Effect of Local Immunotherapy of Syngeneic Rat Fibrosarcoma with Hapten and Anti-hapten-Tumor Serum upon Nonlocally Treated Tumor

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ABSTRACT

Previously, we produced complete regression of allogeneic and syngeneic rat tumors by in situ tumor modification with a hapten, L-phenylalanine mustard (PhM), followed by i.v. administration of an antisera developed against PhM-tumor extract conjugate.

To evaluate the effect of this antisera, we treated two syngeneic methylcholanthrene-induced rat fibrosarcomas in rats treated with the conjugate of PhM and fibrosarcoma extract containing PhM-specific antibody and a barely detectable level of antibody specific to the conjugate.

All of the paired tumors in untreated rats progressively grew and killed the hosts. Intratumoral (i.t.) injection of PhM only to the left tumor followed by i.v. administration of the antisera produced uniform regression of all tumors, with disappearance of both treated (6 of 11 animals) and untreated (9 of 11 animals) tumors and prolonged survival time. Of the tumors which disappeared, three treated and five untreated tumors did not recur, and two rats were cured. The substitution of antisera with normal serum or the substitution of the i.t. injection of PhM with s.c. PhM injection resulted in a significantly lower effect on both paired tumors, and there were no cures, indicating that the combination of i.t. injection of PhM and antisera is important for successful tumor regression.

Regression of the untreated tumor was significantly greater in the PhM i.t.-antisera group than in any other PhM-injected group. This difference cannot be explained by a systemic effect of PhM. In addition, the effect of PhM i.t.-antisera on the untreated side tumor was markedly reduced by the excision of PhM-injected left side tumor. These results suggest that treated and untreated regressing tumor tissue may be beneficial for the regression of the untreated tumor.

Interestingly, each pair of tumors synchronously changed its volume with a highly significant positive linear correlation, regardless of treatment modality, differences in tumor growth patterns, or differences in initial sizes of the paired tumors. In the PhM i.t.-antisera group, this correlation seems to suggest the induction of systemic effects by the local treatment.

INTRODUCTION

There is mounting evidence on the potential usefulness of tumor-associated antigens for immunotherapy of cancer. However, these antigens appear to be generally ineffective not only in inducing immune response but also in acting as a potential target for selective immunological attack.

After demonstrating the haptenic activity of PhM,3 Burke et al. (8) injected PhM i.v. into mice with established ependymoma to conjugate it to the tumor tissue and followed this with an i.v. injection of anti-PhM serum. This approach produced inhibition of tumor growth, although a relatively high incidence of anaphylactic-like immediate mortality was noted. Ineffectiveness of either PhM or antiserum alone and the immunological nature of the inhibition mechanism were also demonstrated.

This suggested to us that the problem of ineffective tumor antigen in immunotherapy might be overcome by the use of a hapten. To explore this possibility, we confirmed the haptenic activity of PhM (2, 3). The rat antisera induced with PhM-conjugated extracts of allogeneic (3) or syngeneic (2) tumor or of normal tissue (3) and the rabbit antisera produced against PhM-conjugated rat tumors (2, 3) or human tumors (3) were specific to PhM. These antisera also sometimes contained low titers of antibodies independently specific to hapten-carrier conjugate and/or carrier.

In our immunotherapy model, an i.t. injection of PhM was followed by an i.v. injection of antisera produced against the conjugate of PhM and tumor extract. Although the induction of a hapten-mediated immune injury of the tumor tissue was the purpose of this approach, carrier protein derived from the target tumor was used in antisera production to take advantage of carrier (tumor)-specific antibody in addition to the hapten-specific antibody. PhM was given i.t. to avoid the mortality noted by Burke et al. (8), which might be due to a reaction between a PhM-plasma protein conjugate created by the i.v. PhM injection and subsequently administered anti-PhM antibody. A single treatment with this approach produced complete regression of established allogeneic tumor and cured most animals (4). Subsequent studies with a syngeneic rat tumor and a syngeneic antisera yielded similar regression (2), ruling out the theoretical possibility that the tumor regression might be caused by histocompatibility antigens. Since syngeneic antisera has no clinical relevancy, a xenogeneic antisera absorbed with normal rat tissue was then studied in the same syngeneic tumor model. This also produced a similar response without recognizable side effects (2). In all studies, regression following PhM plus antiserum treatment was always significantly greater than that of PhM i.t. injection, although antiserum alone was generally ineffective. Thus, the effectiveness of this treatment system is consistently reproducible. It is due to neither PhM nor antiserum alone but probably to the interaction

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3 The abbreviations used are: PhM, L-phenylalanine mustard; it., intratumoral; MC1, methylcholanthrene-induced rat fibrosarcoma; AC33: Undifferentiated rat mammary carcinoma appeared after dimethyl-β-aziridinopropionamide treatment. I.d., intradermal; BCG, Bacillus Calmette-Guerin.
of both at the tumor site along with the local cytotoxic effect of PhM.

Interestingly, the injection of PhM into only one of bilateral tumors, followed by an i.v. administration of antiserum, caused complete regression of both tumors in our allogeneic rat tumor model (5).

The present study, which uses syngeneic antiserum and rats with bilateral syngeneic tumors, was performed to confirm the response of the untreated tumor. In addition, an attempt was made to determine if there is any relationship between growth or regression patterns of the treated and untreated tumors.

MATERIALS AND METHODS

Animals. Inbred male Lewis rats (LEW/Mai), weighing approximately 200 g (Microbiological Associates, Inc., Walkersville, Md.), were used throughout the experiment. The age of rats used in the immunotherapy experiment differed by no more than 1 week.

Tumors. Fibrosarcoma (MC1) syngeneic to Lewis rats (2) was used. The weak antigenicity of this tumor in Lewis rats was indicated in the work by Ziegler et al. (23). However, immunization of Lewis rats with an extract of MC1 tumor did not produce any detectable tumor-specific antibody (2). 

Preparation of Carrier Proteins. Carrier proteins were prepared from MC1 tumor, AC33 tumor, and rat normal tissue as described previously (2, 3, 8).

AC33 syngeneic to Lewis rats (2) was also used as one of the carrier protein sources. The antigenicities between MC1 and AC33 tumors are distinct as determined by the amputation of AC33-bearing tails of rats and challenging the animal with either tumor.

Haptenic Agent. PhM was used (courtesy of Dr. Wayne Brencmann, Jr., Burroughs Wellcome Co., Research Triangle Park, N. C.), as in our previous studies (2-5). The haptenic activity of this agent has been established (2, 3, 8). PhM directly conjugates with proteins in vitro (3, 8) and seems to conjugate in vivo with the tissue at the injection site (2). We showed previously that i.t. or i.d. injections of PhM solution without carrier are able to produce anti-PhM antibody in rats (3). Taking advantage of the altered vascular permeability in the tumor tissue, Burke et al. (7, 8) were able to haptenize the cells of malignant ependymoma by i.v. injection of PhM. Although this is an alkylating anticancer agent as well as a hapten, it has been confirmed that the effect produced by this agent and specific antiserum is not due to the effect of PhM alone (2, 3, 8).

Preparation of Carrier Proteins. Carrier proteins were prepared from MC1 tumor, AC33 tumor, and rat normal tissue as described previously (3). The pooled normal tissue included liver, heart, spleen, and muscle. The tissue was minced, homogenized in an ice bath, and extracted with distilled water, and the protein-rich supernatant was lyophilized. The extracts of MC1 tumor, AC33 tumor, and normal tissue are designated accordingly.

PhM Conjugation. The lyophilized extracts of MC1, AC33, and normal tissue were conjugated with PhM by the method of Burke et al. (8) utilizing a 3.5-mg/ml PhM solution rather than the 1.75-mg/ml dose that we used previously (3). After evaluating different concentrations of PhM, this concentration was found to be the most effective for production of a potent syngeneic antiserum, probably due to the lower immunogenicity of the syngeneic carrier compared to the previous allogeneic carrier (2).

Production of Antiserum. A mixture composed of 20 mg of PhM-MC1 antigen in 0.5 ml of 0.9% NaCl solution and 0.5 ml of Freund’s complete adjuvant was divided equally and injected s.c. into each gluteal area of the rat. One month later, 20 mg of antigen were given i.p. The animals were bled by cardiac puncture 2 weeks after the last injection.

PhM Solution for Injection. PhM solution was freshly prepared before use as described previously (4). Briefly, 100 mg of PhM were dissolved in 2 ml of 0.1 N HCl with minimum warming and cooled to 25°C, and 3 ml of 0.9% NaCl solution were added. The concentration of solution was adjusted to 5 mg/ml (final pH, 6.6) with 0.15 M phosphate buffer (pH 8.0).

Specificity and Titer of Antiserum. The specificity and titer of the pooled anti-PhM-MC1 serum were determined by microdouble gel diffusion as described previously (3). 

Immunochemistry. A mixture composed of 2 mg/ml (final pH, 6.6) with 0.15 M phosphate buffer (pH 8.0).

Specificity and Titer of Antiserum. The specificity and titer of the pooled anti-PhM-MC1 serum were determined by microdouble gel diffusion as described previously (3). Agar (agarose; Sigma Chemical Co., St. Louis, Mo.), 0.8% in 0.02 M phosphate buffer (pH 7.2), was placed between a plastic template with 2-mm-diameter wells and a glass slide precoated with agar. The antigen and antiserum were applied in 15-µl amounts into the wells. The concentration of all antigens was 30 mg, dry weight, per ml. The titer was determined by applying the antiserum in serial dilution against PhM-conjugated or unconjugated MC1, AC33, and normal tissue antigens.

The specificity was also determined with concentrated fractions of the antiserum prepared by Rivanol-ammonium sulfate fractionation (12) to supplement the low sensitivity of the gel diffusion method. As described previously (2), after removal of Rivanol by the addition of NaCl (final concentration, 5%), the supernatant and sediment were further fractionated with ammonium sulfate (final concentration, 50%). The precipitates were dialyzed and lyophilized. All of the supernatants from the ammonium sulfate fractionation were also pooled, dialyzed, and lyophilized.

The 3 lyophilized fractions, a Rivanol supernatant-ammonium sulfate sediment fraction, a Rivanol sediment-ammonium sulfate sediment fraction, and the remaining fraction of antiserum, were reconstituted with distilled water to 30 mg/ml. The electrophoretic characteristics and the specificity of these fractions were determined by microzone electrophoresis (Beckman Instruments, Inc., Palo Alto, Calif.) and gel diffusion.

Immunotherapy Experiment. Both thighs of the rats were given s.c. injections of 2 x 10⁶ viable cells (trypan blue exclusion) in 0.5 ml of Hanks’ balanced salt solution. Ten days after transplantation, the maximum diameters of the tumors were measured 3-dimensionally (2) with Vernier calipers. The diameter of the diameters was expressed as a tumor volume and used as an index of tumor volume change. PhM was injected either i.t. into the left tumor or s.c. at a site 1 cm from the edge of the left tumor. Three hr later, anti-PhM-MC1 serum or normal serum (0.005 ml/g body weight) was administered i.v. through the tail vein.

Both sera had previously been incubated at 56°C for 30 min. The antiserum was concentrated by lyophilization to double the titer against PhM-MC1 antigen (1:32). The normal serum was similarly concentrated. The tumor volume was measured for 9 consecutive days and on the 12th, 17th, 21st, and 34th post-treatment days. Daily observation of the tumors was continued.

and the survival time of the animals was recorded. All tumor measurements were performed by a single person throughout the experiment.

Fifty rats with established tumors (left tumor average size, 3.3 cu cm; right tumor average size, 2.6 cu cm) were divided into 5 groups. There were no significant differences between the groups with respect to body weight or tumor volume of either side (Table 2).

The experimental groups were as follows: PhM-antisemum group, 11 rats that received PhM i.t. into the left tumor followed by antiserum i.v.; PhM-normal serum group, 11 rats that received PhM i.t. into left tumor followed by normal serum i.v.; PhM s.c.-antisemum group, 9 rats that received PhM s.c. at a site 1 cm from the left tumor followed by antiserum i.v.; PhM-antisemum-excision group, 9 rats that received PhM i.t. into the left tumor followed by antiserum i.v. Immediately after the antiserum injection, the PhM-injected tumor was surgically excised under anesthesia (i.p. sodium pentobarbital). The wound was irrigated with sterile 0.9% NaCl solution, sutured, and shielded with a plastic covering (Aeroplast dressing: Park, Davis and Co., Detroit, Mich.). The no-treatment group consisted of 10 rats.

The tumor growth and survival times of each group were compared by one-way analysis of variance and linear contrast. In comparisons of incidences of tumor regression (>50%) and complete tumor disappearance, the χ² test was used. Differences were considered significant when p was smaller than 0.05.

**RESULTS**

**Specificity and Titer of Antiserum**

In double-gel diffusion, the pooled anti-PhM-MC1 serum did not show any reaction with the unconjugated MC1, AC33, and normal tissue antigens but produced a common precipitation line specific to these antigens conjugated with PhM. This antiserum also frequently produced an additional very weak line specific only to PhM-MC1 in repeated determinations. Among the 3 fractions obtained by the Rivanol-ammonium sulfate fractionation, only the Rivanol supernatant-ammonium sulfate sediment fraction was immunologically active and showed electrophoretic migration peaks at β and γ positions. The specificity of this fraction was identical to the original anti-PhM-MC1 serum. The maximum dilution giving a positive reaction was 1:16 against all PhM-conjugated antigens in gel diffusion. The characteristics of this antiserum are similar to another batch of syngeneic anti-PhM-MC1 serum determined in a previous study (2).
Results of Treatment

No-Treatment Group (Chart 1A). Paired tumors in all rats progressively grew and killed the hosts between the 12th and 15th posttreatment days.

PhM-Antiserum Group (Chart 1B). All paired tumors, regardless of whether PhM was injected, began regressing between the third and fourth posttreatment day, and all of them showed more than 50% reduction of volume. The average volumes reduced to 18.6% of the pretreatment volume in the injected tumors and to 16.5% in uninjected tumors by the ninth day. Although paired tumors in 2 rats resumed growth early, the remaining 9 rats showed continued regression of their tumors with disappearance of both tumors in 6 rats and one tumor in 3 rats between the 8th and 16th posttreatment days. In the 3 rats in which one tumor disappeared, the other tumor was barely palpable. Of the tumors which disappeared, 3 of 6 injected and 5 of 9 uninjected tumors did not recur. Finally, 2 rats survived without any recurrence of paired tumors up until the time of sacrifice, 100 days after the transplantation. The injected tumors and uninjected tumors showed a similar pattern in growth curves.

PhM-Normal Serum Group (Chart 1C). All of the paired tumors showed some degree of regression, but the onset of the regression seemed to begin slightly later (4th or 5th posttreatment day) than those of the PhM-antiserum group. The paired tumors in 10 of 11 rats quickly resumed growth during the 6th to 12th posttreatment days. In one rat, the uninjected tumor disappeared on the 21st posttreatment day, but the rat eventually died with slowly growing bilateral tumors. Only 3 of the 11 animals showed a tumor volume reduction greater than 50%.

PhM s.c.-Antiserum Group (Chart 1D). Seven of 9 rats showed some degree of tumor regression, while 2 others showed only slight growth inhibition of their paired tumors. Those temporarily regressed tumors quickly resumed their growth. Only one treated tumor showed greater than a 50% reduction in size.

PhM-Antiserum-Excision Group (Chart 1E). The early excision of tumors treated with PhM-antiserum markedly reduced the effect on the uninjected tumors. A reduction of tumor volume greater than 50% occurred in 5 of 9 tumors. One of these 5 tumors disappeared on the ninth posttreatment day but reappeared 1 week later and ultimately killed the host with a slight prolongation of survival time.

Comparison of Groups

The tumor volumes of each group on each day were compared by one-way analysis of variance and linear contrast (Table 1). Compared with the no-treatment group, all 4 PhM-injected groups showed a significant growth inhibition of both treated and untreated tumors after the fourth posttreatment day. The cytotoxicity of PhM might be responsible for this general inhibition. However, inhibition of the treated tumor in the PhM-antisera group was significantly greater than that of the PhM s.c.-antisera and PhM-normal serum groups. In addition, the inhibition of the untreated tumor was also significantly greater in the PhM-antisera group than in any other PhM-injected group. Except for a transient difference of treated tumor volumes between the PhM-normal serum group and the PhM s.c.-antisera group on the fourth to fifth posttreatment days, there were no significant differences among the PhM-normal serum, PhM s.c.-antisera, and PhM-antisera-excision groups in comparison of either treated or untreated tumors.

The PhM-antisera group also showed a significantly greater response than any other group in comparing the incidence of tumor volume reduction greater than 50% (p < 0.001) and of tumor disappearance (p < 0.001), regardless of whether the treated or untreated tumor is considered. The differences among other groups were statistically insignificant (Table 2).

The survival time of the PhM-antisera group was significantly longer (p < 0.01 to 0.001) than any other group, and cures of animals occurred only in the PhM-antisera group. The differences among the survival times of the PhM-normal serum, PhM s.c.-antisera, and PhM-antisera- excision groups were not significant, but these 3 groups showed significantly (p < 0.05) longer survival times than the no-treatment group (Table 2).

Relation between the Paired Tumors

A highly significant (p < 0.001) positive linear correlation between the volumes of the paired tumors of each individual rat was found in all animals. The correlation was also significant if determined with the growth rate (given tumor volume/pre-
The previous observations by us (2) and Burke et al. (8), strongly suggest that the antiserum may cause an immunologic local reaction to PhM-injected tumor tissue. The marked difference in titer between PhM-specific and PhM-MC1 conjugate-specific antibodies contained in the antiserum suggest that the PhM-specific antibody plays a major role in this reaction.

The regression of untreated tumors in PhM-injected groups might be explained by the systemic effect of PhM. However, the significantly different response of un.injected tumors between the PhM-antisemum group and other PhM-injected groups is difficult to explain by a systemic effect of PhM. If it is assumed that PhM leaked from the injected tissue, modified the un injected tumor, and subsequently reacted with antiserum to cause enhanced regression, then the response of the un injected tumor should have been the same in the PhM-antisemum group and the PhM-nomnalserum group. On the other hand, the response of the un injected tumor to PhM-antisemum treatment was markedly reduced by the removal of the injected tumor. This is unlikely to be due to removal of injected tumor as a depot of PhM, since the excision group produced rather greater response on the untreated tumor than the PhM-nomnal serum and PhM s.c.-antisemum groups which retained PhM did (Table 2). Thus, retention of treated tumor tissue appears to be responsible for the enhanced regression of untreated tumor in the present study demonstrates that the treatment of only one of bilateral MC1 tumors with PhM plus anti-PhM-MC1 serum is able to produce complete regression of not only the treated but also the locally untreated tumor.

Although PhM itself was effective in the PhM-injected groups, the response of PhM-injected tumor was significantly greater in the PhM-antisemum group than in the PhM-nomnal serum group. Thus, the effect of PhM-antisemum treatment is due not to the effect of PhM alone but to the combination of PhM and antiserum. However, the effect of this combination was markedly reduced when PhM was injected into an adjacent site rather than into the tumor tissue, demonstrating the importance of the i.t. injection of PhM in this combination and the ineffectiveness of antiserum by itself. These results, in concert with the previous observations by us (2) and Burke et al. (8), indicate the possibility that the treated and untreated tumors of the same animal may concurrently disappear if conditions are appropriate.

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the PhM-antisem group. The regression of guinea pig hepatocarcinoma caused by i.t. injection of PhM (6) or BCG (11) induces systemic tumor immunity, and the immunity increases with retention time of BCG-treated tumor (11). The animals treated with i.t. injection of Mycobacterium bovis (22), local hyperthermia (19), and local electrocoagulation (21) inhibited the growth of metastases (9, 22) or increased resistance against secondary challenge (21), whereas the animals from which tumors were excised did not (19, 21, 22). These observations and the present data seem to suggest that retention of locally treated tumor tissue in an appropriate condition may be beneficial to induce the systemic effect.

The regression or inhibition of untreated tumors by local treatment of another tumor has been reported following i.t. injection of Corynebacterium parvum (14), a hapten, 2,4-dinitrophenylmethacrylate (18), neuraminidase (20), and local hyperthermia (19) in animals. A similar phenomenon has also been observed in humans after cryosurgery of tumors (1, 10) and intralesional BCG therapy (15–17). Although the incidence is far from uniform and the mechanisms are virtually unknown, it is interesting that this phenomenon may occur regardless of the type of local treatment. In the present study, a highly significant positive linear correlation between volume changes of paired tumors was found. The data obtained from the no-treatment group may or may not indicate the real relationship between the paired tumors, because of a possible spurious correlation created by the independent growth of paired tumors with similar growth rates. However, in the PhM-antisem group, the paired tumors took the same course with a significant correlation in continuous regression or in growth resumption. The statistical correlation in this group indicates the relationship between the paired tumors and suggests the development of systemic factors which influence the course of the pair. We previously observed a similar correlation in the same type of experiment with rats bearing 2 allogeneic tumors (5). The existence of such a correlation is also indicated from the growth curves of tumors following i.t. neuraminidase treatment of one of 2 mouse fibrosarcomas (20).

The least square regression lines obtained from the PhM-antisem group (Chart 2) suggest the possibility that the paired tumors may disappear almost concomitantly, regardless of whether they were treated or untreated and of differences in their initial sizes, if conditions are appropriate. Although no such analysis has been reported previously, similar events were observed in the neuraminidase experiment cited above (20) and in canine venereal tumors following i.t. BCG injection of one of bilateral tumors (13). Nearly concurrent regression of BCG-injected dermal melanoma and untreated pulmonary metastases has also been observed (15).

The previous observations of Arai et al. (4) and Burke et al. (8) suggest that a PhM-specific antibody may elicit an immunological tissue injury, possibly an Arthus-like reaction (9), at the site of the PhM-treated tumor tissue. Borsos et al. (6) demonstrated a nonspecific killing effect of antibody and complement against the tumor cells pretreated with alkylating anticancer agents. Both may give some clue to the regression mechanisms of the treated tumor in the PhM-antisem group. However, the exact mechanisms of regression of either treated or untreated tumor are unknown.

Further studies of the relationship of growth patterns between locally treated and untreated tumors and elucidation of the mechanisms involved in the regression of the untreated tumor will provide useful information for the treatment of cancer with metastases.

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