The Concept of Immunotherapy of Human Sarcomas: The Need for an in vitro Monitoring Assay*

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ABSTRACT

Patients with sarcomas circulate a population of lymphocytes that exert cytotoxicity to cultured sarcoma cells; these lymphocytes behave as if presensitization to sarcoma-distinctive antigen(s) occurred already in vivo. This cytotoxic effect can be abrogated by sarcoma-specific blocking serum factors. Another “noncommitted” population of lymphocytes may gain de novo immune reactivity to allogeneic cells in vitro but this reaction requires several days of cocultivation. This cytotoxic effect cannot be abrogated by sarcoma-specific serum blocking factors.

A well standardized and well controlled in vitro assay for the monitoring of tumor-specific immune reactions of patients would be of great value in the selection of those therapeutic modalities that favor antitumor immune reactions.

Introduction

Tumor distinctive neoantigenicity of human sarcomas has been clearly demonstrated first by indirect immunofluorescence and other antibody-mediated reactions and subsequently by cytotoxic lymphocytes. Immunological cross-reactions between cells of different types of sarcomas implied a viral causative agent probably of the type C oncoma virus class. In contradistinction with the murine leukemia-sarcoma systems, in which the well defined type C oncoma viruses are the causative agents of both hematopoietic neoplastic diseases (leukemias, malignant lymphomas) and sarcomas (soft tissue and osteogenic sarcomas), no serological cross-reaction between malignant hematopoietic diseases including malignant lymphomas and the sarcomas (soft tissue and bone or cartilage sarcomas) could be demonstrated in the human system. In this respect, the human system displays the dichotomy found in the avian leukosis complex where type C viruses cause most neoplastic processes but the weight of evidence favors the etiologic role of a herpes type virus in Marek’s visceral and neurolymphomatosis. Indeed, benign and malignant lymphoproliferative diseases of man, such as infectious mononucleosis.

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and Burkitt’s lymphoma, are thought to be caused by the Epstein-Barr herpes virus.10 Recently, Hodgkin’s disease4,22 and Kaposi’s sarcoma5 have been found to be associated with a herpes-type virus resembling Epstein-Barr virus. Thus, it appeared important to determine whether or not patients with lymphoproliferative diseases or with Kaposi’s sarcoma exhibited any tumor-distinctively specific immune reactions also to cells of soft tissue or chondrosarcomas or osteosarcomas. If there are antigens coded for by etiologically related viruses in all these tumors, an immunological cross reaction may be expected. However, if a herpes type virus is the etiologically important agent in lymphoproliferative malignant diseases or in Kaposi’s sarcoma and a type C oncornavirus codes for tumor-specific neoantigens in soft tissue, chondrosarcoma or osteosarcomas, no such cross-reaction is expected to take place. Furthermore, there appeared to be a controversy concerning the relationship of Ewing’s sarcoma to reticulum cell, i.e. lymphoproliferative, tumors versus osteogenic sarcomas. Thus, immunological re-actions of patients with Ewing’s sarcoma to either reticulum cell sarcomas or to soft tissue (rhabdomyosarcoma) or osteogenic sarcomas could provide information concerning the taxonomy of Ewing’s sarcoma. Investigations of this type show extensive immunological cross-reactions between soft tissue, chondrogenic and osteogenic sarcomas of man.8,16,18,23,29,30 Furthermore, patients with Ewing’s sarcoma circulate lymphocytes that appear to be presensitized to tumor-distinctive antigens of a rhabdomyosarcoma cell line.21 In this paper, an example of an apparently tumor-specific immune reaction of a patient with Kaposi’s sarcoma to rhabdomyosarcoma will be shown. All these data, however, remain preliminary until more information becomes available. In particular, more controls are needed, such as reactions of healthy donors as compared with those of patients to a battery of sarcoma, carcinoma and other cell lines. These preliminary results seem to indicate that Ewing’s and Kaposi’s sarcoma are within the group of soft tissue, osteosarcomas and chondrosarcomas and are not within the group of hematopoietic or lymphoproliferative neoplastic diseases.

These types of investigations have become possible by means of newly devised in vitro assays, such as the colony inhibition assay,7,8 the mixed lymphocyte target interaction test26 and the lymphocyte cytotoxicity assay.16,23,24 In the latter assay, it was shown that patients with sarcomas circulated a population of lymphocytes that interacted promptly upon encounter in vitro with cultured sarcoma cells and actually killed these target tumor cells. Thus, these lymphocytes behaved as if they were already presensitized to sarcoma-specific antigen in vivo. Other noncommitted populations of lymphoid cells could gain de novo immune reactivity to carcinoma and other allogeneic target cells but actual killing of these target cells became demonstrable only after several days of cocultivation. Sarcoma-specific serum factors could block the cytotoxic effect of lymphocytes specifically cytotoxic to sarcoma cells but these serum factors could not block those lymphocytes that acquired cytotoxic potency de novo to allogeneic tumor cells, including sarcoma cells.18,19 Thus, the former effect was thought to represent a reaction to sarcoma-distinctive tumor antigens, whereas the latter effect appeared to be directed to transplantation antigens.

Beyond the immediate scope of demonstrating tumor-distinctively specific immune reactions to sarcomas lies the distant aim of utilizing these immune reactions for therapeutic benefits: Would active immunization of patients with X-ray inactivated cultured sarcoma cells increase the compartment of tumor-specifically cytotoxic lymphocytes? Would serum factors antagonistic to or synergistic with cytotoxic
lymphocytes appear? Would the \textit{in vitro} monitoring system be capable of selecting donors of sarcoma-specific transfer factor\(^1\) and how would the administration of transfer factor alter the immunological relationship of the human host with its neoplasm? How would surgical excision, chemotherapy and radiotherapy alter the human host's immune reactions to its tumor? Most of these questions remain unanswered for the present. In this communication, preliminary studies concerning some of the outlined problems will be reported.

**Materials and Methods**

**The Cytotoxicity Assay**

Single cell suspensions were prepared from established cultures of tumor cells by trypsinization using a 0.25 percent solution of commercial trypsin at room temperature until the cells floated from the glass (approximately 5 to 10 minutes). The tumor cells were washed and resuspended by centrifugation using a cooled (+5°C) International Centrifuge Model PR6 at 90 g in 10 minutes; the resuspended tumor cells were then counted in hemocytometers. The leukocytes or purified lymphocytes derived from defibrinated venous blood. The red blood cells were sedimented at room temperature for 20 minutes after adding equal volumes of 6 percent dextran 100,000 to 200,000 MW in phosphate buffered saline. The white blood cells were obtained by centrifugation (as described for tumor cells) from the supernatant; the white blood cells were resuspended in tissue culture medium. For the preparation of pure suspensions of lymphocytes, the leukocytes were passed through a cotton fiber column packed in a syringe\(^6\) and the lymphocytes were collected. The trypsinized and washed tumor target cells were suspended in fresh tissue culture medium and were dispensed into the wells of the 8-well LabTek chamber/slide cultures. Leukocytes or purified lymphocytes of patients were added in a proportion to favor the white blood cells at least 100:1 (see legends of figures for experimental data). For dispensing tumor target cells and leukocytes or lymphocytes into the wells, Eppendorf micropipettes were used; precisely 0.2 ml volumes of each target cells and leukocytes or lymphocytes were delivered into each well. The actual cell counts will be given in the legends of each figure. In some wells, tumor cells pre-treated with decomplemented (heating at 56°C for 30 minutes) patients' sera were dispensed. Preparations of leukocytes or purified lymphocytes were then added to the wells. The cultures were incubated in a humidified 5 percent CO\(_2\) atmosphere for four to nine days; fresh medium was added on the third or fourth day. The medium was Ham's F10 with 150 mg per 1,000 cc medium of fresh glutamine added. It was supplemented with 20 percent heat-inactivated (56°C for one hour) fetal calf serum and it contained penicillin G 100 units per ml, streptomycin 125 µg per ml and neomycin 50 µg per ml. The medium was filtered through a 0.45 and 0.22 micron pore size Millipore membrane under pressure and was tested for bacteriological sterility before use. Daily, slides were stained according to Wright and the target tumor cells adherent to glass were counted in 10 fields using a \(\times 10\) ocular and \(\times 10\) objective in each duplicate well. The counts were averaged to one field and this value was plotted as a growth curve. Growth curves of tumor target cells alone and exposed to leukocytes or lymphocytes or sera and leukocytes or lymphocytes were then compared. Statistical analyses\(^25\) indicated that 2-fold differences between two points on two curves were significant and 3-fold differences were highly significant; occasionally a 20 percent difference was significant. Cytotoxicity could also be observed under high magnification. As shown earlier,\(^{16,18,19,20}\) lymphocytes were seen to surround and vacuolize target tumor cells which then floated from the glass and dis-
integrated. Thus, the number of tumor target cells adherent to the glass after exposure to lymphocytes expressed the number of target tumor cells missing in comparisons with growth curves of control target tumor cells.

Established Target Tumor Cell Lines

Protocol #2043. This culture was established from a woman with squamous cell carcinoma of the uterine cervix. The cell line has been maintained in the authors' laboratory in Ham's F10 medium enriched with 10 percent fetal calf serum through 76 passages.

Protocol #2089. This culture was established from the rhabdomyosarcoma of a child and has been maintained in our laboratory in Ham's F10 medium with 10 percent fetal calf serum through 100 passages.

Protocol #2117. This culture was established from a patient with neurofibrosarcoma and has been maintained in our laboratory in Ham's F10 medium containing 10 percent fetal calf serum through 90 passages.

Protocol #2291. This culture was established in the authors' laboratory from a patient (Mr. C.H. #85890) with disseminated undifferentiated sarcoma, probably rhabdomyosarcoma, and it has been maintained through 96 serial passages in Ham's F10 medium with 20 percent fetal calf serum.

Protocol #2322. This culture was established in the authors' laboratory from a patient (Mrs. B.L. #86191) with slowly metastasizing chondrosarcoma. It has been carried through 80 serial passages in Ham's F10 medium with 10 to 20 percent fetal calf serum.

Case Histories

Mr. O.A. #87659

This 30 year old man developed embryonal rhabdomyosarcoma of the left arm in 1971. He received 6,000 r radiotherapy and chemotherapy with cyclophosphamide, vincristine and actinomycin D. Finally, left shoulder disarticulation was done in January 1972. At the end of 1972, the patient was free of clinically recognizable metastases.

Miss P.D. #86260

This 17 year old girl developed an undifferentiated, widely disseminated neoplasm thought to be embryonal sarcoma. The first clinical remission, lasting six months, was achieved with adriamycin and imidazole carboxamide. The second clinical remission, lasting three months, was achieved with cyclophosphamide, vincristine and actinomycin D. This patient circulated serum factors blocking the cytotoxicity of her lymphocytes to sarcoma cells. Although blocking serum factors became undetectable after the administration of cytosine arabinoside, rapid tumor growth continued until the death of the patient. The patient received active immunization with cultured allogeneic rhabdomyosarcoma cells in the last two months before her death (after regrowth of tumors despite chemotherapy occurred). The tumor cells were inactivated by 12,000 r X-ray irradiation before intracutaneous inoculation. The inocula contained 1 to 5 million irradiated cells.

Mr. M.M. #89868

This 48 year old man developed a pleomorphic sarcoma consistent with pleomorphic rhabdomyosarcoma of the left buttock (originally diagnosed histologically as alveolar rhabdomyosarcoma). After excision, 5,040 r Co60 radiotherapy was given. During treatment, a metastasis in the right pectoral muscle appeared. The patient now receives chemotherapy with cyclophosphamide, vincristine and actinomycin D and is clinically free of tumors.

Mr. R.M. #89730

This 29 year old man developed a large retroperitoneal neurofibrosarcoma which was inoperable. He received chemotherapy with cyclophosphamide, vincristine and actinomycin D and with adriamycin and imidazole carboxamide but failed to respond. He died after an approximately 12 month course.

Mr. C.P. #87561

This 50 year old man developed a fibrosarcoma of the left arm. After amputation, he developed bilateral pulmonary metastases. He received chemotherapy with adriamycin and imidazole carboxamide with mixed response. Radiotherapy with Co60 irradiation to one group, and neutron beam irradiation to another group of metastases was given. Finally, large doses of methotrexate were given with leukovorin rescue. The patient failed to respond and died after a two year course.

* Two tests involving sera and lymphocytes of this patient (figures 9 and 10) were reported but the case history of this patient was inadvertently omitted from that publication.
Mr. R.W. #87609

This 64 year old man developed Kaposi's sarcoma which responded well to treatment according to the MOPP (mustargen, vincristine, procarbazine, prednisone) protocol. After recurrence in three months, actinomycin D was given with partial success. Finally, vinblastine and bleomycin were given; vinblastine was omitted owing to toxicity. Complete regression of all lesions occurred on continuous bleomycin treatment. While in remission, the patient developed myocardial infarction at home and was not seen at our clinics in the past two months.

Results

In figure 1 is shown a cytotoxicity assay performed with the leukocytes of a patient with rhabdomyosarcoma (experiment #3031). The leukocytes (the lymphocytes were the cells visibly active in this reaction) inhibited promptly upon encounter and, in a sustained fashion, the growth of allogeneic rhabdomyosarcoma cells, while the growth of the allogeneic squamous carcinoma cells was not inhibited until after the fourth day of cocultivation. Inhibition of growth of squamous carcinoma cells became evident only on the sixth day of cocultivation; pretreatment with heated (decomplemented) serum had no effect on this reaction. The presence of heat inactivated serum in the reaction mixture prevented to a small extent the killing of rhabdomyosarcoma cells by lymphocytes ("blocking" effect).

In figure 2 is depicted the reaction of the leukocyte preparations of a patient with Kaposi's sarcoma, a patient with rhabdomyosarcoma and a normal donor to two established rhabdomyosarcoma cell lines (experiment #2504). The leukocytes of both patients inhibited the growth of both cell lines promptly; the inhibition remained highly significant on days 2, 3, 5 and 6. The leukocytes of the normal donor exerted no significant inhibition to rhabdomyosarcoma cell line #2089 until after the second day, and to rhabdomyosarcoma cell line #2291 until after the third day of cocultivation. By the fifth and sixth days, the leukocytes of the normal donor exhibited strong inhibitory power to both target tumor cell lines.

In figure 3 are shown tests with the buffy coat leukocyte preparations of a patient with Kaposi's sarcoma against two sarcoma and one squamous carcinoma cell lines. The purified lymphocytes of a normal donor were also tested against these cell lines (experiment #3147). The leukocytes of the patient with Kaposi's sarcoma

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**Figure 1.** In the wells of the LabTek chamber/slides, 4 x 10^5 cells per well of the following target tumor cells were placed: #2089 rhabdomyosarcoma and #2043 squamous carcinoma, respectively. In some wells the tumor target cells were pretreated with the patient's (Mr. M.M.) heat-inactivated serum. From this patient with rhabdomyosarcoma, 1.48 x 10^6 leukocytes were added to each well. The growth of the rhabdomyosarcoma cells was inhibited throughout the six days, whereas the squamous carcinoma cells suffered damage only by the sixth day. Se = heated serum; Ly = leukocytes (including lymphocytes).
FIGURE 2. In the wells of LabTek chamber/slides, $4 \times 10^5$ target tumor cells per well of the following cultures were added: #2089 rhabdomyosarcoma and #2291 rhabdomyosarcoma. From a patient with Kaposi's sarcoma (Mr. R.W.), from a patient with rhabdomyosarcoma (Mr. O.A.) and from a normal donor (Mr. J.C.), $4 \times 10^6$ leukocytes were placed into each appropriate chamber. The leukocytes of the two sarcoma patients inhibited the growth of both sarcoma cultures from the first to the sixth day; the leukocytes of the normal donor acquired cytotoxic potency only after the fourth day of cocultivation.

strongly inhibited the growth of both sarcoma cell lines but not that of the squamous carcinoma cell line. The slight protective, i.e. blocking, effect of the patient's decomplemented serum is not significant. The lymphocytes of the normal individual failed to exert any inhibitory effect to these cell lines.

In figure 4 are shown the reaction curves between leukocytes of two patients with fibrosarcoma (or malignant fibrous histiocytoma) and two sarcoma and one squamous carcinoma cell lines (experiment #3143). Both patients yielded lymphocytes cytotoxic to the sarcoma cell lines but not to the squamous carcinoma cell line; both patients circulated serum factors that blocked the cytotoxic effect of their lymphocytes on the sarcoma cell lines.

In figure 5 is shown the case of a 17 year
old girl with undifferentiated sarcoma. The cytotoxic effect of her lymphocytes to two sarcoma cell lines was clearly evident by the second day of cocultivation; however, cytotoxic reaction to squamous carcinoma cells manifested itself only after the third day of cocultivation. Both the chondrosarcoma and rhabdomyosarcoma cell lines appeared to outgrow the cytotoxic effect of the lymphocytes as evidenced by the upward tendency of the growth curves by the fourth day. The decomplemented serum exerted a slight but not significant blocking effect (experiment #2635).
In the wells of LabTek chamber/slides, $4 \times 10^3$ cells per well of the following target tumor cultures were added: #2089 rhabdomyosarcoma, #2291 rhabdomyosarcoma and #2043 squamous carcinoma. From a patient with undifferentiated sarcoma (Miss P.D.), $2 \times 10^5$ leukocytes per well were added. Cytotoxic effect of leukocytes to the sarcoma cells remained highly significant throughout the five-day experiment; squamous carcinoma cells were not affected. Se = heated serum; Ly = leukocytes (including lymphocytes).

It is shown in figure 6 that after repeated intracutaneous immunizations with X-ray (12,000 r) inactivated #2089 rhabdomyosarcoma cells, the cytotoxic effect of the lymphocytes to the two sarcoma cell lines was highly significant by the second day of cocultivation and remained sustained for the five days of the experiment. Blocking factors were not detectable at this time in the serum. No cytotoxic effect to squamous carcinoma cells was evident for three days and only a slight cytotoxic effect developed to this cell line by the fifth day (experiment #2901).

Discussion

Previous studies demonstrated that sarcoma-distinctive cytotoxicity of lymphocytes from patients with sarcomas occurred even in allogeneic combinations and that blocking serum factors also displayed a sarcoma-distinctive specificity. Thus, it appears to be possible to monitor in vitro the tumor-specific immune reactions of a patient to his tumor even if the autologous system requiring the patient's tumor established in culture is not available, inasmuch as cultured tumor cells, particularly those deriving from sarcomas, express tumor-distinctive antigens which cross-react within related histological classes of these tumors.

The availability of a standardized in vitro monitoring system for the measurement of specific antitumor immune reactions would be of extraordinary value. An assay of this type not only would contribute to the understanding of host-tumor relationship but also would permit prognostication. It would enable the clinical oncologist to pursue therapeutic courses that are beneficial to the host's antitumor immune reactions or to abandon those that promote tumor growth by suppressing or unfavorably altering the host's immune reactions. For example, if an expansion of the lymphocyte compartment expressing antitumor cytotoxicity occurs without the development of serum factors blocking this effect during a given course of treatment, this modality of treatment should be pursued further in correlation with the clinical status of the patient. The same judgment would apply if serum factors (complement-dependent cytolytic antibodies; "unblocking" serum factors) developed during the course of a treatment modality. On the contrary, if another form of treatment results in sustained reduction of lymphocytes that
are cytotoxic to tumor cells and/or in the development of serum factors that abrogate the antitumor cytotoxicity of lymphocytes, this treatment modality should be abandoned. Thus, instead of assessing the patient's immunological status by a battery of nonspecific antigens, a tumor-distinctive immune reaction would be utilized.

In addition, the in vitro assay may reveal the morphological features of cytotoxic effect of lymphocytes on tumor cells, enlighten the role of molecular mediators released during the process of target cell damage, and/or establish how tumor cells can gain resistance to cytotoxic lymphocytes (by releasing excessive amounts of soluble tumor antigens; by not expressing any tumor-distinctive neoantigens; by developing sialomucin coats, etc.).

For the understanding of the very complex immunological relationship between host (treated or untreated) and tumor, an in vitro assay that is well standardized (tumor target cells established in cultures that constantly express tumor antigens; purified, viable lymphocytes, etc.) and well controlled (such as a battery of tumor target cell lines to be used; reaction patterns of patients with similar and dissimilar tumors and reaction patterns of normal individuals to cultured autologous and allogeneic cells to be observed; and the exclusion of potential activators of non-specific immune reactions, such as calf proteins and antibiotics, from the reaction mixture, etc.) is needed. The assays presently in use approach but do not as yet fulfill this goal.

References

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