RESEARCH IN RADIOBIOLOGY

Final Report of Work in Progress in
Immunobiology of Experimental Host-tumor
Relationships

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FINAL PROGRESS REPORT

I. OVERALL PROJECT SUMMARY

It is well established that among the numerous types of cancers now plaguing mankind, some are more easily treated than others. Tumors can have a viral, chemical, genetic, or environmental etiology, while for other tumor types no direct cause has yet been established. The radionuclides used by the energy industry, primarily the heavy-metal α -emitters can pose a serious threat to society since the tumors induced by these agents appear to be highly virulent. Because of the potential for human exposure to α -emitting particles, one must agree that the development of appropriate experimental models which are able to investigate the host-tumor relationships of radiation-induced malignancies, at both the cellular and physiological levels, are important. In our laboratory, we have accrued a considerable amount of expertise in describing the immunobiology of the host-tumor relationship employing a number of murine model systems. It is well established that the immunologic responses elicited by normal syngeneic hosts to most rodent tumors are initiated and/or effectuated by T lymphocytes. Furthermore, the recent elucidation of the biochemical structure of the antigen-specific receptor on T cells has given us new insight into how tumors possessing unique tumor antigens or displayed peptides might evade the immune system. The antigen-specific receptors of T cells evolved with an inability to recognize native antigen. Instead, these lymphocytes recognize a cell surface bound macromolecular complex consisting of the foreign antigen (or small processed antigen peptide) noncovalently attached to a self-MHC molecule. It is presently believed that the peptide antigens of tumor, like peptide antigens produced by virally infected cells, are presented to the immune system primarily in the context of a MHC Class I molecule. Therefore, a tumor may circumvent immune recognition by fluxes in the expression of either "foreign" tumor antigens or through the modulation of normal MHC molecule expression.

Our work on the immunobiology of tumors induced in normal mice by non-ionizing radiation and chemical carcinogens has previously demonstrated a correlation between MHC molecule expression (Class I only) and the immunogenicity of tumors in a transplanted syngeneic host. That is, immunogenic or regressive tumors were found to demonstrate higher constitutive or inducible levels of MHC expression, while most virulent, aggressive tumors exhibited a low level of MHC Class I expression. We attributed much of the control of MHC molecule expression by antigen-bearing tumors and normal cells to the immunological status of the host since the host must provide the appropriate stimulus to enhance MHC antigen expression by the invading tumor. Our published results with UVR-induced tumors suggested that a significant role is played by the T-cell lymphokine, γ -interferon (γ IFN), in the modulation of MHC molecule expression *in vivo*. We originally hypothesized in this proposal, that virulent tumors induced by bone-seeking radionuclides may be refractory to γ IFN stimulation of MHC molecule expression. Alternatively, it is also possible that certain tumors might be fully responsive to the Class I modulatory influences by γ IFN, but exhibit a reduced capacity to stimulate the synthesis of this important lymphokine by appropriate subpopulations of host T cells. Within our original proposal we presented experiments designed to : 1) describe the virulence, latency period, and transplantation characteristics of ^{238}Pu , ^{241}Am , and ^{228}Th tumors arising as osteogenic sarcomas and hepatic carcinomas, 2) determine the relationship between inducible expression of MHC Class I molecules by γ IFN and *in vivo* immunogenicity of these radioisotope-induced tumors, and 3) elucidate any molecular mechanisms responsible for a lack of responsiveness to γ IFN or a failure by the host to induce host γ IFN production.

II. RECENT RESEARCH PROGRESS

1. The major problem encountered following the induction of our first set of ^{238}Pu -induced tumors in C3H/HeN strain mice (0.075 μCi) represented a difficulty in ability to

successfully propagate the tumors *in vitro*. During this past year, we have evaluated a large number of tissue culture protocols, medium preparations, and dissociation procedures in an attempt to resolve this difficulty. While the problem is far from totally resolved, limited success was achieved with a culture medium consisting of RPMI 1640, +7.5% prescreened FCS and 7.5% donor horse serum. Mechanical dissociation, rather than enzymatic dissociation, of tumor fragments, has also proven to enhance our chances for successful tumor cell propagation *in vitro*. The incorporation of L-cell conditioned medium, the inclusion of a variety of steroid hormones, lymphokines or cytokines, into the growth medium has met with minimal success. Although our attempts to culture the ^{238}Pu -induced murine osteosarcomas that were generated from the first group of injected animals was limited to only a few of the isolates, we were able to establish the following consistent patterns:

- a. All of the tumors induced by ^{238}Pu are capable of progressive growth in secondary transplant recipients, and maintain their histologic type following excision from the secondary transplant recipients.
- b. Those tumors capable of successful *in vitro* propagation, are all susceptible to the influences of γIFN . Significant elevations in cell surface expression of both K^{k} and D^{k} molecules have been observed following a 24-hour exposure to only 25 units/ml of recombinant γIFN *in vitro*. Further, no murine osteosarcoma thus far tested could be induced by γIFN to express MHC Class II molecules. It therefore appeared that ^{238}Pu -induced osteosarcomas, similar to most normal cell types, respond to γIFN by enhancing their cell surface expression of MHC Class I molecules. This finding indicates that our original hypothesis, that virulent bone tumors induced by bone-seeking radionuclides may be refractory to the inductive influences of γIFN , is probably incorrect.
- c. It was our hope to provide an accurate quantitation to the number of tumor cells injected into normal recipients for the studies designed to question tumor cell

immunogenicity. This has proven to not provide a reliable means for tumor cell vaccination or tumor cell challenge to "tumor-immunized" hosts due to the difficulties in *in vitro* propagation. Therefore, we decided to employ an alternative approach to question this aspect of the proposal. Appropriate experiments were set up where tumor fragments of defined size (1 mm^3) were isolated from tumor-bearing donors and surgically implanted subcutaneously into normal recipients. After 2-3 weeks, when the tumor size approximated 1 cm^3 , the tumor was carefully and completely excised, followed by a 2-week rest. These "tumor-immunized" animals were then challenged by implantation of a second tumor fragment (obtained from a donor of the same tumor, or another tumor induced by the same radionuclide) and the implanted tumor evaluated (quantitatively) for growth or rejection. Our studies in this area established that ^{238}Pu -induced osteogenic tumors were weakly immunogenic as all secondary challenges were found to grow progressively with no significant signs of rejection.

2. Three new groups of normal C3H/HeN strain mice (50 animals each) were injected with either ^{238}Pu ($0.075 \text{ } \mu\text{Ci}$), ^{241}Am ($0.075 \text{ } \mu\text{Ci}$) or ^{228}Th ($0.7 \text{ } \mu\text{Ci}/\text{mouse}$). This protocol yielded new ^{238}Pu -induced tumors (BAP-1 – BAP-11) having latency periods from 9-15 months. We were also able to induce 7 new ^{228}Th -induced tumors, BAT-1 – BAT-7 (latency period 7-11 months), and 1 ^{241}Am -induced malignancy, BAA-1 (latency period 14 months). All of the tumors were capable of growing progressively in normal and immunosuppressed syngeneic recipients, most were osteogenic sarcomas and all tested tumors were only weakly immunogenic. BAP-8 was a hemangiosarcoma, BAT-3 a trichoepithelioma, and BAP-4 and BAT-2 were lymphomas. FACS analysis of BAT-2, which is capable of metastasizes to all peripheral lymph nodes, suggested that it is of the immature B-cell lineage. BAP-4 was not analyzed.

3. Our work to define the role(s) played by non-tumor host cells which are normally present in solid tumors has led to a number of conclusions. Macrophages, especially

macrophages that reside in an inflammatory environment, are well appreciated to produce prostaglandins. PGE₂ has recently been reported to depress the ability of T cells to produce IL-2, probably through its ability to stimulate elevations in neighboring T lymphocytes' cAMP levels. Elevations in cAMP are now known to depress lymphocyte IL-2 production with minimal inhibition to their capacity to secrete IL-4 following activation. Such changes would be predicted to depress primary host responsiveness to neoantigen stimulation. The responsible mechanisms have not yet been reported. Our data, which extends the presently published work, can be summarized as follows:

- a. PGE₂, ACTH, or epinephrine (all of which cause elevations in lymphocyte cAMP levels), cAMP or other cAMP elevating agents (which directly increase intracellular levels of this second messenger), plus inhibitors of PKC and PLC, all share the same properties on activated T cells. Each causes a marked reduction in γ IFN and IL-2 production, exert no appreciable effect on the secretion of IL-4 or IL-5, and their effects actually promote lymphocyte production of IL-6. Enhanced plasma levels of IL-6 have recently been observed in animals bearing various tumor types, and the IL-6 found was established to be of lymphoid cell (probably macrophage) origin. Collectively, these findings suggest that progressively growing tumors (including the osteogenic sarcomas we are presently evaluating) may be capable of exerting pronounced influences on the capacity of host lymphoid cells to produce lymphokines, the molecules which serve to direct the adaptive immune response towards, or away from, the types of responses that can promote protective immunity.
- b. Very preliminary work, evaluating the ²²⁸Th-induced osteogenic sarcoma BAT-1, has established that T cells from peripheral lymph nodes which drain the tissue site of tumor inoculation, totally lack the capacity to secrete γ IFN following activation, and are markedly reduced in their capacity to produce the lymphokine IL-2 following activation. Interestingly, T cells from the spleens of these same animals

behaved normally. This highly reproducible result indicates that BAT-1, as a progressing tumor, appears to be producing or influencing the production of substances that exert immunomodulatory influences on the host's T cells. Such changes in T-cell function might depress the antigen-specific T cells' ability to generate essential lymphokines necessary for protective forms of anti-tumor immunity. Additional studies, where the BAT-1 tumor was allowed to progress *in vivo* for a longer period of time, and consequently reach a larger size, determined that the immunomodulatory influence became systemic, and severely affected splenic T-cell function following activation. Further work in this area is necessary before any firm conclusions can be made.

4. A series of experiments have been initiated to question whether definable changes in the immune status of radionuclide-injected animals takes place prior to the development of overt neoplasms. This was predicted to represent a fruitful new area of investigation, predicated upon the finding that all of the osteosarcomas thus far tested respond to γ IFN (by enhancing their MHC Class I molecule expression), their weak immunogenicity, coupled to the very reproducible observation that T cells isolated from normal mice injected with ^{238}Pu or ^{228}Th 7-10 months previously were suppressed in their ability to produce γ IFN and IL-2, while exhibiting an enhanced ability to produce IL-4. Three groups of mice (60 mice/group) were set up for these studies. One group received 0.075 μCi ^{238}Pu , and a second 0.075 μCi of ^{228}Th . At biweekly intervals, animals from both of the experimental groups, plus an equivalent number of control age-matched mice, were sacrificed and their lymphoid tissue removed and the T cells analyzed for ability to produce IL-2, IL-4, IL-6 and γ IFN following T-cell stimulation. The findings from these experiments are summarized below:

Lymphokine analysis at selective time points are shown in Figure 1a-d. Spleen cells from animals 2, 4, and 6 weeks post-exposure with either radionuclide did not show only consistent changes in their ability to produce the T-cell cytokines IL-2 and IL-4

(Figure 1a-b) when activated *in vitro*. Likewise, spleen cells removed from animals 6 weeks post-exposure, did not show any significant changes in their ability to produce γ IFN. When we examined spleen cell activation with anti-CD3 9 weeks post-exposure, however, we found that IL-6 production by spleen cells from animals previously exposed to either ^{238}Pu or ^{228}Th was greatly elevated compared to normal controls; even though their production of IL-2, IL-4, and γ IFN was not significantly altered (Figure 1a-d). The observation that activated spleen cells from ^{238}Pu and ^{228}Th produced IL-6 at a greater than 2-3 times the concentration produced by spleen cells from control animals persisted until week 21, at which time the amount of IL-6 produced by individual animals began to fluctuate. At 21 weeks post-exposure, the IL-6 production by the exposed groups was not significantly different from controls (data not shown). However, at 24 weeks post-exposure, the IL-6 production by spleen cells from ^{228}Th or ^{238}Pu exposed animals was again greatly increased. By 34 weeks post-exposure, however, the IL-6 levels were not significantly different between radionuclide exposed animals and control animals. No spontaneous IL-6 was found in the serum from these animals at any time, post exposure nor were unactivated spleen cells from these animals producing IL-6 *in vitro* (data not shown). Whether T cells, macrophages, or both populations are responsible for the increased IL-6 production has yet to be differentiated because spleen cells from actinide exposed animals produced increased amounts of IL-6 when activated with either anti-CD3 (which primarily activates T cells) or with LPS, which activates primarily macrophages.

Interestingly, the ability of activated spleen cells from actinide exposed animals to produce IL-2, IL-4, and γ IFN when activate *in vitro* began to decrease at 21 weeks following actinide-exposure. This correlated with the time period where constant fluctuations in IL-6 production were observed. By 24 weeks post-exposure, the IL-2, IL-4, and γ IFN production by activated spleen cells were greatly decreased, a trend that continued until the last data time point taken at 34 weeks. We also examined the ability of anti-CD3 activated spleen cells from animals exposed to ^{228}Th or ^{238}Pu 34 weeks earlier to

produce the T-cell cytokines IL-3 (Figure 2) and GM-CSF (Figure 3) *in vitro*. both IL-3 and GM-CSF production was decreased in radionuclide exposed animals at this single time point. Osteogenic sarcomas arose in the surviving ^{238}Pu and ^{228}Th exposed animals beginning 39 weeks post-exposure. Therefore, the results of this study confirms our initial hypothesis that radionuclide exposure may impair T-cell production of cytokines important to the successful generation of cellular immune responses, prior to the development of overt malignancies.

We believe that this study represents the first time that any type of analysis has been made on the more subtle changes in immune status caused following animal exposure to particular species of bone-seeking radionuclides. We feel that these preliminary findings are provocative since they suggest that detrimental influences on the immune capabilities of animals exposed to radionuclides may significantly precede the development of overt neoplasms. Furthermore, these findings also suggest that detrimental changes in the capacity of the mammalian immune system to function normally following radionuclide exposure, could alter host susceptibility to many types of infectious agents, as well as, malignancies of etiologies not directly caused by the mutogenic activities of the radionuclide. Altered immune function may also contribute to an enhanced susceptibility to autoimmune diseases, especially those which might be precipitated by abnormalities in IL-6.

Over the past year, the laboratory has gained a considerable enhancement in expertise in the accurate quantitation of cytokines. We now have, on line, ELISA assays for IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, GM-CSF, γIFN and TNF and are developing a very reliable quantitative PCR procedure to parallel the cytokine analysis. It is our plan, in spite of the fact that this DOE project officially terminated this past year, to conduct an additional set of experiments. We plan to repeat the experiments which have evaluated immunocompetence following selected actinide exposure in the murine system. Additionally, we plan to assess whether actinide-exposed animals maintain normal

capacities to be effectively immunized to clinically relevant vaccines such as diphtheria and tetanus toxoid.

We realize that our DOE funding has expired for this project. However, the very provocative preliminary results obtained during the final Contract year, coupled to the fact that the original working hypothesis proved to be both technically difficult and probably incorrect, make it important for us to formally repeat our final set of experiments using the new assay systems now available. Once completed, the DOE will be made aware of our experimental results, and be provided copies of any publications that might result from such studies.

IL-2 production by activated spleen cells from mice exposed to Thorium or Plutonium

gIFN production by activated spleen cells from mice exposed to Thorium or Plutonium

IL-4 production by activated spleen cells from mice exposed to Thorium or Plutonium

IL-6 production by activated spleen cells from mice exposed to Thorium or Plutonium

**IL-3 produced by activated spleen cells
from mice 34 weeks after exposure to Thorium or Plutonium**

**GM-CSF produced by activated spleen cells from mice
34 weeks after exposure to Thorium or Plutonium**

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