Detection of Metastatic Tumor Cells by Intraperitoneal Inoculation of Organ Brei from Tumor-bearing Mice*

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As early as 1889 the pioneer of animal tumor transplantation, A. Hanau (18), recorded the detection of metastases in lymph nodes of a rat by subcutaneous transfer into other rats. Gierke (14) in 1908 attempted "not always successfully" to transfer tumor metastases from the lung of the mouse. The interest in this method increased in 1927 after Blumenthal and Auler (1) interpreted their induction of a subcutaneous tumor by spleen brei from a tumor-bearing rat as indicating the presence of "cancer virus" in apparently normal tissue. Experiments in this direction were continued by several authors (9, 12, 13, 17, 26, 29, 34). Finally, Woglom (40) showed by a crucial demonstration that the presence of even a few metastatic tumor cells in apparently normal tissues accounts for growth of tumors by their inoculation.

It occurred to us that by using the intraperitoneal route for inoculation, it might be possible to avoid the nonspecific tissue reactions often observed in skin injected with organ brei. We have reported previously (15) that intraperitoneally injected mashed organs disappeared from the peritoneal fluid within a few days, while tumor cells of mashed tumors multiplied in this fluid as free cells. Accordingly, we have used the peritoneal cavity as a location that selectively promotes the growth of tumor cells inoculated together with a high number of normal cells. This would seem to make it a favorable site for detection of tumor cells in the blood and organs of mice bearing tumors at various sites of their bodies.

MATERIAL AND METHODS

Tumors and mouse strains.—Two sarcoma strains—S-37 and S-180—were carried in CFW mice in serial intraperitoneal transfers as free cells growing in the peritoneal fluid (15). Carcinoma E 0771 was transferred serially in C57Bl/6 strain of mice by the same method; and mouse carcinoma strains Barrett (C3H/Am) and H-2712 were transferred in C3H mice by using mashed peritoneal implants (owing to a scarcity of tumor cells in the fluid).[1]

Pattern of the experiment.—Groups of mice inoculated with tumor cells of the same strain, by the same route, and designated as "donors of organs" were sacrificed serially, at various time intervals, and bled. Their livers, kidneys, spleens, lungs, and brains were mashed separately, and the brei of each organ, as well as the withdrawn blood, were injected intraperitoneally into new mice. These mice recipients of organ brei were periodically examined for gross evidence of tumor growth in their peritoneal cavity, for the presence of tumor cells in their peritoneal fluid, and, finally, at the autopsy, for tissue characteristics of any implant. Positive results of these examinations—i.e., evidence of tumor growth in the peritoneal cavity—were interpreted as a demonstration of the presence in inoculated material of viable tumor cells spread from the primary tumor growth by implantation and by metastasis.

Inoculation of tumor cells into "donors."—Large numbers of tumor cells (50 to 100 million) were inoculated into prospective "donors of organs" to obtain abundant primary growth. Technics of intraperitoneal and intrapleural inoculation of requisite numbers of cells were described elsewhere (15, 16). For the site of subcutaneous inoculation, we used two areas, topographically distant: (*a*) the flank (left) and (*b*) the scalp. The site of intramuscular injection was the left thigh.

Inoculation of mashed organs from "donors" into "recipients."—Blood of each sacrificed donor was withdrawn with a capillary pipette (about 0.2 ml.) from the right heart and injected intraperitoneally, while still fluid, into the recipient. Organs from serous cavities were rinsed twice in 0.85 per cent NaCl solution before mashing in order to avoid any contamination of the organ brei with serous fluid which might contain tumor cells. In several instances samples of the third rinsing fluid were injected intraperitoneally into new mice, and specimens of peritoneal fluid from these mice were examined at various intervals. In no instance were tumor cells found in these smears. It was presumed, therefore, that any growth from twice-rinsed organs could hardly be attributed to contamination with tumor cells from serous exudate. Each organ was mashed in about 3 ml. of 0.85 per cent NaCl solution, and about 1 ml. of the brei suspension was injected intraperitoneally into the mouse.

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[1] Tumor Strains E 0771 and H-2712 and Mouse Strains C3H and C57Bl/6 were obtained from R. Jackson Memorial Laboratory, Bar Harbor, Maine; CFW Strain from Carworth Farms, New City, New York; Barrett Carcinoma (C3H/Am) through the courtesy of Dr. M. K. Barrett, National Cancer Institute.

Recording of the results.—Recipients of organ brei were usually examined after 10 days, after 15 to 20 days if they survived, and, in rare instances, later. The examination included withdrawal of a specimen of peritoneal fluid for microscopic test, palpation of the abdomen, and, ultimately, the autopsy. We described previously (15) the "auto-inoculation" of the abdominal wall with tumor cells from peritoneal fluid—i.e., tumor tissue growth at the sites of inoculating or exploratory abdominal punctures. The sharply localized ingrowth of tumor cells from the fluid into the serosa and subcutaneous tissue made easier the detection of positive results of organ transfers, in particular, if only a small number of tumor cells were inoculated with the organs. Occurrence of either free tumor cells in the peritoneal fluid or of implants in the peritoneal cavity was recorded as a positive result.

RESULTS

In control experiments none of the CFW, C57, or C3H mice injected intraperitoneally with mashed organs of normal mice presented, at the autopsy (after 7 days), any evidence of new growth or of a tissue reaction. However, their abdomens were often distended by about 0.5 ml. of peritoneal fluid containing mostly macrophages; while in new mice the amount of fluid was not above 0.2 ml. and the proportion of macrophages not more than 15 per cent. These results do not support the contention of some authors (4, 22) that the gross picture of ascites and the resulting increase in body weight may be accepted as criteria for the demonstration of tumor cell growth in peritoneal fluid.

In control CFW mice inoculated with free tumor cells from the peritoneal fluid, i.e., a suspension of tumor cells without any trace of tissue, the peritoneal cavity, after 4–6 days, generally contained a copious amount (about 1 ml.) of fluid containing tumor cells, and eventually a small tumor appeared at the site of puncture. However, in mice inoculated with a mixture of free cells and mashed organs there were, in addition to the above types of growth, several small circular tumors on visceral serosa (20).

In summarizing our experiments, we have recorded for each group of mice bearing primary tumors of the same strain, same age, and same localization, the frequency of intraperitoneal growth from their organs inoculated into new mice; i.e., the frequency in these organs of tumor cells transported from the site of primary growth by implantation and by metastasis. The data on all groups were tabulated separately for each tumor strain. It appears from Tables 1–5 that each tumor strain showed, under similar experimental conditions, a difference in the rate of growth and the frequency and localization of tumor cell spread into organs. Thus, in all groups of mice with primary growth of Sarcoma 37, these cells were detected at an earlier stage after inoculation, in a higher percentage of animals, and in a greater variety of organs than in mice with other tumors (Table 1 as compared to Tables 2–5). The difference between the two sarcoma strains, 37 and 180 was significant, but only quantitative (Tables 1 and 2). However, carcinoma strains (Tables 3, 4, and 5) showed, besides considerable delay in appearance of metastases, some peculiarities of their distribution, such as extreme scarcity of brain metastases from the primary carcinoma E 0771 (Table 3), lack of metastases from the scalp for the primary Barrett carcinoma (Table 4), absence of metastases in abdominal organs from intrapleural carcinoma H-2712 (Table 5), etc.

On the other hand, within this wide range of variations, the following common factors appeared to be significant for metastatic growth from primary tumors of all strains:

a) Site of primary tumor growth.—Organs from donors bearing their primary growth in serous cavities, particularly in the peritoneal cavity, induced intraperitoneal tumors in the majority of recipients. The next highest percentage of tumor growth from organs was recorded for donors with the primary neoplasm in subcutaneous tissues of the flank; a lower frequency was found in bearers of intramuscular tumors, and the poorest results in donors having primary subcutaneous tumors in their scalps, in spite of the large size of these tumors.

b) Age of primary tumor growth.—As a rule, the percentage of positive results of organ inoculation increased with the age of primary tumor growth in donors. For instance, metastatic cells were detected only in later stages of primary carcinoma Barrett or carcinoma H-2712 growth (Tables 4 and 5).

c) Number of tumor cells in organs.—Organs of mice bearing large S-37 tumors in their scalps induced a considerable number of tumors in recipients, but only following a long interval (20 days) after inoculation (Table 1). Since S-37 grows fast, this delay in the growth can be attributed only to the scarcity of viable tumor cells in inoculated organs. Late positive results of organ inoculation were recorded also in some groups with other tumors, in particular for Ca H-2712 (Table 5). Thus, the scarcity of tumor cells in organs was reflected by the delay of their growth into sizable tumors but, apparently, without decrease in their frequency. However, it was noted that slowly growing tumors in recipients had a tendency to become necrotic and to regress. Thus, some instances of initial tumor growth from organs could have been missed.

DISCUSSION

The purpose of this paper was to describe a practical method for serial detection in tumor-bearing mice of metastases in various organs with a minimum of pseudopositive or pseudonegative results; to study with the aid of this method the spread of metastases from primary tumors grown at various sites of the body; and to outline, on the basis of these results, the pattern of localization and frequency of metastases for five different tumor strains.

The principle of detection of metastatic tumor cells by inoculation of organ brei is not new, as it was pointed out in our introduction, but only subcutaneous and intramuscular routes of inoculation were explored and for the most part in a very small series of animals (1, 2, 12, 13, 14, 17, 18, 26, 27, 29). The use of the intraperitoneal route in our

TABLE 1

GROWTH OF TUMOR CELLS FROM THE BLOOD AND THE ORGANS OF S-37-BEARING CFW MICE

Tumor growth in mice donors of organs			Frequency of tumor growth in mice (Recipients of i.p. injected blood and mash from the following organs of mice donors)					
Site of tumor growth	Age of growth at the date of the autopsy (days)	Interval between inoculation of mice recipients and their examination (days)	Blood	Liver	Spleen	Kidney	Lung	Brain
Peritoneal cavity	1	10	0	1	2	0	0	0
	2	11	0	5	6	4	0	0
	3	11	2	8	8	6	4	2
	3	11	4	10	7	8	6	5
	5	11	3	10	8	10	7	4
Pleural cavity	1	10	0	0	0	0	4	0
	2	10	0	0	0	1	4	0
	3	10	4	10	3	9	10	3
	5	11	2	9	4	8	8	2
Subcutaneous tissue:	2	10	0	0	0	0	0	0
	5	11	2	0	3	2	4	0
Flank	10	11	5	6	8	8	4	0
Scalp	11	10	0	0	0	0	0	0
	11	20	3	6	3	3	6	6
Muscle of the thigh	5	10	0	0	0	0	0	0
	15	10	2	0	3	2	4	0
	15	15	5	0	6	8	5	0

Ten mice were used in each group. For criteria of growth see "Material and Methods." Mice were examined more frequently than indicated, but slight changes in the frequency of tumor growth at successive examinations were not recorded.

TABLE 2

GROWTH OF TUMOR CELLS FROM THE BLOOD AND THE ORGANS OF S-180-BEARING CFW MICE

Tumor growth in mice donors of organs			Frequency of tumor growth in mice (Recipients of i.p. injected blood and mash from the following organs of mice donors					
Site of tumor growth	Age of growth at the date of the autopsy (days)	Interval between inoculation of mice recipients and their examination (days)	Blood	Liver	Spleen	Kidney	Lung	Brain
Peritoneal cavity	1	10	0	0	0	0	0	0
	2	10	0	0	0	0	2	0
	4	10	2	8	6	8	9	4
	6	10	2	4	3	3	7	2
	6	15	0	6	4	3	9	1
Pleural cavity	4	10	0	1	0	0	1	0
	8	10	5	6	3	9	10	2
Subcutaneous tissue:	6	10	0	1	0	1	3	0
Flank	10	10	0	4	6	6	4	0
	12	10	0	6	4	3	6	0
Scalp	8	10	0	0	0	0	2	2
	11	20	0	1	0	0	4	3
Muscle of the thigh	4	10	0	0	0	0	4	0
	4	17	2	0	0	0	6	0

Ten mice were used in each group. For criteria of growth see "Material and Methods." Mice were examined more frequently than indicated, but slight changes in the frequency of tumor growth at successive examinations were not recorded.

TABLE 3

GROWTH OF TUMOR CELLS FROM THE BLOOD AND THE ORGANS OF CARCINOMA E 0771-BEARING C57BL/6 MICE

Tumor growth in mice donors of organs			Frequency of tumor growth in mice (Recipients of i.p. injected blood and mash from the following organs of mice donors)					
Site of tumor growth	Age of growth at the date of the autopsy (days)	Interval between inoculation of recipients and their examination (days)	Blood	Liver	Spleen	Kidney	Lung	Brain
Peritoneal cavity	2	15	0	0	0	0	1	0
	4	15	0	0	0	0	1	0
	10	14	0	4	8	3	7	0
	10	20	0	4	9	3	8	0
Pleural cavity	7	10	0	2	7	6	8	0
Subcutaneous tissue:	3	20	0	0	0	0	0	0
Flank	5	20	0	0	0	0	0	0
	12	10	0	0	0	0	6	0
	12	20	0	6	3	0	7	0
Scalp	13	10	0	0	0	0	4	2
Muscle of the thigh	10	10	0	0	0	0	4	0
	10	20	0	0	0	0	9	0

Ten mice were used in each group. For criteria of growth see "Material and Methods." Mice were examined more frequently than indicated, but slight changes in the frequency of tumor growth at successive examinations were not recorded.

TABLE 4

GROWTH OF TUMOR CELLS FROM THE BLOOD AND THE ORGANS OF BARRETT CARCINOMA-BEARING C3H MICE

Tumor growth in mice donors of organs			Frequency of tumor growth in mice (Recipients of i.p. injected blood and mash from the following organs of mice donors)					
Site of tumor growth	Age of growth at the date of the autopsy (days)	Interval between inoculation of recipients and their examination (days)	Blood	Liver	Spleen	Kidney	Lung	Brain
Peritoneal cavity	12	12	0	8	6	7	7	3
	15	10	0	5	7	8	7	1
	20	10	0	6	4	6	8	0
	30	10	0	8	7	6	8	0
	30	10	0	8	9	9	10	0
Pleural cavity	8	10	0	2	0	0	10	0
	12	10	0	3	1	8	10	0
	12	20	0	7	1	8	10	0
Subcutaneous tissue:	5	10	0	0	0	0	3	0
Flank	12	10	0	0	2	0	8	0
Scalp	15	15	0	0	0	0	0	0
Muscle of the thigh	20	10	0	2	0	0	7	0

Ten mice were used in each group. For criteria of growth see "Material and Methods." Mice were examined more frequently than indicated, but slight changes in the frequency of tumor growth at successive examinations were not recorded.

TABLE 5

GROWTH OF TUMOR CELLS FROM THE BLOOD AND THE ORGANS OF CARCINOMA H-2712-BEARING C3H MICE

Tumor growth in mice donors of organs			Frequency of tumor growth in mice (Recipients of i.p. injected blood and mash from the following organs of mice donors)					
Site of tumor growth	Age of growth at the date of the autopsy (days)	Interval between inoculation of recipients and their examination (days)	Blood	Liver	Spleen	Kidney	Lung	Brain
Peritoneal cavity	20	10	0	3	4	2	8	0
	20	20	0	8	5	7	9	0
Pleural cavity	20	20	0	0	0	0	5	0
Subcutaneous tissue:	20	10	0	0	0	0	0	0
Flank	20	20	0	1	0	2	6	0
Scalp	20	20	0	2	0	0	5	0
Muscle of the thigh	20	20	0	1	0	0	3	0

Ten mice were used in each group. For criteria of growth see "Material and Methods." Mice were examined more frequently than indicated, but slight changes in the frequency of tumor growth at successive examinations were not recorded.

experiments eliminated nonspecific reactions in subcutaneous or muscle tissue (swelling, necrosis, granulations, scar formation) which frequently obliterate growth from small inocula or simulate a tumor in negative cases. Moreover, the growth of tumor cells from intraperitoneally inoculated material could be identified, in the majority of mice, by rapid examination of smears from peritoneal exudate. Thus, it was technically easy to obtain clear-cut results in a large number of mice serially inoculated with brei of various organs. Furthermore, primary tumors were grown in mice donors of organs at various body sites selected to produce lymph-borne metastases (by inoculation of the flank), blood-borne metastases (from scalp tumor), or serous fluid-borne implantation and metastases (from free tumor cell growth in the peritoneal or the pleural exudate).

It should be pointed out that our positive results constitute some evidence that a sufficient number of cells from primary neoplasms have reached several organs (7), but they do not indicate whether these tumor cells started to proliferate before their transfer with the organ brei into new mice. Moreover, our negative results do not exclude the occurrence in the organ of a very low number of tumor cells or their inhibition by a defense reaction in the recipient (3, 19, 37).

Our data illustrate the spread of metastatic cells from various initial points (various sites of primary neoplasms of various strains) to various end points (various organs) and suggest the analogy between these results and the data of human pathology.

a) Fluid-borne implantation and metastases.—It is understandable that intraperitoneally inoculated tumor cells floating in the peritoneal fluid penetrated into open stomata between the cavity and the lymphatics (10) or nestled below the visceral peritoneum by damaging the lining, inducing a coagulum and stimulating an ingrowth of fibroblasts (30). It is possible that tumor cell implantation from serous fluid on the surface of abdominal organs resulted eventually in metastases comparable to transcoelomic metastases of human tumors (39), while in other cases there was only local invasion of the organ surface. This problem is under investigation.

b) Lymph-borne metastases.—Serial autopsies of mice with flank tumors showed progressive infiltration of lymphatics and lymph nodes in the abdominal wall with tumor growth reaching peritoneal serosa in most animals. This process seemed to be analogous to the spread of human metastases by "lymphatic permeation" or by embolization (39).

c) Blood-borne metastases.—Tumors were grown from the blood of mice bearing S-37 cells at any site of their bodies, mostly in later stages of their growth (Table 1), less often in mice with S-180 growth, and never in animals inoculated with carcinoma strains. Previous reports on occurrence of S-37 cells in the blood (21, 29) and of cells from other sarcoma strains (24, 25) showed extensive variations in the frequency of these results, depending apparently on the age and the site of primary tumors (Table 1). Our negative results with carcinoma strains did not exclude an intermittent occurrence, at long time intervals, of tumor emboli in the blood. Indeed, metastases in the lungs and in the liver from carcinoma growing in the scalp were obviously blood-borne, and so, probably, were the metastases from the thigh. The observation that they were localized frequently in the lung, rarely in the liver, and not at all in other organs (Tables 3, 4, and 5) is in agreement with the known tendency of human tumor emboli liberated into the blood stream to be arrested by the first capillary bed of the systemic blood in the lung and those of portal blood by the liver (28, 35, 39).

Our results agree with observations of early workers on the localization of metastases from slowly growing subcutaneous mouse tumors mainly in the lungs, and exceptionally in the liver (14, 27), on the distribution of metastases in animals after intravenous injection of tumor cells (23, 31–33, 36), and on high frequency of implantation and metastases from intraperitoneally inoculated sarcoma (25). While our results support the hypothesis that local tissue invasion is a prerequisite to the formation of blood-borne metastases (8) and that localization of metastases depends primarily on the availability of tumor emboli and the anatomical distribution of the vascular system (6, 7, 8, 11, 28), our data may suggest that the availability of tumor emboli depends on the opportunities for tumor cells or clumps to get separated, owing to their reduced adhesiveness (5), from the neoplastic tissue, as in serous fluid or in edematous connective tissue. Moreover, our results may be interpreted as illustrating a parallelism between the growth rate of neoplasms and their tendency to induce metastases.

The method outlined above for detection of metastatic cells may be used for the study of the mechanism of metastatic processes in transferable mouse tumors. Moreover, it may be applied for screening the effect of various physical and chemical agents on tumor cells transported into organs from the primary growth. We are studying the effect of radioactive colloidal gold on metastatic

tumor cells in mice, and the results will be reported separately.

SUMMARY AND CONCLUSIONS

1. Intraperitoneal inoculation of mashed organs or blood from tumor-bearing mice resulted, in numerous instances, in peritoneal growth in new mice. These results were attributed to the presence in the inoculated material of viable tumor cells spread from the primary growth by implantation and by metastasis.

2. By the use of this method in mice bearing tumors of various strains (two sarcomas: S-37 and S-180; three carcinomas: E 0771, Barrett's [C3H/Am] and H-2712) at various sites of their bodies, it was found that the frequency and distribution of metastatic cells in various organs showed different patterns for various tumor strains and depended, for the same tumor strain, on the site and the age of primary tumor growth. The number of metastatic cells in an organ was reflected by the rate of growth of intraperitoneal tumors induced by inoculation of this organ.

3. Discussion of the data correlating the site of localization of metastatic cells (certain organs) with the site of their origin (site of the primary neoplasm) indicated the spread of these cells from the primary tumor by routes of serous fluids, lymph, and blood.

4. Application of the outlined method of detection of metastatic cells for the study of metastases from transferable tumors and for the screening of metastases-inhibiting agents is suggested.

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