Neoplasm Immunity:
Experimental and Clinical

Ray G. Crispen, Editor

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NEOPLASM IMMUNITY: EXPERIMENTAL AND CLINICAL

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Editor:

RAY G. CRISPEN*
Director of ITR Biomedical Research
904 W. Adams, University of Illinois, Chicago, Illinois, U.S.A.

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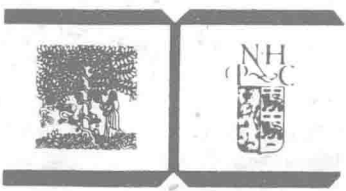

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DEVELOPMENTS IN CANCER RESEARCH

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Volume 2—Tumor Progression, edited by Ray G. Crispen, 1980

Volume 3—Neoplasm Immunity: Experimental and Clinical, edited by Ray G. Crispen, 1980

EDITOR'S NOTE

The symposium was very informative and demonstrated recent advancements made in our understanding of the role of the immune system, the role of tumor antigens and the importance of measuring immunologic parameters in clinical trials.

Dr. Fidler has shown that the pathogenesis of metastasis is a complex biological phenomenon which is highly selective. His results suggest that in a weakly immunogenic tumor system the constant interaction of tumor cells with syngeneic lymphocytes may lead to tumor growth and spread. Metastasis occurs when these unique tumor cells can evade the host's defense mechanisms. Dr. Kollmorgen demonstrated that a fat diet in animals allows progression of tumor growth and may be related to the carcinogenicity of various antigens when they are covalently linked with lipid moieties.

The integration of viral DNA into human neoplastic cells was illustrated by the research work of Dr. Kit. The fact that viral c-type particles can have a normal physiologic function during gestation in addition to their pathogenic role was investigated. Dr. Panem demonstrated that an antigen which reacts with antisera prepared to the placenta antigen can also be found during the pathogenesis of systemic lupus erythematosus. And the isolation of tumor-related proteins in the urine of cancer patients by Dr. Rudman may shed new light on the relation between proteolysis and tumorigenesis.

Many studies have demonstrated that tumor-associated antigens and tumor-associated immune responses are present in all experimental animal and human tumors. These responses include cell-mediated and humoral immunity as well as nonspecific host defense mechanisms. A model for selectively stimulating cellular immunity without stimulating detectable antibody formation was presented by Dr. Hunter and cell-mediated immunity assays for breast cancer were reported by Dr. McCoy. The relationship of tumor induced cellular immunity and cellular suppression in tumor bearing animals, as well as, passive transfer of tumor immunity using *in vitro* sensitized cells were explored.

Immunologic manipulation of the host has laid the foundation for clinical immunotherapy, and the necessity for the immune monitoring of the patient. In 1964 Professor Georges Mathé proposed one of the first clinical trials of systemic immunotherapy. He reported on its efficacy in the maintenance of chemotherapy induced remissions in patients with leukemic lymphosarcomas, resulting in enhanced survival. Currently he is conducting experiments on second generation immune adjuvants searching for those which do not induce suppressor cells and at the same time possess the favorable effects of BCG for antitumor activity. Various approaches to

immunotherapy including immunization with tumor cells or tumor antigen, and immunorestorative immunotherapy with thymic hormones and levamisole are presented.

It is apparent that this conference has clearly demonstrated the large experimental basis for establishing immunotherapy programs in humans and gives a better understanding of the mechanisms involved in manipulating the immune system.

NEOPLASM IMMUNITY

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Effect of Syngeneic Lymphocytes on the Vascularity, Growth and Induced Metastasis of the B16 Melanoma*

Isaiah J. Fidler[1]
D.M. Gersten[2]

SUMMARY—We investigated the possible mechanism(s) by which syngeneic lymphocytes participate in the establishment and growth of experimental metastases. In the weakly immunogenic B16 melanoma system, host lymphocytes can increase the arrest of circulating tumor emboli in pulmonary capillaries. In the present studies we wished to determine whether syngeneic lymphocytes also can affect the process of cancer metastasis at steps which occur after tumor cell arrest. Lung metastases were produced in C57BL/6 mice by injecting B16 melanoma cells i.v. Fewer lung metastases resulted from the injection of tumor cells into immunosuppressed mice than normal syngeneic mice. The mice were immunosuppressed by adult thymectomy and sublethal (450R) whole-body X-irradiation (ATX mice). Normal and ATX mice were also injected i.v. with a single dose of 10^7 syngeneic lymphocytes at 24 hr before, or 24 or 48 hr after the i.v. injection of tumor cells. All mice were killed 3 weeks after tumor cell injection and the number of artificial metastases was determined. The data demonstrated that a) the number of pulmonary metastases in ATX mice was significantly lower than in normal mice; b) reconstitution of ATX mice with 10^7 lymphocytes reversed this decrease; and c) the enhancement of metastasis in ATX mice by the injection of 10^7 lymphocytes occurred at all times of reconstitution. Even in ATX mice injected with lymphocytes 48 hr after tumor cell arrest had already taken place, and tumor cells invaded the parenchyma, a dramatic increase in metastases was observed. In subsequent studies the B16 melanoma was injected s.c. into normal and immunosuppressed mice which were or were not reconstituted with the i.v. injection of 10^7 syngeneic lymphocytes 24 hr before tumor cell injection. Tumor growth and vascularity were monitored and found to be significantly decreased in the ATX mice. In lymphocyte-reconstituted ATX mice, however, tumor growth and vascularity were pronounced and indistinguishable from control groups. Therefore, the present studies indicate that lymphocyte enhancement of metastasis can occur at several steps of the process which follow initial tumor cell arrest.

[1]Head, Biology of Metastasis, Cancer Biology Program, NCI Frederick Cancer Research Center, Frederick, Maryland.

[2]Department of Pathology, Georgetown University School of Medicine and Dentistry, Washington, D.C.

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INTRODUCTION

Since the initial proposal by Prehn(1) that the immune response could play both a stimulatory and inhibitory role in the progression of tumor growth, several investigations on the participation of immune factors in experimental metastasis have been reported (2-4). Two types of evidence suggest that lymphocytes may have a profound effect on the outcome of experimental metastasis in mice. First, injection of lymphocyte:tumor cell aggregates containing varying ratios of lymphocytes to tumor cells altered the number of lung colonies formed by constant numbers of tumor cells. The extent and direction of the alteration was dependent both on the immune status of the lymphocytes and their number. Second, lymphocyte depletion of animals by thymectomy and X-irradiation prior to i.v. injection of weakly immunogenic tumor cells produced a significant decrease in the number of lung colonies, which could be reversed by reconstitution of mice with syngeneic lymphocytes 24 hr prior to i.v. tumor cell injection. Again, the number of metastases was dependent on the source and number of syngeneic lymphocytes (2).

The mere presence of tumor cells in the circulation does not constitute metastasis. Metastases must result from the arrest of circulating tumor cells in a capillary bed, extravasation into the organ parenchyma and subsequent growth of tumor cells into visible colonies. Since tumor cell arrest in the microvasculature must precede the subsequent development of tumor cells, the formation of an heterotypic circulating embolus of some critical size is a crucial event in the pathogenesis of metastasis. Since the formation of such an embolus would ensure the initial arrest of tumor cells in the capillary bed of an organ, such arrest would influence the ultimate number of visible metastases (5). To test this proposal, experiments evaluating the effects of lymphocytes on the initial arrest of radioisotopically labeled tumor cells injected i.v. in the lung as well as their subsequent kinetic distribution throughout the body were performed (6). The results of those studies demonstrated that the ultimate outcome of experimental metastasis could not be predicted from the initial arrest data of tumor cells. While the critical embolus size probably has a significant role in the initial arrest of circulating emboli and therefore some effect on their subsequent survival and growth, it was clear that additional factors were involved.

In the present study, we wished to determine whether lymphocytes could influence the outcome of metastasis by interacting with tumor cells at steps other than initial arrest in the sequence of experimental metastasis. Specifically we investigated the influence of normal and sensitized syngeneic lymphocytes on the vascularity surrounding tumors growing s.c., as well as on tumor growth s.c. and in the lungs. In addition, we asked whether the outcome of such interactions is influenced by properties of host lym-

phocytes and of the tumor cells themselves.

MATERIALS AND METHODS

Animals

Inbred C57BL/6 mice were obtained from the Animal Production Area, Frederick Cancer Research Center, Frederick, Maryland. At 4 wk of age, mice were either thymectomized or sham-thymectomized as described previously (6). One week later and 4 wk before the i.v. tumor cell injection these animals were given a single, acute exposure to 450R, whole body X-irradiation. The animals were given drinking water which contained 10 ppm chlorine (7).

Tumor Cells and Cell Culture

B16-F1 (with low metastatic potential) and B16-F10 (with high metastatic potential) tumor cells were isolated originally by sequential i.v. passage through C57BL/6 mice (1) and have been maintained in culture as described previously (8). The tumors were checked and found free of mycoplasma and pathogenic murine viruses including LDH virus.

Sensitization of C57BL/6 Mice to B16 Melanoma

C57BL/6 mice, 6 to 8 wk old, were given a s.c. injection of 100,000 viable B16 cells. Three weeks later when the tumors were 10 to 30 mm in diameter the animals were killed. Their lymph node and spleen lymphocytes were prepared as described below and designated as tumor-bearing lymphocytes.

Preparation of Normal and Sensitized Lymphocytes

Axillary, cervical, and mesenteric lymph nodes and spleens were collected aseptically from normal or tumor-bearing mice, placed in cold Hanks' balanced salt solution (HBSS) and forced through a wire mesh sieve. The resulting suspensions were filtered through a glass wool column and centrifuged, and the cellular pellets were resuspended in serumless complete minimum essential medium (CMEM). Erythrocytes were removed from the preparation by hypotonic lysis as described previously (6). Viability was about 95% as determined by the trypan exclusion test. Practically all cells were determined morphologically to be lymphocytes.

Procedures for Study of Experimental Metastasis

For studies of experimental metastasis we routinely harvested cells from nonconfluent monolayers by overlaying the cultures with a thin layer of 0.25% trypsin–0.02% EDTA for 1 min. The flask was tapped to facilitate removal of cells from the plastic, and fresh medium was immediately added. The cells were washed and resuspended in HBSS. Tumor cell viability as

determined by the trypan blue exclusion test usually exceeded 95%. The suspension was diluted to yield 5 x 10^4 single cells/0.2 ml, the inoculum volume per mouse. This dose was selected because that number of cells could produce enough metastases to be easily counted.

Tumor cells were injected i.v. into the tail vein of C57BL/6 mice and mice were killed 18 days later. The number of resultant pulmonary tumor colonies was determined with the aid of a dissecting microscope by two independent observers.

Procedure for Study of Experimental Metastasis in Immunosuppressed C57BL/6 Mice with or without Lymphocyte Reconstitution

Mice were divided into three major groups: adult thymectomized X-irradiated (450R) (ATX); sham-thymectomized X-irradiated (STX) and normal mice. In addition, each of these groups was subdivided into four lymphocyte treatment groups: no lymphocyte reconstitution, (0); lymphocyte reconstitution 24 hr prior to i.v. tumor cell injection (−24); lymphocyte reconstitution 24 hr following i.v. tumor cell injection (+24); and lymphocyte reconstitution 48 hr following tumor cell injection (+48). Thus, each experiment represents a 3 x 4 array using 10 animals for each of the three experiments. The number of tumor cells injected i.v. was always 50,000 single viable cells. The number of i.v.-injected lymphocytes varied among the different experiments. We injected mice with either 1 x 10^7 or 1 x 10^8 lymphocytes from either normal or tumor-bearing mice. All mice were killed 18 days following the i.v. injection of tumor cells, and the number of lung metastases was determined as described above.

Influence of Syngeneic Lymphocytes on the Growth and Vascularization of Tumor Cells Injected Subcutaneously

The B16 melanoma F10 cell line was used throughout these experiments. Fifty thousand viable cells were injected in 0.1 ml HBSS s.c. lateral to the midline on the abdomen. The study included the following groups of mice (15/group): 1) normal mice; 2) ATX mice, and 3) STX mice. Each major group of mice consisted of three subgroups (5/group): 1) unirradiated unreconstituted, 2) irradiated (450R) unreconstituted, and 3) irradiated (450R) and lymphocyte-reconstituted (1 x 10^7 normal C57BL/6 spleen and lymph node lymphocytes). Fourteen and 20 days later the mice were killed and the skin was reflected to expose s.c. implants and vessels in the flank (9). Tumor size was measured and the degree of vascularization surrounding the growing tumors was compared to that on the uninjected side and recorded as mild or pronounced.

Statistical Analysis

Statistical significance was analyzed using Colhran's modified t-test.

RESULTS

We have shown previously that the initial arrest of i.v.-injected tumor cells in the pulmonary vasculature does not correlate with the final number of visible metastases. However, both initial arrest and development of metastases can be altered by the interaction with host lymphocytes (6,10). The first experiment asks whether post-initial arrest events in the metastatic process (see Discussion) can be affected by the presence of tumor-bearing lymphocytes. In Table 1 it is shown that, as reported previously (2), ATX significantly reduced the number of tumors following the i.v. injection of an equal number of B16-Fl cells from 42 ± 5 to 16 ± 2 ($p < 0.01$). Prior injections (−24 hr) of recipients with 10^7 tumor-bearing lymphocytes (so that lymphocytes were present at the time of tumor cell injection) increased the number of tumors to 246 ± 10 in the normal group, 239 ± 18 in the STX group and 166 ± 23 in the ATX group. This implies that 10^7 lymphocytes from tumor-bearing mice are sufficient to abrogate the decrease in the

Table 1—NUMBER OF LUNG TUMOR COLONIES IN CONTROL, THYMECTOMIZED X-IRRADIATED AND SHAM-THYMECTOMIZED X-IRRADIATED C57BL/6 MICE

Group of C57BL/6 Mice Given i.v. Injection	Av. No. of Pulmonary Metastases[a]			
		Lymphocyte-treated		
	Untreated	-24 hr	+24 hr	+48 hr
Normal Controls	42 ± 5[b] (20 - 72)	246 ± 10 (213 - 243)	44 ± 4 (26 - 60)	55 ± 6 (38 - 90)
Thymectomized X-Irradiated[c]	16 ± 2 (6 - 25)	166 ± 23 (99 - 240)	60 ± 11 (35 - 118)	39 ± 3 (24 - 51)
Sham-thymectomized X-Irradiated	60 ± 11 (32 - 140)	239 ± 18 (170 - 300)	47 ± 8 (20 - 88)	90 ± 14 (28 - 150)
Normal Mice Injected With Lymphocytes Alone	—	0	0	0

Mice were either Untreated or Treated Once by i.v. Injection of 10^7 Syngeneic Lymphocytes from Tumor-Bearing Donors at 24 hr prior to and 24 hr or 48 hr following i.v. Injection of 50,000 Viable B16-F1 Melanoma Cells.

[a]Ten mice/group; pulmonary metastases were counted 21 days after i.v. injection with the aid of a dissecting microscope.

[b]Mean ± SEM (Range).

[c]Thymectomized at 4-5 weeks of age; 450R total body irradiation given 10 days after surgery and 4 weeks prior to tumor cell injection.