension flowed into the splenic vein with virtually no leakage of fluid. The injection completed, the needle was removed, and a period of 1 min was allowed to elapse to permit all tumor cells to be flushed into the portal circulation. The splenic pedicle was then clipped with a medium hemoclip (Edward Weck and Co., Inc., Research Triangle Park, NC), the spleen was removed, and the splenic pedicle was then dropped i.p. The abdominal wall musculature and skin were then closed in one layer using 9 mm autoclip wound clips (Clay Adams, Parsippany, NJ). The animals were then randomized to their respective therapy groups and allowed to recover. By Day 3 after tumor injection, micrometastases were apparent histologically (Fig. 1).

Adoptive Immunotherapy Model. For treatment of micrometastases, LAK cells were suspended at 1 x 10^6 cells in 1 ml of HBSS and injected into the tail vein on Days 3 and 6 after tumor injection. Mice were injected with injections of HBSS or RIL-2 i.p., 0.5 ml/injection every 8 h. These injections were given from Days 3 through 10 for HBSS and RIL-2 doses ≤ 25,000 units/injection and from Days 3 through 7 for RIL-2 doses > 25,000 units. The units of RIL-2 activity were determined using an IL-2 dependent cell line as described previously (9, 14, 15). The amount of RIL-2 injected (in units) varied depending upon the design of each experiment and is noted in the legends to the charts and tables.

At least 6 mice were included in each treatment group. At 14 days after tumor injection, the mice were ear tagged and randomized. The mice were then given a tail vein injection consisting of 0.5 ml of a 15% solution of India ink (Higgins Black No. 4417; A. W. Faber-Castell) in phosphate-buffered saline. The carbon particles from the ink were found to be phagocytized by the Kupffer cells of the liver, rendering the liver black. The mice were then killed by cervical dislocation, and their livers were harvested and bleached by Fekete's solution (16), allowing the metastases to become easily countable as they formed discrete white nodules on the surface of the liver, since they did not incorporate the India ink (Figs. 2 and 3). Nodules were counted in a blind fashion without knowledge of the treatment of the mouse. Liver metastases too numerous to count upon autopsy were assigned an arbitrary value of 250, since we were able to count reliably only numbers of metastases approaching 250/liver. After all data were recorded, the code was broken.

Statistical Analysis. The significance of differences in numbers of liver metastases between groups was determined by the Wilcoxon rank sum test (17). Two-tailed P values are presented in all experiments.
RESULTS

Effective Reduction of the Number of Established Hepatic Metastases by RIL-2 and LAK Cells Plus RIL-2. Mice with established Day 3 liver metastases were treated with varying doses of RIL-2 alone or in combination with LAK cells. LAK cells (10^6) were given i.v. on Days 3 and 6, and RIL-2 was given i.p. every 8 h as described in “Materials and Methods.” Results of 2 characteristic experiments are shown in Table 1 and Chart 1. Mice receiving RIL-2 alone had significantly fewer numbers of established liver metastases by Day 14 when treated with doses greater than 5000 units of RIL-2 (P < 0.02) compared to mice receiving HBSS alone. Treatment with LAK cells alone (combined with HBSS) was not usually capable of significantly reducing the number of liver metastases. However, when treatment with LAK cells was combined with the administration of RIL-2, the greatest reductions in liver metastases were seen. Combining LAK cells with RIL-2 also produced significant-reduction in liver metastases when compared to RIL-2 alone. The extent of reduction of liver metastases was dependent upon the dose of RIL-2. The appearance of the livers at the time of harvest from a representative experiment is shown in Fig. 3.

Dose Titration of RIL-2 in Vivo. The reduction in the number of liver metastases resulting from RIL-2 administration alone was highly dose dependent. Results of 42 determinations from 20 consecutive experiments are shown in Chart 2. The reduction in metastases was variable at RIL-2 doses less than or equal to 25,000 units with a percentage reduction ranging from 0 to 97% (mean, 42% in 28 determinations). At doses from 1,000 to 5,000, 10,000 to 15,000, and 25,000 units, the percentage reduction over HBSS was statistically significant (P < 0.05) in 12, 2 of 4, and 8 of 12 determinations, respectively. At doses greater than 25,000 units, the reduction in liver metastases was highly reproducible with a range of percentage reduction of 66 to 95% (mean, 83% in 14 determinations); 14 of 14 determinations had a statistically significant decrease in the number of metastases (P < 0.0005). At doses equal to or greater than 100,000 units given i.p. approximately every 8 h, significant toxicity was seen by 5 days that limited the amount of RIL-2 that could be given in any one experiment. This toxicity was manifested as weight gain of the animals and ascites with pleural effusions seen at autopsy.

When 1 × 10^6 LAK cells/ml were given i.v. on Days 3 and 6 after tumor injection in addition to RIL-2, the percentage reduction of liver metastases was markedly increased over the corresponding reduction when RIL-2 alone was used. Results of 23 determinations from 16 consecutive experiments are shown in Chart 3. At doses of 5,000 to 25,000 units, the corresponding range of percentage reduction was 6 to 99% (mean, 75% in 17 determinations). At doses of 5,000, 10,000, and 25,000 units, the percentage reduction over HBSS control was statistically significant (P < 0.05) in 3 of 7, 2 of 2, and 6 of 8 determinations, respectively. At doses greater than 25,000 units, the range in percentage reduction was 95 to 99% (mean, 97% in 6 determinations); 6 of 6 determinations resulted in a statistically significant reduction in the number of metastases compared to the HBSS control. Statistically significant differences were seen between mice treated with LAK cells plus RIL-2 compared to RIL-2 alone, both in the ≤25,000 unit group (P < 0.0005) and >25,000 unit group (P < 0.0005).

Necessity of In Vitro Activation of Fresh Splenocytes by RIL-2 to Achieve Immunotherapeutic Effect on Liver Metastases. The increased reduction in liver metastases obtained by LAK cells plus RIL-2 compared to RIL-2 alone was dependent upon the activation of LAK cells. The i.v. adoptive transfer of 1 × 10^6 fresh splenocytes or splenocytes cultured for 3 days without RIL-2 failed to achieve any significant antitumor effect when given in combination with RIL-2 compared to RIL-2 given alone (Table 2). However, when LAK cells were combined with RIL-2, a significant reduction in the number of metastases over RIL-2 alone was achieved (P < 0.01 in both experiments).

Elimination of Therapeutic Effect of RIL-2 Alone but not of LAK Cells Plus RIL-2 by Irradiation of the Host. We examined the effect of a sublethal dose of irradiation upon the reduction of liver metastases by RIL-2 and LAK cells plus RIL-2. Mice were given 500 rads total body irradiation from a ^137Cs source. One h later, they were given injections of tumor cells intrasplenically, and therapy was begun 3 days later (Chart 4).

In the normal nonirradiated host, treatment with 50,000 units RIL-2 i.p. 3 times a day significantly reduced the number of liver metastases when used alone (mean number of metastases in HBSS group was 247 compared to 17 in the RIL-2 group; P < 0.0005) and when given with LAK cells (mean, 0.2 metastases; P < 0.004). However, in irradiated mice, treatment with RIL-2 alone was ineffective in reducing the number of liver metastases (250 in the HBSS group compared to 227 in the RIL-2 group), whereas...
REDUCTION OF LIVER METASTASES IN VIVO BY RIL-2 AND LAK PLUS RIL-2

Chart 1. Effect of RIL-2 and LAK cells on therapy of MCA-105 liver metastases. The decrease in experimentally induced MCA-105 liver metastases caused by the injection of RIL-2 given i.p. every 8 h (left), was compared to the same doses of RIL-2 administered concomitantly with 1 x 10^6 syngeneic LAK cells generated in vitro. RIL-2 was given i.p. beginning on Day 3, and LAK cells were given i.v. at Days 3 and 6 posttumor injection (right). The number of metastases were counted on a coded fashion at Day 14 after MCA-105 injection. •, measure of the number of liver metastases in an individual mouse.

Chart 2. Dose titration of the ability of RIL-2 to reduce liver metastases in 20 consecutive experiments. Each animal was injected with 3 x 10^6 MCA-105 cells and treated with RIL-2 i.p. every 8 h starting on Day 3. Increasing doses of RIL-2 led to increasing reduction in the number of hepatic metastases. •, separate experimental determination.

Chart 3. Effect of LAK cells plus increasing doses of RIL-2 on MCA-105 liver metastases in 16 consecutive experiments. Each animal was injected with 3 x 10^6 MCA-105 cells and treated with RIL-2 i.p. every 8 h starting on Day 3. Increasing doses of RIL-2 led to increasing reduction in the number of hepatic metastases when 10^6 LAK cells were administered i.v. concurrently on Days 3 and 6. •, separate experimental determination.

DISCUSSION

In work reported previously from our laboratory (3–6), we have established that normal murine splenocytes or human peripheral blood lymphocytes cultured in vitro with the lymphokine IL-2 developed lytic activity for fresh autologous, noncultured, syngeneic, or allogeneic primary and metastatic tumor cells, regard-
REDUCTION OF LIVER METASTASES IN VIVO BY RIL-2 AND LAK PLUS RIL-2

less of their natural killer susceptibility, but not to normal cells, when tested in an in vitro cytotoxicity 51Cr release assay. Detailed studies in the mouse and human have characterized the nature of the phenotype of both the precursor and effector cells involved in this phenomenon. These cells have been called LAK cells.

Recently, Chang et al. (14) reported that systemically administered RIL-2 resulted in the generation of spleen cells in vivo that were cytotoxic in vitro in a 4-h 51Cr release assay against fresh tumor targets. Further studies by Ettinghausen et al. (18) have also shown that RIL-2 given systemically will result in the appearance of proliferating cells in vivo in multiple organs (lung, liver, spleen, kidney, lymph nodes) that resemble activated lymphocytes on histological sections and bear the Thy-1.2 antigen. When the organs harboring such cells were harvested and these cells were separated on Ficoll gradients, these activated lymphocytes were cytotoxic in vitro in a 4- and 18-h 51Cr release assay against fresh tumor targets. Thus, it appears that RIL-2 administration leads to LAK cells generation in vivo.

Recently Mule et al. (10) reported on the therapy of established pulmonary micrometastases with LAK cells plus RIL-2. In that model, LAK cells given alone had no effect. In a separate study, Rosenberg et al. (11) have also reported on the successful therapy of established lung metastases and subdermal solid tumor with high doses of RIL-2 alone, whereas sodium dodecyl sulfate given alone had no effect. LAK cells are larger than resting lymphocytes and tend to lodge in the first capillary bed they traverse (the lung) shortly after i.v. injection. It was thus of interest to study the therapeutic effects of LAK cells in organs other than the lung to determine whether LAK cells could mediate therapeutic effects systemically. Because no reproducible model existed for the study of isolated liver metastases, we first developed a model for the induction of artificial metastases in this organ that could be easily quantitated.

By injecting tumor cell suspensions into the spleen, we avoided the difficulties of injecting directly into the portal vein and were able to generate metastases with a single operative procedure. The spleen was removed to prevent the development of a large organ that could be easily quantitated. The spleen was removed to prevent the development of a large organ that could be easily quantitated. The spleen was removed to prevent the development of a large organ that could be easily quantitated. The spleen was removed to prevent the development of a large organ that could be easily quantitated.

In this study, we have analyzed some of the requirements necessary for the successful treatment of established Day 3 liver metastases with RIL-2 and LAK cells plus RIL-2. The production of RIL-2 in large quantities by isolating the gene coding for IL-2 and expressing it in E. coli (9, 12) has made it possible to test the in vivo effects of this lymphokine. As shown in Charts 1 and 2 and Table 1, the repetitive administration of large doses of RIL-2 was able to achieve reduction of established liver metastases. This requirement probably reflects the necessity to achieve sustained bioavailability of the lymphokine in vivo due to its short serum half-life after i.p. injection (14, 15). The fact that the systemic administration of RIL-2 was capable of significantly reducing liver metastases in vivo is in accord with the investigations of Ettinghausen et al. (3) who showed that IL-2 can generate cytotoxic cells in the liver in vivo.

The percentage reduction in the number of liver metastases by RIL-2 was seen in a dose-dependent fashion; doses greater than 25,000 units i.p. 3 times a day were necessary to achieve a reproducibly significant effect. These doses, however, carried with them significant toxicity, thus limiting the amount of RIL-2 that could be given in any one experiment. Our results are in accord with studies of the therapy of lung metastases by Mule et al. (10) who showed that the i.v. transfer of LAK cells given concomitantly with RIL-2 could significantly reduce the number of established metastatic foci at doses of RIL-2 that had no effect when administered alone.

In the course of multiple experiments in the treatment of liver metastases, it was found that the systemic transfer of LAK cells given alone at Days 3 and 6 after tumor induction rarely was able to mediate a significant reduction of metastases. However, as shown in Chart 3, when concomitant administration of RIL-2 was given, we obtained a significant reduction in the number of metastases, especially at RIL-2 doses greater than 5,000 units i.p. 3 times a day. The percentage reduction was significant in all 16 experiments when greater than 5,000 units of RIL-2 was given in conjunction with LAK cells. Consistent reduction of hepatic metastases with treatment by RIL-2 alone required doses greater than 25,000 units and was always less effective than when combined with LAK cell administration. Thus, the administration of LAK cells significantly reduced the number of metastatic foci in the liver when combined with RIL-2 at RIL-2 doses that were much lower than those required without LAK cells. In addition, RIL-2 and LAK cells plus RIL-2 have been shown recently to prolong survival and effect cures in animal bearing murine sarcoma hepatic metastases.4

The mechanism(s) of RIL-2-mediated antitumor effects in vivo is currently unknown. However, as indicated by the data in Chart 4, the effectiveness of high doses of RIL-2 against liver metastases was abrogated when the host was given a sublethal dose of total body irradiation prior to RIL-2 therapy. This suggests that the RIL-2 acts through a host lymphoid component rather than by direct tumor cytotoxicity. The effects of LAK cells when given with RIL-2 were sustained despite host preirradiation. It is unknown at this time whether LAK cells mediate their effects in vivo by direct tumor toxicity or whether the host contributes a relatively radioresistant component to the response, as has been shown in other tumor systems (20, 21). This finding, however, does suggest that LAK cells do not require full host immunocompetence to mediate antitumor effects in vivo and suggests that this treatment may lend itself to combination with other antican

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4 R. Lafreniere and S. A. Rosenberg. Successful therapy of hepatic metastases from several murine tumors using lymphokine-activated killer (LAK) cells and recombinant interleukin 2, submitted for publication.
cer therapies. The requirement for IL-2 in conjunction with LAK cells suggests that the LAK cells divide in vivo and that this proliferation is required for manifestation of the therapeutic effect. Using the transfer of LAK cells from congenic mice, Ettinghausen et al.\(^1\) have demonstrated that this is indeed the case.

Work in our laboratory (22) had shown previously that phytohemagglutinin-activated lymphocytes and LAK cells were cleared by the lungs early after i.v. injection and were then distributed mainly to the liver and spleen. The finding that LAK cells plus RIL-2 therapy could successfully reduce liver metastases may be related to this hepatic distribution of LAK cells.

Some of the mice treated with LAK cells plus RIL-2 when autopsied at Day 14 failed to show any evidence of liver metastases, but most mice did have small numbers of metastatic deposits. It is possible that the migration of LAK cells to the liver is not optimal and that more RIL-2 and/or the transfer of additional LAK cells would cure additional mice. The presence of suppressor cells within the infused LAK cell population is another possible problem (23, 24) that could be overcome by the removal of tumor-enhancing cells (25).

The findings reported here provide a rationale for the performance of clinical trials of RIL-2 alone or in conjunction with LAK cells in the therapy of liver metastases in humans. We have recently begun these clinical trials.

REFERENCES


Fig. 1. Liver section at Day 3 posttumor injection showing clumps of tumor cells growing within the liver sinusoids. H&E, × 860.

Fig. 2. Section of Day 14 liver metastasis showing its relationship to the liver surface and parenchyma. Note the black particles throughout this section which represent carbon particles within Kupffer cells from the i.v. injection of India ink given prior to sacrifice. H&E, × 54.
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