The Effect of Antibody to L-Phenylalanine Mustard Conjugate on Malignant Cells Selectively Marked through "Early Inflammatory-like" Vascular Permeability

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Summary

Antibody was produced against the haptene L-phenylalanine mustard by conjugating the mustard with human γ-globulin and using the conjugate plus Freund's adjuvant to immunize rabbits. In mice with transplanted ependymomas, the haptene L-phenylalanine mustard was concentrated in the region of the tumor cells by virtue of the "early inflammatory-like" permeability of tumor vessels to the mustard. Malignant ependymomas in mice treated by giving the mouse an injection of a minimal dose of L-phenylalanine mustard followed in 0.5 hr by specific antibody showed decreased growth over the control groups. Haptene-specific antibody was shown by fluorescent technics to localize in the region of the cell membrane of L-phenylalanine mustard-treated tumors.

Introduction

The concept of destroying a malignant cell in situ while leaving its normal neighbor intact and functioning has many parallels among well-known immunologic phenomena. It is, therefore, not surprising that for well over 50 years an immunologic approach has held a logical attraction for students of cancer. Immunologic studies have, for a large part, concentrated on the antigenic structure of the cancer cell, considering the question of the presence or absence of tumor-specific antigens. There is now ample evidence for antigenic loss (15, 17) as well as antigenic fortification (23) and gain (6), and, further, it is now clear that these features may be seen simultaneously. Although there is no doubt that specific antigens exist in certain experimental tumors, as documented by Old and Boyse (16), and probably also in spontaneously occurring human tumors (5, 7), there is no complete explanation for the lack of an effective immunologic response by the host. Whether this is because of a lack of antigen release, immunologic paralysis, tolerance, or still another factor, remains to be seen. In recent years, a persistent effort has been made to discover a method which would produce an effective host reaction against cancer cells. Nevertheless, it is reasonable to say that such attempts to use immunologic processes in the treatment of cancer in man have not proved effective (20). Of considerable interest at this time, however, are the attempts to treat malignant disease through the use of immunologically competent cells (24) and by production of the antibody to cancer cells in animals made tolerant to normal cells (10).

It is, at the same time, worthwhile to consider the possible significance of the interaction between malignant cells and their supporting structures, for malignant tumors are not made solely of cancer cells; there are, in addition, the usual stroma and vessels. But recent investigative effort aimed at the destruction of malignant disease in situ has placed the greatest emphasis on the behavior and composition of the malignant cell itself, while studies have accorded little attention to the vascular and connective tissue supporting these malignant cells. Nevertheless, information obtained from studies of vascular permeability in the region of early inflammation (3, 14) and of malignant neoplastic disease (19) has shown that the vascular supporting structure in the region of inflammation and of at least some malignant cells behaves in an unusual way, i.e., there is a loss of selective permeability. Theoretically, malignant tumors or areas of early inflammation may be separated from normal tissue on the basis of the behavior of their vascular supply. The current advances in tumor identification and localization using radioactive metals such as arsenic 74 (13), mercury 203 (11), and copper 64 (1) are cases in point.

In an attempt to utilize this "early inflammatory-like permeability" of tumor vessels and, in addition, to utilize the potentially powerful tool of selectivity inherent in immunologic reactions, the following experimental program was tested.

It was assumed that if malignant neoplastic tissue could be preferentially marked using the nonselective permeability in their supporting vessels to concentrate a labeling substance in the region of the tumor cells, and if this group of malignant cells were changed by the incorporation of the specific marking substance, they would become antigenically distinguishable from normal cells. Further, if the above could be achieved, antibody against the marking compound, when introduced into an animal with marked cells, would selectively react with the marked sites, possibly causing damage to the population of marked cells while leaving normal host cells intact.

The practical problems involved in attempting to test these designs are, first the localization of a marker onto the cells selected for tagging, and, second, production of an antibody against the marking substance. The compound used for a marker is, therefore, of considerable importance: it must pass through tumor
vessel walls in large quantities, fix to the malignant cells, and be antigenic. In addition to its tagging function, the marking compound could be of further use if it also had properties producing specific injury to the marked cell, thus enhancing the antitumor system by a separate cytotoxic mechanism. The malignant cells would thereby be not only marked, but damaged as well. It is important to emphasize that the primary concept being tested does not require the use of a cytotoxic agent to mark the malignant cells. Any damage caused by the marker is, in a manner of speaking, a dividend. The use of a cytotoxic chemical agent coupled with the use of a cytotoxic immunologic system can be looked upon as a 2-fold attack on the integrity of a malignant cell.

L-Phenylalanine mustard was chosen as the marking compound because it was found to concentrate in the area of malignant tumor (19), was rapidly fixed in the tissue (2) close to the site of migration from the blood stream, and, although not known to be antigenic itself, was found to act successfully as a hapten when conjugated to a protein. In addition, it in itself damaged the cell.

Methods and Materials

Preparation of the L-Phenylalanine Mustard Protein Conjugate

L-Phenylalanine mustard, 35 mg, was (Alkeran; obtained as a powder from Burroughs-Wellcome and Co.) dissolved in 2 ml of 0.1 N HCl over heat. This solution was allowed to cool to room temperature and 5 ml of saline were added. The pH was adjusted to pH 6.6 immediately before using with 0.15 M phosphate buffer to a final concentration of 1.75 mg of PAM per ml.

Human γ-globulin, 75 mg, was then dissolved in 21 ml of 0.15 M phosphate buffer, pH 6.6, and agitated slowly at 37°C. Four ml of the above PAM solution were added dropwise slowly to the buffered HGG solution and the mixture was agitated slowly overnight. The resulting L-phenylalanine mustard human γ-globulin conjugate (PAM-HGG) was then dialyzed at 4°C in order to remove unconjugated mustard. Following conjugation, the solution was stored in the frozen state until used as an antigen.

The character of the protein mustard complex was studied in dialysis experiments and by paper electrophoresis using 14C-tagged L-phenylalanine mustard. Four ml of a solution containing 7 mg of p-(di-(β-chloroethyl)-2-14C)-amino-L-phenylalanine (18) with a total activity of 1.7 μc were added dropwise to a solution of 75 mg of HGG in 25 ml of phosphate buffer (pH 8.0). The mustard solution was adjusted to a pH of 6.8 immediately prior to its addition. The mixture was stirred slowly for 20 hr at room temperature.

One ml was removed for 14C counting and 28 ml were exhaustively dialyzed against phosphate buffer. The buffer was replaced at the end of each 12 hr. A total of 6 liters of buffer was used over a 72-hr span. The total number of counts in the dialyzed contents of the bag gave an incorporation efficiency of approximately 5% of the nitrogen mustard into protein. This binding is consistent with the highly labile nature of the mustard group at pH 6.8 indicating a firm binding of the hapten to the protein. Experiments in which the PAM-HGG conjugate was subjected to paper electrophoresis showed that the conjugated material has the mobility of human γ-globulin.

Animals

Female New Zealand white rabbits weighing 2–3 kg, in individual cages and fed rabbit chow ad libitum, were used throughout for the production of antibody. Adult mice of the strain C3H, weighing 20–30 gm, and obtained from Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Me., were used throughout as tumor test animals.

Immunization

Adjuvant mixtures were prepared by emulsifying the above-described (PAM-HGG) conjugate with an equal volume of Freund's complete adjuvant (1.5 parts Arlacel, 8.5 parts mineral oil, 5 mg/ml mycobacterium). The initial injections were in toe pads of all 4 feet and in both thighs. In total, approximately 3 mg of conjugate were given throughout the 6 sites. An i.v. booster of 2 mg of conjugate was given 3 weeks after the initial immunizing injections and the animals were bled between the 5th and 7th day following the booster.

Analysis of Antibody

Quantitative analysis of antibody against HGG and the hapten PAM was carried out using the microprecipitation method of Heidelberger and MacPherson (8, 9). Aliquots were chosen to give values in the 1–4 mg protein range. Antibody against HGG was determined by using 1 ml of diluted rabbit anti (PAM-HGG) serum in all tubes. Increasing amounts of the antigen HGG were added to each tube. All tubes were incubated at 37°C for 1 hr and then kept at 4°C for 5 days. Specific precipitates were washed twice with cold saline and analyzed using the biuret reagent (12). The amount of antihapten antibody (anti-PAM) was estimated using the above procedure except that the antigen added was a bovine serum albumin-L-phenylalanine mustard (PAM-BSA) conjugate instead of the PAM-HGG conjugate used to immunize. Increasing amounts of PAM-BSA conjugate were added to 1 ml of rabbit anti (PAM-HGG) serum and the antigen antibody reaction processed as above.

Experimental Tumor

The malignant tumor used in these experiments was a mouse ependymoma with high vascularity originally produced in the brain of a C3H strain mouse by implantation of a pellet of the carcinogen 2-methylcholanthrene. The tumor was obtained from the Roswell Park Memorial Institute, Buffalo, New York.

The tumors were implanted in successive groups of C3H mice by injecting 0.1 ml of tumor suspension i.m. into the right hind leg under light general anesthesia. The tumor suspension was made by grinding 1 gm of tumor in 5 ml of sterile saline. The animals were allowed to recover from anesthesia and thereafter were housed for 10–14 days following tumor injection. In this time, a tumor developed in the thigh of the mouse which was
Effect of Antibody to l-Phenylalanine Mustard Conjugate

Fluorescent Antibody

Fluorescent antibody studies were carried out by conjugating fluorescein isothiocyanate to specific anti-l-phenylalanine mustard serum according to the method of Coons et al. (4). Tissue was prepared for fluorescent antibody studies from the frozen state using the cryostat and also by paraffin embedding (21). Fluorescent sections were studied using a Reichert ultraviolet microscope.

Experimental Results

Chart 1 demonstrates the results of quantitative microprecipitation analysis of the antibody against the hapten PAM in the serum of rabbits immune to the conjugate PAM-HGG. The optimal precipitation of antibody was seen in the region of slight antigen excess. Qualitative determinations were carried out on serum to be used in the test experiment to be sure of the presence of anti-hapten antibody. The amount of anti-hapten antibody contained in the serum was considerably less than that produced against HGG.

In an attempt to determine the effect of specific antibody on an intact tumor host system, multiple experiments were carried out. In each experiment, C3H mice with tumors implanted in the thigh as described above were divided into 4 groups. Each group contained 10–15 mice. On the day of experiment, all mice in each group were given an i.v. injection via the tail vein according to the following 4 schedules. The 1st group was given 0.25 ml of saline and returned to their cage to act as untreated controls. The 2nd group were given 3 µg of PAM/gm of body weight and returned to their cage. (Dose-response curves had previously determined that this dose of PAM would have no detectable effect on the tumor growth.) The 3rd group of mice received an i.v. injection of 0.25 ml of specific rabbit antiserum. This group of mice acted as a control for the action of antibody alone. Mice in the 4th group were given an i.v. injection of 3 µg PAM/gm of body weight followed in 0.5 hr by a 2nd i.v. injection of 0.25 ml of specific antiserum.

The pattern of tumor growth was reasonably consistent in the 6 experiments reported. The groups in each experiment that received PAM and/or antiserum were injected with the same mustard solution and the same batch of specific antiserum. For each experiment, the PAM solution was freshly prepared. While several batches of specific antiserum were used in these experiments, each experiment was conducted with a single batch.

The effect these 4 regimes had on tumor growth in the 6 studies is illustrated in Chart 2. Since the pattern of tumor growth was consistent in these experiments, the data including the range of response within each group on the 6th day after treatment are given in Table 1. The tumor growth in mice treated with PAM alone, specific antibody alone, and control mice given injections of saline was identical. However, the group of mice treated with PAM followed in 0.5 hr by specific antiserum showed a marked decrease in the rate of tumor growth as compared with the 3 other groups. The decrease in rate of growth was most marked shortly following the single injection of PAM followed by specific antiserum. After this initial decrease in growth rate, however,
the tumor again began to grow at a rate more like the rate of growth seen in the control animals.

There was no immediate mortality in the saline control group or in the groups treated with L-phenylalanine mustard alone or with specific antibody alone. There was a considerable immediate mortality amounting to 10% in the overall group treated with mustard followed by specific antibody. A reaction occurred immediately following injection of the specific antibody consisting of respiratory distress and occasionally prostration and death. However, this reaction resembling anaphylaxis appeared to have no lasting effect on the surviving mice and in this group treated by L-phenylalanine followed by specific antibody, life was prolonged for 2 days beyond that of the various control groups. There is, however, no firm evidence eliminating the possibility of a nonspecific Schwartzman or Arthus effect on the tumor nor is there evidence eliminating the nonspecific effect of hypotension on tumor growth.

In order to gain information concerning the specificity of the antiserum against PAM, and to detect nonspecific effects of antiserum following a nonrelated cytotoxic agent, experiments were carried out in which the PAM-specific antiserum was injected following a primary injection of 2,2'-dichloro-A'-methyl-diethylamine rather than PAM. In this experiment, HN2 was given to the control group in a dose of 3 μg of HN2 per gm of body weight i.v. followed in 0.5 hr by an i.v. injection of 0.2 ml of PAM specific antiserum. Examination of Chart 3 reveals that anti-PAM-specific antiserum given following a primary injection of HN2 does not affect tumor growth over the effect of HN2 given alone.

To obtain evidence bearing on the exact site of localization of the haptene PAM and also on the site of antibody localization, mice containing implanted tumors in their thighs 10-12 days before experiment were sacrificed 0.5 hr following the i.v. injection of 3 μg of PAM per gm of body weight. Tumors were immediately removed from the thigh and prepared for fluorescent antibody study (21). Sections were stained with anti-PAM serum that had been conjugated with fluorescein isothiocyanate (4). Fig. 1 is a black and white reproduction of a color photomicrograph of a typical field demonstration of the distribution of fluorescence. (Control slides stained with specific antiserum followed by specific fluorescent antiserum showed no specific fluorescent staining.) Most of the immunofluorescence is localized in the area of the tumor cell wall. An occasional cell, however, contains considerable fluorescent material throughout its cytoplasm. Sections of muscle and liver from the same animal show little immunofluorescence when stained with fluorescent antibody. The fluorescence that is present in these control tissues is faint and localized mostly in the region of the endothelium of small vessels with almost no localization detectable on the muscle or hepatic cell walls. These studies may be interpreted as indicating that the concentration of PAM in tumor tissue is considerably in excess of that in muscle or liver and that the mustard is localized for the most part in the region of the tumor cell wall.

Histologic study of the tumors obtained from mice from each of the 4 treatment groups outlined above and examined 6 and 8 days following treatment demonstrated that there is little morphologic difference between the control group treated with saline

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<th>TABLE 1</th>
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<td><strong>RESULTS OF INDIVIDUAL EXPERIMENTS WITH RANGE OF RESPONSE AT DAY 6</strong></td>
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<tr>
<th>EXPERIMENT NO.</th>
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CHART 3. The effect of a single i.v. dose of HN2 followed in 0.5 hr by antiserum against PAM-HGG conjugate compared to the effect of HN2 alone.
and the groups of mice given PAM alone or specific antibody alone. In these 3 groups, there were large areas of tumor cells tending to arrange themselves in cords, together with patchy areas of necrosis and only scant connective tissue supporting structure. Fig. 2 illustrates a section taken at 8 days following the injection of sterile saline alone. Fig. 3 illustrates a tumor taken from an animal treated 8 days previously with PAM alone.

The above histologic picture differs considerably from that seen in a tumor 8 days after treatment with PAM followed in 0.5 hr by specific antibody. The viable tumor cells present tend to be localized in nests surrounded by fusiform cells taking a pale stain. Figs. 4 and 5 show such an area. The tumor cell nests tend to be small and there is little necrosis. Figs. 6 and 7 are higher power views of a similar nest of viable tumor cells. The fusiform cells occupy large areas and, in places, greatly outnumber the viable tumor cells. It is important to emphasize, however, that the changes seen are more a change in the relative numbers of cells than difference in cell types, for the same fusiform cells seen in great profusion in tumors treated with PAM followed by specific antibody are also seen as stroma of untreated tumors or tumors treated with mustard alone (see Fig. 2).

Discussion

The experimental work described has been restricted in these experiments to the use of a single dose of L-phenylalanine mustard, at a level known to have no gross effect on the tumor, followed by a single injection of specific antibody 0.5 hr after the mustard. These experiments were designed to provide information concerning the specificity and the degree of cytotoxicity of an antibody system directed against normal cell. The experimental results appear to indicate that a considerable degree of both specificity and cytotoxicity are present in the system as described, although nonspecific Arthus or Schwartzman effects are not ruled out. There are, however, a multitude of questions left unanswered, as well as a number of possibilities which might increase the specific cytotoxic reaction of the system: what is the effect, for example, of increasing doses of PAM, specific antibody, or both on the extent of killing of tumor cells? What is the possibility of a treatment schedule consisting of multiple doses of a haptene marking compound followed by haptene-specific antibody staged at varying intervals? Also of interest, what is the effect of this form of cytotoxic therapy employed subsequent to or simultaneous with radiation therapy?

In the system described above, there are 2 separate steps, each of which is indispensable for the success of this theoretical form of therapy. The 1st is the localization of a substance on the cell to be marked. The 2nd is the production of a heterologous, cytotoxic antibody to the substance used as a marking compound. Localization has been accomplished by utilizing the biologic properties present in tumor vessels, i.e., their "early inflammatory-like" vascular permeability. However, any factor which will increase the concentration of marking substance deposited in the tumor would presumably increase the efficiency of the system as a whole. Concentration of marking compound is largely dependent on the extent of vascular supply to the selected area. Any form of inflammatory reaction created within the selected area of the tumor would serve not only to increase further the permeability to the marking substance, but would also serve to increase the volume of blood flow, thus bringing to the tumor an increased amount of marking compound. An inflammatory reaction localized to a tumor-bearing area might be conveniently induced with a dose of X-radiation causing erythema. It might also be accomplished with direct perfusion using a permeability-inducing agent such as histamine. We have already seen in our preliminary experiments that the tumor inhibiting effect is greater on highly vascular tumors than on relatively avascular ones.

Further, with respect to increasing the efficiency of localization and also that of the biologic effect of the total system, it is worth noting that the experiments conducted thus far have shown concentration of a compound within a tumor to be closely related to the solubility of the compound. These studies have involved only the transfer into the interstitial space. Information concerning the solubility characteristics important in passage across the cell or nuclear membrane may also prove to be of considerable value.

The 2nd component of the total system, that of antibody production, is of obvious importance to the efficiency of the reaction. In work, thus far, we have found that the haptene carrier has considerable bearing on the antihaptene antibody titer obtained. HGG has been found to be considerably more efficient than BSA in this regard. Since the cytotoxic efficiency of the system depends on the quantity and type of antibody present as well as on quantity and localization of the antigenic marking compound (and probably the presence of complement), the efficiency of the carrier is of some importance.

The experiments presented above suggest the possibility of a combined approach to tumor therapy, utilizing the biologic specificity of an immunologic system where none exists in the natural state through passive provision of both an antibody and a specifically localized antigen.

References

J. F. Burke, V. H. Mark, A. H. Soloway, and S. Leskowitz


Fig. 1. Immunofluorescence photomicrograph of tumor cells showing the localization of fluorescein-labeled rabbit serum in the region of cell wall reacted 0.5 hr following i.v. injection of PAM. H & E, × 250.

Fig. 2. Photomicrograph of tumor 8 days following single i.v. injection of saline alone. H & E, × 120.
Fig. 3. High power photomicrograph of tumor 8 days following single i.v. injection of PAM alone. H & E, × 200.
Fig. 4. Photograph of tumor 8 days following single i.v. injection of PAM followed in 0.5 hr by specific antiserum. H & E, × 30.
Fig. 5. Medium power photomicrograph of tumor 8 days following single i.v. injection of PAM followed in 0.5 hr by specific antiserum. H & E, × 60.
Fig. 6. High power photomicrograph of tumor 8 days following single i.v. injection of PAM followed in 0.5 hr by specific antiserum showing tumor cell nest. H & E, × 150.
Fig. 7. High power view of pale-staining fusiform cells surrounding tumor cell nest in tumor taken 8 days following single i.v. injection of PAM followed in 0.5 hr by specific antiserum. H & E, × 300.
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