Induced Immunologic Response to Tumors

John F. Burke

Department of Surgery, Harvard Medical School, Boston, Massachusetts 02115, and the Surgical Service of the Shriner's Burns Institute and the Massachusetts General Hospital, Boston, Massachusetts 02114

Summary

A tumoricidal system has been described utilizing a two-step process which, first, specifically alters the antigenic nature of a tumor cell and, second, adds a heterologous cytotoxic antibody without affecting normal cells. The antigenic structure of the tumor cell is altered by the nitrogen mustard, L-phenylalanine mustard, and a specific antibody is made to this mustard by injecting it into the rabbit as a hapten conjugated to human gamma-globulin. Selectivity in the system concentrating the hapten and later the specific antibody on the tumor cell is obtained by utilizing the loss of selective permeability in the tumor vessels. L-phenylalanine mustard is introduced intravenously as a primary injection followed in a half-hour by an intravenous injection of the antibody. The system has been demonstrated to be tumoricidal but, as yet, has low efficiency and high toxicity.

Experience indicates that the antigenic similarities between normal cells and malignant cells are such that the usual creation of effective antibody against malignant cells arising in a host does not occur. This is not to say that there is exact antigenic similarity between normal and cancer cells, for there is now ample evidence demonstrating wide differences in the antigenic structure of the two groups. There is evidence for antigenic gain (8), antigenic loss (17, 19), and antigenic fortification (25), and there is no doubt that specific tumor antigen exists in certain experimental tumors (18) and surely also exists in spontaneously occurring human tumors (7, 9).

However, there is no explanation for the lack of effective immunologic response to these tumor antigens by the host. This could be because the specific tumor antigen is not released by the tumor cell, because the host was subjected to immunologic paralysis or tolerance, or still another factor. A number of interesting attempts have been made to amplify the antigenic differences between normal and malignant cells through the use of antibody produced against cancer cells in animals made tolerant to normal cells (12) and through the use of immunologically competent cells (26). It is, however, fair to say that, at this time, attempts to use the immunologic system in this treatment of malignant disease in man has not proved effective.

The following report describes a further attempt to utilize the specificity of immunologic reaction to destroy malignant cells in situ. It was felt that, if the antigenic structure of a malignant cell could be altered without altering the structure of the somatic cell, antibody could then be induced against the altered portion of the malignant cell and, therefore, would not react in the host with the unaltered somatic cell. Also, if the process of altering the antigenic structure of the malignant cell was in itself cytotoxic, a chemically medicated cell damage would be added to the immunologic mediated one, the sum of which might be considerably increased over immunologic damage alone. The above system would theoretically have the additional advantage of a treatment regime utilizing cytotoxic and immunologic substances which would not inhibit the host's own immunologic mechanism, so that the therapeutic regime would be acting in concert with whatever attempts the host may make to suppress immunologically the growth of malignant cells. In order to accomplish the above in practical terms, it is necessary to alter selectively the antigenic structure of malignant cells with a substance that is (a) capable of inducing antibodies in a second system and (b) cytotoxic. In addition, a method is needed to deliver this substance to the malignant cells without contaminating normal cells.

The vascular system provides a method for selectively delivering the substance to malignant cells in far greater concentration than that delivered to normal tissue. Information obtained from studies of vascular permeability in regions of early inflammation (4, 16) and of neoplastic disease (21) has demonstrated that the vascular-supporting structure in the region of inflammation or malignant change has lost its usual selective permeability. Therefore, malignant tumors or areas of inflammation may be separated from normal tissue on the basis of vascular permeability. The concept of identifying tumors by localizing radioactive substances in the extravascular space by way of this specific loss of selective permeability has been widely exploited. The use of radioactive metals, such as Arsenic 74 (15), Mercury 203 (13), and Copper 64 (1), are cases in point.

A more difficult problem is the selection of a substance which will specifically alter the malignant cell, be toxic to the cell, pass through tumor vessel walls in large quantities, fixed to the malignant cell, and in addition be antigenic. The use of a nitrogen mustard is an obvious candidate, and, although nitrogen mustards have not been found to be antigenic in themselves, it was found that they act successfully as hapten when conjugated with protein. The specific nitrogen mustard

1This investigation was supported by Grant A1-02392 from the National Institutes of Allergy and Infectious Diseases, and Grant CA-07368 from the National Cancer Institute and the American Cancer Society.
chosen was PAM\(^2\), known to be concentrated in the area of malignant tumors (21), rapidly fixed to the tissue close to the site of migration from the bloodstream (2), and cytotoxic (24).

The following description will include comments on the immunologic feasibility of the above system, the effect on tumor host systems, and the possible future usefulness of such a system.

**Immunologic Feasibility**

The demonstration of antibody formed against the hapten PAM is essential. This was done by conjugating PAM with HGG. This HGG conjugate (PAM-HGG) was then emulsified with complete Freund’s adjvant and injected into rabbits using the usual immunization technic. The resulting antiserum was analyzed quantitatively against HGG and against the hapten PAM using the micro-precipitation technic. Aliquots were chosen to give values in the 1 to 4 mg protein range, and specific precipitants were analyzed using the Biuret reagent. The amount of anti-hapten antibody was estimated using a PAM-BSA conjugate. Increasing amounts of the PAM-BSA conjugate were added to 1 ml of rabbit anti-PAM-HGG serum. Results of this quantitative micro-precipitation analysis for antibody against the hapten PAM in rabbits immunized against the conjugate PAM-HGG show that the optimal precipitation of antibody was seen in the region of slight antigen excess. The titration gave a typical curve of the antibody precipitated as increasing amounts of hapten conjugated with BSA were added to a maximum precipitation; it then fell with the addition of further antigen.

To determine the feasibility of this system, it is also necessary to demonstrate that the anti-hapten antibody localizes specifically and in high concentration in the area of the tumor cell and, in addition, does not localize on normal somatic cells. In order to demonstrate the concentration of anti-hapten antibody on the tumor cells, the fluorescent antibody technic was used. These studies were carried out by conjugating fluorescein isothiocyanate to the specific anti-PAM serum. The fluorescent antiserum was flooded on cryostat sections of tumor, as well as normal liver and muscle, all taken from animals sacrificed one half-hour following the intravenous injection of 3 mg of PAM per gram of body weight. Fluorescent studies showed that there was no detectable fluorescence in any of the normal tissues studied. On the other hand, studies of tumor tissues stained with fluorescent antiserum showed that there was considerable immunofluorescence localized in the area of the tumor cell wall. An occasional tumor cell, however, contained a considerable amount of fluorescent material throughout its cytoplasm. It was of interest to note that in the tumor tissue there was minimal staining of the vascular- and connective tissue-supporting structures. These studies may be interpreted as indicating that the concentration of PAM in tumor tissue is greatly in excess of that in muscle or liver and that PAM is localized for the most part in the region of the tumor cell wall. Furthermore, it may also be interpreted to indicate that the specific antibody to the hapten PAM is localized at the antigen.

**Effect on Tumor Host Systems**

Following the demonstration of ability to create antibodies to the hapten PAM, the ability to localize this hapten in the tumor cell, and the ability to produce a specific antigen-antibody reaction at the site of localization of the antigen, it is possible to test the *in vivo* effectiveness of the system in a tumor-bearing animal. In the attempt to determine the effect of specific antibody on the intact tumor host system, experiments were carried out using three experimental tumors in inbred mice. An ependymoma and mammary carcinoma in the C3H strain and leukemia in the AK4 strain were used. The theoretical antitumor activity of the above cytotoxic system rests on the damaging effects of specific heterologous antibody which is localized to the tumor cell because of the selective concentration of antigen on the cell wall. This selective concentration of antigen is accomplished through the loss of selective permeability in tumor vessels. Chart 1 demonstrates this process diagrammatically. A single dose of 3 mg/gm of body weight of PAM is given to the animals intravenously and allowed to localize in the tumor. One half-hour following the intravenous injection of the hapten PAM, specific anti-hapten antibody is injected intravenously. As demonstrated in the diagram, the loss of selective permeability in the tumor vessels allows both the hapten and the anti-hapten antibody to concentrate in the region of the tumor causing extensive cytotoxic reactions, whereas the concentration of hapten in the region of the normal tissue is not sufficient to cause perceptible damage.

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2The following abbreviations are used: PAM, L-phenylalanine mustard; HGG, human gamma-globulin; BSA, bovine serum albumin; and HN2, 2,2'-dichloro-N-methyl-diethylamine.
If the above is true, the severity of the antitumor effect should be directly proportional to vascularity of the tumor since both the concentration of the antigen in the region of the tumor cell and the delivery of the antibody are directly dependent on this vascularity. To test this hypothesis, a highly vascular tumor in the C3H strain, the ependymoma, and a poorly vascularized tumor, the transplanted mammary carcinoma in the C3H strain, were chosen as test tumors. Leukemia was also chosen because it provided an intravascular tumor where there was no possibility of concentration of antigen or antibody by way of vascular permeability. In the case of the leukemia, there should be no enhancement of cytotoxic effect between tumor cells and normal cells.

The therapeutic effect of antigen followed by specific antibody on the pattern of tumor growth (3) is demonstrated in Chart 2. The tumor growth in mice treated with PAM alone, specific antibody alone, and control mice given an injection of intravenous saline is identical. The group of mice treated with PAM followed by a specific antibody showed a marked decrease in rate of tumor growth as compared with the three other groups. Moreover, the decrease in rate of growth was most marked shortly after the injection of PAM followed by specific antiserum. However, following this initial decrease in growth rate, the tumor again began to grow at a rate more consistent with the rate of growth seen in the control animals.

There was no immediate mortality or morbidity in the saline control group or in the group treated with PAM or specific antibody alone. There was, however, considerable immediate morbidity and a 10% mortality in the group treated with specific antibody followed by specific antibody. A reaction consisting of respiratory distress and occasionally prostration and death occurred immediately following injection of the specific antibody. The reaction resembled anaphylaxis and, in the animals that survived, appeared to have no lasting effect. It is postulated that the antitumor effect is mediated by specific immunologic action. There is, however, no evidence eliminating a nonspecific Schwartzman or Arthus effect on the tumor, nor is evidence eliminating the possibility of nonspecific effect of the treatment regime available.

In order to gain information concerning the specificity of the antiserum against PAM and to attempt to detect nonspecific effects of antiserum following a nonrelated cytotoxic agent, animals were treated with PAM specific antiserum following a primary injection of HN2 rather than PAM. Chart 3 shows a comparison of the tumor growth in animals treated with HN2 followed by PAM specific antiserum and HN2 alone, demonstrating that the treatment regime following a primary injection of HN2 has no specific or nonspecific effect on tumor growth over the effect of HN2 alone.

Histology of the tumors obtained from the animals examined 6 and 8 days after treatment with PAM followed by specific antiserum shows no histologic difference among the group treated with saline, with PAM alone, or with specific antibody alone. In these control groups, there are large areas of tumor cells tending to arrange themselves in cords together with patchy areas of necrosis. Only a scant connective tissue-supporting structure was seen. The histologic picture seen in animals treated with PAM followed by specific antibody shows considerable differences from that seen in controls. Small nests of viable tumor cells are seen surrounded...
by large areas of fusiform cells taking pale stain. This histologic picture is more of a change in relative numbers of cells than in different cell types. The fusiform cells seen in great profusion in tumors treated with PAM followed by specific antibody were also seen in the stroma of untreated tumors.

Having established the cytotoxic nature of the above test system, it is important to gain information concerning the effectiveness of the system on other tumors (5) and in particular with tumors of various vascularity. For that reason, as noted above, the transplanted mammary carcinoma which is relatively avascular and leukemia which resides in the vascular system itself were chosen. The results of experiments comparing the effectiveness of the above antitumor system on vascular and avascular tumors are demonstrated in Chart 4. From this evidence it appears that the effect of PAM followed by specific antibody on solid vascularized tumors is related to the degree of vascularity found in the tumor itself. The highly vascularized ependymoma was inhibited to a far greater degree than was the mammary carcinoma. Furthermore, when leukemia-bearing mice of the AK4 strain were treated with PAM followed by specific antibody, there was no detectable effect of the treatment regime on the course of the leukemia.

The experiments reported were designed to provide information concerning the specificity and cytotoxicity of antibody directed against cells which had been specifically altered. The experiments indicate that there is a considerable degree of both cytotoxicity and specificity in the system, as discussed, although a nonspecific Arthus or Schwartzman effect cannot be ruled out. The exact role of delayed hypersensitivity in this system is not understood. It is possible that at least part of the tumoricidal effect could be derived from specific sensitization of the host by a tumor cell mass which had been damaged by nitrogen mustard followed by an antigen-antibody reaction. If this is so, one might expect further regression of the tumor with time. To date, however, there is no evidence of a delayed hypersensitivity reaction to the altered tumor cell although this possibility has not been systematically examined.

 Attempts to improve the cancer cell-destroying efficiency of the system described above could either come from: (a) increased efficiency of localization of antigenic substance on the tumor cell wall; (b) the production of a more effective heterologous, cytotoxic antibody; or (c) a mechanism which produces stimulation of the host's inherent immunologic system to react to the altered tumor cells. Considerable work is now in progress examining the possibilities of host stimulation and more effective heterologous antibody. With respect to efficiency of localization, it is worth noting that the experiments conducted have shown the concentration of the compound within tumors to be related to the solubility of the compound itself. These studies have involved only the transfer of substances from the vascular system to the interstitial space. It is also of interest to obtain information concerning the solubility characteristics of the substance in passage across the cell or nuclear membrane. It will also be important to gain information concerning the effectiveness on short-acting compounds and on use of "single armed" mustards.

In order to utilize the above system to maximum efficiency, it is clear that the frequent toxic reactions must be controlled. The 10% mortality in mice treated with nitrogen mustard followed by specific antibody prevents its use elsewhere until this toxicity is controlled. This might be accomplished by fractionating the antiserum so that pure gamma-globulin is obtained. Fractionation, however, has created logistic problems in the experimental laboratory thus far because the anti-haptene titer is low and any chemical manipulation tends to decrease specific activity.

REFERENCES

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