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A New Reproducible Model of Hepatic and Peritoneal Metastases from Colonic Carcinoma

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Abstract—*A new technique for the generation of tumour nodules in both liver and peritoneal cavity has been developed in Wistar wag rats. The cell line* 192 *NRc was derived from a* 1,2-*dimethylhydrazine (DMH)-induced colonic carcinoma and was cultured on positively charged ion-exchange polystyrene microspheres. The tumour grew to confluence on the spheres, adhering firmly by pseudopodial extensions, enabling washing and injections of spheroids without significant dislodgement of cells.* 5×10^4 *tumour spheroids injected into the portal vein produced a mean of* 35 ± 24 *(S.D.) nodules in the liver with an average diameter of* 1.5 ± 0.55 *(S.D.)* mm *at* 14 *days. The spheroids did not enter the hepatic veins and therefore did not produce pulmonary metastases. Similarly,* 5×10^3 *tumour spheroids injected into the peritoneal cavity, after gently abrading the peritoneum with gauze, produced a mean of* 65 ± 37 *(S.D.) nodules with an average diameter of* 1.6 ± 0.39 *(S.D.)* mm *at* 14 *days. The tumour continued to grow as discrete nodules in both locations until near the time of death at approx.* 36 days *from inoculation.*

This animal model is reproducible and will allow the study of a number of treatment modalities for discrete neoplastic lesions in both the liver and peritoneal cavity at any stage of tumour growth without interference from tumour at unwanted sites.

INTRODUCTION

THE LIVER and peritoneal cavity are the main sites of disease recurrence in large bowel cancer, together accounting for approx. 85% of all treatment failures [1–4]. Surgical excision is the only treatment option proven to influence survival in patients with this disease, the role of adjuvant chemotherapy remaining controversial [5].

In order to investigate adjuvant intra-peritoneal chemotherapy in colonic carcinoma, we needed to develop a model of peritoneal and hepatic metastases which was reproducible and with a pattern of tumour growth which allowed easy measurement of end points. Many of the animal models of hepatic metasases reviewed in the literature were unsuitable because they employed portal or superior mesenteric vein injections of single-cell suspensions, which produced a high density of colonies in the liver with tumour confluence after only a few days [6–10]. Intra-portal injections also have the potential to produce lung metastases from cells entering the hepatic veins during injection. Intra-splenic cell injection has been used by several investigators in mice, but has the disadvantages of necessitating splenectomy and producing pancreatic metastases in 20% of animals [11, 12]. We were unable to produce hepatic metastases in rats using this technique and considered a murine model too small for the surgical cannulations to be undertaken.

Models in which hepatic and peritoneal metastases develop spontaneously from DMH-induced colonic carcinomas are cumbersome because there is a lead-in time of 20–30 weeks and the majority of tumours induced do not spontaneously metastasize before the animal dies [13]. Primary lesions have been produced by caecal wall injections of tumour cells to increase the incidence of dissemination, but still only approx. 45% of animals develop metastases in both the liver and peritoneal cavity [14–17].

To overcome these problems, we developed a technique in which polystyrene microspheres with a surface-adherent DMH-induced cell line were used to produce discrete tumour nodules in the liver and peritoneal cavity.

MATERIALS AND METHODS

Rats

Inbred male Wistar wag rats weighing between 250 and 280 g were obtained from the Animal Resources Centre at Murdoch University, Western

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Australia. The animals were fed standard rat pellets and water *ad libitum* and allowed a period of 7 days acclimatization.

The cell line

The cell line 192 NRc is one of several which has been derived from DMH-induced colonic carcinomas in Wistar wag rats in the department. The supply of tumour for culture was established by intramuscular hindlimb implantation and then freezing down a large quantity of solid material at −70°C from the second passage generation.

Culture of tumour cells on microspheres

The microspheres were positively charged polystyrene ion-exchange resins (Dowex 2, 200–400 mesh). Spheres were graded into three size ranges for the initial experiments (32–45, 45–75 and 75–120 μm in diameter) by dry sieving with Endocotts test sieves and the number of particles per mg of dry powder determined. Aliquots of microspheres were suspended in 5 ml of Hank's solution (without Ca^{2+}, Mg^{2+}) in glass vials, autoclaved and then washed in RPMI 1640 media. Plastic pipettes were used for all work to minimize loss of microspheres [18–20]. 2×10^5 microspheres were added to each 100 ml polystyrene flask containing 10 ml RPMI 1640, penicillin 100 U/ml, streptomycin 100 μg/ml and 5% foetal bovine serum. After adding a harvested suspension of tumour cells and gently mixing the contents, cells initially bonded loosely to spheres causing adherence of spheres to each other and to the floor of the flask. Over the course of 3–4 days, the cells covered each particle as a firmly attached monolayer and the resultant spheroids gradually separated out again.

The spheroids were centrifuged at 200 rpm for 2 min and the supernatant removed. This was repeated three times to remove free tumour cells from the suspension, the spheroids then being diluted to the appropriate concentration in RPMI 1640 for injection. Trypan blue exclusion showed that all adherent cells were viable.

Sphere binding capacity

Estimation of cell numbers per sphere was assessed by dissociating a suspension of tumour spheroids with 0.05% trypsin and 0.02% EDTA in Hank's solution and then counting the number of denuded spheres and cells in known volumes on a glass grid and haemocytometer respectively. The cellular binding capacity per sphere was then simply the ratio of cell and sphere counts. These values were confirmed by estimations of cell numbers per sphere from scanning electron micrographs.

Induction of liver and peritoneal metastases

All injections were carried out through an upper abdominal midline incision under halothane/N_2O anaesthesia. A 0.5 ml spheroid suspension was injected into the portal vein at a point distal to the inflow of the splenic vein, over a period of 20 s. In the same animal, visceral and parietal peritoneum were gently wiped with a piece of moist gauze. Tumour spheroids in a volume of 1 ml were then distributed evenly drop by drop over the surface of the abdominal viscera and abdominal wall with a plastic pipette prior to closing the abdomen. (Initial experiments in which spheroids were injected into the peritoneal cavity using a closed technique only resulted in the growth of a single tumour nodule on the abdominal wall at the site of injection, indicating that some degree of peritoneal trauma was required for tumour cell take.)

Measurement of tumour burden

Laparotomy was performed under barbiturate anaesthesia and an injection of 1.0 ml 50% Indian ink (Drawing ink, Reeves Aust. Pty. Ltd.) was given into the inferior vena cava over 1 min to demarcate hepatic tumour [11, 21]. The liver was then removed and bleached in Fekete's solution. All peritoneal nodules >0.5 mm in diameter were excised and fixed in 10% formalin.

The six liver lobes were separated and sectioned at 1 mm intervals. Tumours appeared as white nodules on a dark grey parenchymal background. The diameters of these lesions were measured on one face of each consecutive slice and the total cross-sectional area for the liver calculated.

Peritoneal tumour burden was expressed as the total mass of the excised tumour.

Determination of appropriate sphere size and numbers

Preliminary studies were conducted in nine animals to determine the number and size of spheroids required to achieve the most suitable pattern of tumour growth. Intra-portal injections of 1×10^3, 1×10^4 and 1×10^5 spheroids of three different diameters were performed. Similarly, intra-peritoneal instillations of 1×10^2, 1×10^3 and 1×10^4 spheroids of different sizes were carried out in the same animals. The number of tumour nodules and the total tumour burden in each compartment were determined at 14 days after inoculation as outlined above.

Growth characteristics of the tumour model

Having determined the most appropriate size and number of tumour spheroids for the model, a cumulative study was carried out in 44 rats. Each of these animals was injected with 5×10^4 45–75 μm spheroids into the portal vein and 5×10^3 45–75 μm spheroids into the peritoneal cavity. The animals were then sacrificed in groups of four at 5, 7, 9, 12, 14, 16, 21, 24, 28, 31 and 34 days after

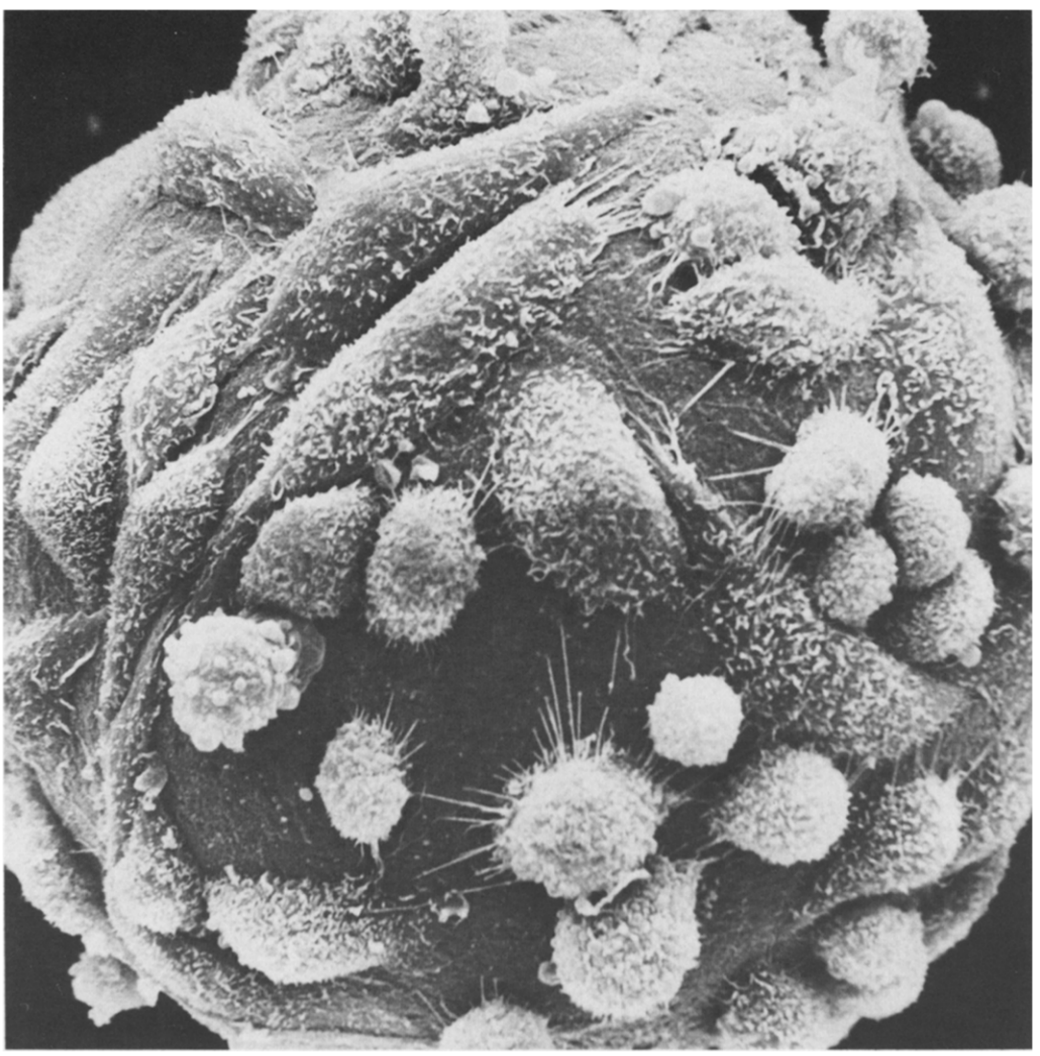

Fig. 1. Scanning electron micrograph showing pseudopodial attachment of 192 NRc carcinoma cells to the surface of an 80 μm diameter polystyrene sphere (mag. 1800×).

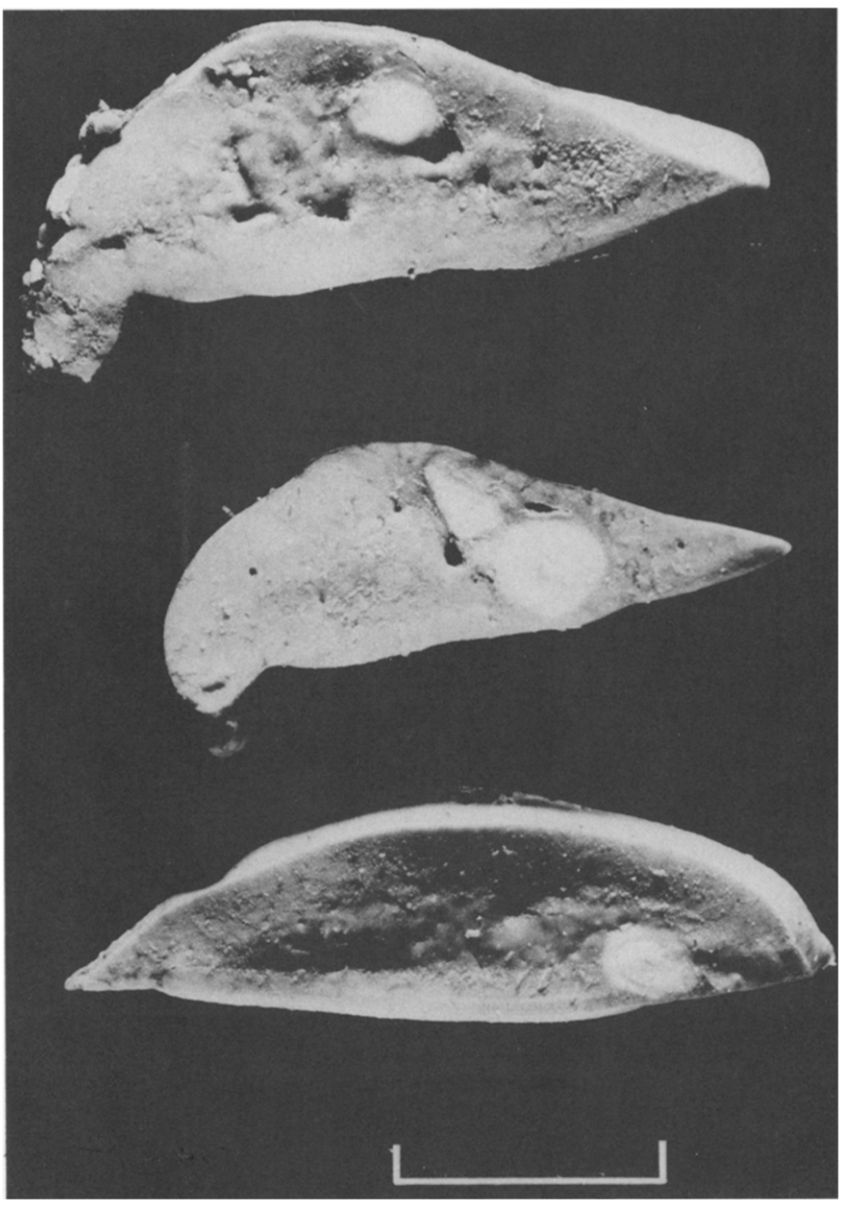

Fig. 2. Section of fixed liver showing discrete tumour nodules (white line represents 1 cm). *Spheroids were injected into the portal vein of this animal* 21 *days prior to sacrifice.*

inoculation respectively and the liver and peritoneal tumour growth measured.

RESULTS

Sphere binding capacity

The calculated cellular binding capacities of microspheres in the three diameter ranges are listed in Table 1. As would be expected, there was an approximately exponential increase in carriage capacity with change in diameter. Scanning electron micrographs confirmed the accuracy of these results (Fig. 1).

The effect of sphere size and number on the pattern of tumour growth.

Injection of the 32–45 μm spheroids into the portal vein did not result in hepatic tumour development in the three animals studied (Table 2a). Injection of the 75–120 μm spheroids resulted in tumour growth at the porta hepatis causing venous obstruction, presumably because the spheroids settled out in the proximal divisions of the portal vein. The 45–75 μm spheroids produced the most appropriate pattern of tumour development in the liver, indicating that they were carried to the peripheral branches of the portal venous system.

Tumour take occurred on peritoneal surfaces with the instillation of spheroids in quantities 10 × less than those required in the liver (Table 2b). The size of the spheres did not make a significant difference to the number of resultant tumour nodules or the total tumour mass in the peritoneal cavity.

Growth characteristics of the peritoneal and liver models

Hepatic tumour take was 100%. Tumour nodules grew in most liver lobes in each animal, although tending to seed the small left lateral lobes less frequently. They grew as well-separated nodules for a large part of the animal's life (Fig. 2). Even when the liver had been largely replaced by tumour at 34 days, individual nodules with intervening compressed normal liver parenchyma could still be identified. The growth curve for the liver model is shown in Fig. 3.

The number and diameter of separate tumour nodules in each animal varied somewhat depending on the pattern of distribution of embolized tumour spheroids. In the liver at 14 days, there were an average of 35 ± 24 (S.D.) nodules with a mean diameter of 1.5 ± 0.55 (S.D.) mm. In the animals studied at 31 days from inoculation, there were an average of 24 ± 11 (S.D.) nodules with a mean diameter of 8.2 ± 4.5 mm.

Table 1. Sphere binding capacity

Size range of spheres (μm)	Number of cells per sphere*	Calculated surface area ratios
32–45	31 ± 5	1.0
45–75	88 ± 7	2.4
75–120	186 ± 12	7.1

*Number of cells per sphere is the mean ± S.D. of five separate trypsinizations in each size range.

Table 2a. Effect of sphere size and number on tumour growth in liver (n = 9 *rats*)

Diameter of spheres (μm)	Number of spheres 10^3	10^4	10^5
32–45	0* (0)	0 (0)	0 (0)
45–75	0 (0)	17 (36)	73 (147)
75–120	confluent mass base of lobe(s); portal vein obstruction porta hepatis		

*Total number of separate hepatic nodules present. Numbers in parentheses refer to the total cross-sectional area of lesions (mm^2) on one surface of consecutive 1 mm liver sections.

Table 2b. Effect of sphere number and size on tumour growth in the peritoneal cavity (n = 9 *rats*)

Diameter of spheres (μm)	Number of spheres 10^2	10^3	10^4
32–45	0* (0)	33 (215)	183 (1485)
45–75	3 (8)	51 (415)	188 (1411)
75–120	22 (96)	45 (133)	82 (656)

*Refers to total number of tumour nodules >0.5 mm excised 14 days after inoculation of spheroids. Numbers in parentheses indicate the total mass of excised nodules in mg.

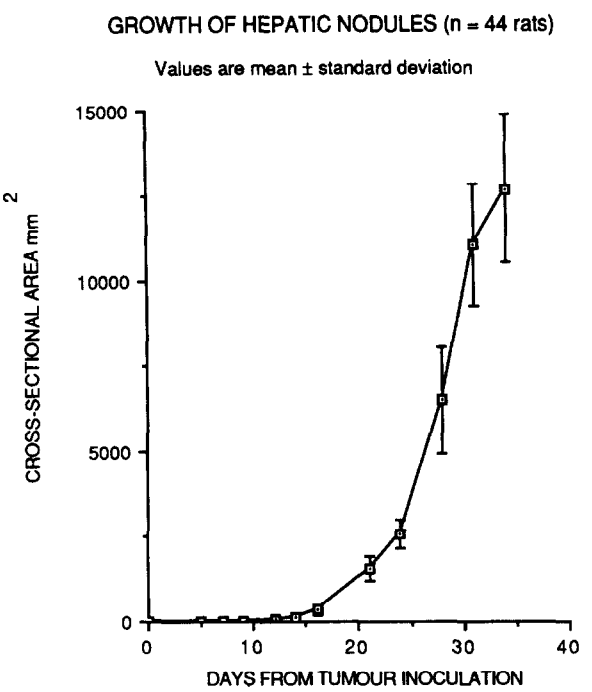

Fig. 3. Total cross-sectional area of tumour nodules in 1 mm liver sections, following injection of 5 × 10^4 45–75 μm spheroids into the portal vein. Each point is the mean of data collected from four animals.

Tumour take in the peritoneal cavity was also 100%. Peritoneal nodules were seen to grow on bowel (Fig. 4), along the abdominal wound and less often on solid viscera and the remaining parietal peritoneal surfaces. Again, tumour nodules remained separate for the majority of the 34 day period of observation. Nodules in the region of the abdominal wound tended to grow at a faster rate than those elsewhere. The peritoneal tumour growth curve (Fig. 5) had similar characteristics to that of the liver model.

In the group of four rats studied at 14 days, there was an average of 65 ± 37 (S.D.) peritoneal nodules with a mean diameter of 1.6 ± 0.39 (S.D.) mm. At 31 days, there was an average of 76 ± 33 (S.D.) nodules with a mean diameter of 5.1 ± 3.2 (S.D.) mm.

Animals sacrificed at 34 days were unwell from their tumour burden and would not have been expected to survive longer than 35–36 days.

Histology

Examination of paraffin sections of the liver and peritoneal lesions revealed a poorly differentiated adenocarcinoma.

DISCUSSION

The purpose of this study was to establish a stable model of hepatic and peritoneal metastases in the rat, with easily measured end points, which could be used to assess adjuvant chemotherapy for colonic carcinoma. To do this, we employed a technique of microsphere cell carriage to produce discrete tumour colonies.

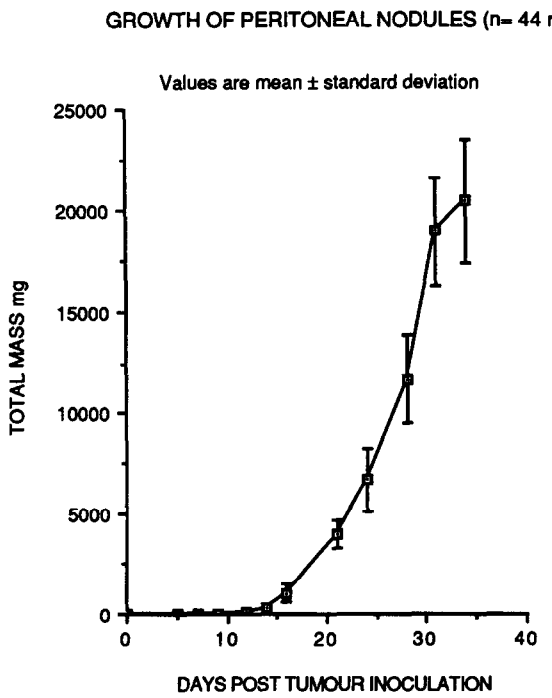

Fig. 5. Total mass of excised peritoneal tumour nodules, following instillation of 5×10^3 *spheroids into the peritoneal cavity. Each point is the mean of data collected from four animals.*

The problems associated with the standard techniques of single-cell injection include variability in tumour take and hence reproducibility, and the high density of tumour nodules produced. The measurement of tumour growth is then made difficult due to the early confluence of nodules. The advantage of the microsphere technique is that it allows the study of a population of discrete nodules in both the liver and peritoneal cavity for a longer period of time than is possible with other tumour models.

Microcarriers of various materials have been used in recent years for tissue culture, mainly for endothelial cell lines, as they increase the surface area available for cellular adherence and make large scale cell culture more cost effective [18, 19]. A further use relates to their ability to promote outgrowth of tumour cell explants from collagen gel by virtue of the fact that they form artificial aggregates of cells which are normally weakly cohesive [20]. We took advantage of the last-mentioned property in developing this tumour model as the cell line 192 NRc does not spontaneously form tight multicellular aggregates in culture. This technique allows closely sized cell packages, each with 100% cell viability to be injected, producing neoplastic lesions in solid organs of interest, without tumour cells entering the venous outflow of that organ and producing metastases at other sites. In a body space such as the peritoneal cavity, it produces a low density population of lesions by virtue of the grouping of cells on the visceral and parietal surfaces.

A problem with human colonic carcinoma is recurrence at sites of trauma, particularly the anastomotic site and the abdominal wound, presumably because the microenvironment of wounded tissue appears to promote tumour growth [8]. The peritoneal model presented here mimics that situation to a large degree in that tumour spheroids adhere and take more readily at sites of trauma. Indeed, injection of tumour spheroids into the peritoneal cavity using a closed technique, to avoid handling of the abdominal viscera, did not produce peritoneal tumour nodules (except at the injection site). In contrast, by gently abrading the peritoneal surfaces we were able to maximize tumour take.

Despite using microspheres to embolize tumour, there still seems to be a crucial number of cells required to overcome the host's defences before tumour take occurs in the liver. Varying the size and number of tumour spheroids injected had a significant effect on the subsequent development of tumour. Even though the sample sizes in the preliminary study were small, it can be seen that approx. 8×10^5 cells had to be injected into the

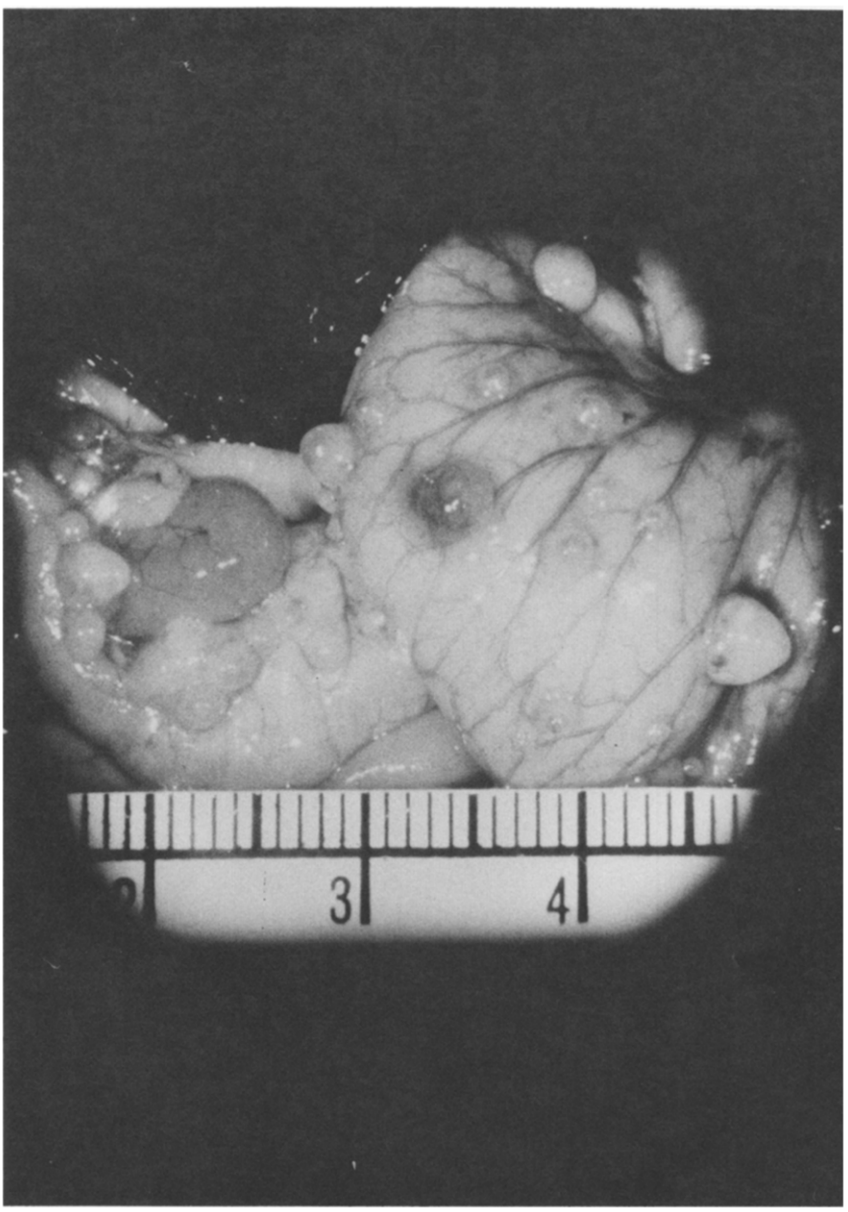

Fig. 4. *Lesions* 2–3 mm *in diameter on the surface of bowel* 16 *days after instillation of tumour spheroids into the peritoneal cavity.*

portal vein before hepatic tumour developed in these animals. In contrast, tumour take in the peritoneal cavity occurred reproducibly with cell numbers 10 × less than those required in the liver. In addition, the size of the tumour spheroid was less important a factor in determining the pattern of tumour growth in comparison to the liver.

The method of improving tumour micronodule demarcation has previously been used by Lafreniere and Wexler [11, 21]. The preferential uptake of carbon particles from the Indian ink into normal liver parenchyma is related to phagocytosis by the Kupffer cells in the reticuloendothelial system. The main use of this technique in our model has again been for the demarcation of small neoplastic nodules.

On average, the animals appeared significantly unwell from disseminated carcinoma at 5 weeks following tumour inoculation. The cell line 192 NRc is particularly rapidly proliferating and therefore the use of a more well-differentiated cell line may extend this time span further.

The microsphere technique should have wider application in the development of tumour models in other organs and in other animal species, provided that a syngeneic cell line is available which has adherent properties. The model outlined here will be useful in the study of a spectrum of secondary malignant lesions, from microscopic nodules to advanced disseminated disease in a pattern not too dissimilar to that of disseminated colonic carcinoma in man.

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