Immunotherapy of Cancer with L-Phenylalanine Mustard as a Hapten

Kazuhiko Arai, Herbert W. Wallace, and William S. Blakemore

Department of Surgery, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104

SUMMARY

An antiserum produced in Wistar rats with an L-phenylalanine mustard (PhM)-conjugated extract of Walker 256 carcinosarcoma, which had been confirmed to be specific against tumor extract, PhM alone, and PhM-tumor conjugate, was used in this study. Seven groups of Wistar rats with established Walker tumor (mean volume, 5.2 cu cm) were treated as follows: (a) by intratumoral (i.t.) injection of PhM plus i.v. injection of antiserum; (b) by injection of PhM (i.t.) plus 0.9% NaCl solution (i.v.); (c) by injection of 0.9% NaCl solution (i.t.) plus antiserum (i.v.); (d) by injection of PhM (i.t.) plus 0.9% NaCl solution (i.v.) plus hydrocortisone (i.m. for 10 days); (e) by injection of PhM (i.t.) plus antiserum (i.v.) plus hydrocortisone (i.m. for 10 days); (f) by injection of PhM (i.m.); or (g) no treatment. All surviving rats were sacrificed 90 days after tumor transplantation. PhM-antiserum treatment induced complete regression of tumor in 7 of 8 rats, with a significant increase in mean survival time. PhM (i.t.) treatment induced regression in some animals, but at a much slower rate, and the mean survival time of this group was significantly shorter than that of the PhM-antiserum group. Antiserum (i.v.) treatment and PhM (i.m.) treatment showed no regressive effect. The addition of an immunosuppressant, hydrocortisone, eliminated the tumor-regressive effect of both PhM and PhM-antiserum treatment. Similar results were obtained in rats with significantly larger tumors. These results indicate that PhM-antiserum immunotherapy can produce complete tumor regression.

INTRODUCTION

The presence of tumor-specific transplantation antigens has been reported in animal tumors induced by chemicals and viruses and in a variety of human cancers, including Burkitt lymphoma (17), sarcoma (10, 20), melanoma (21), and colonic carcinoma (13). However, most of these tumors, if untreated, grow progressively and lead to the death of the host. Possible explanations of this paradoxical phenomenon, that the host fails to reject antigenic tumor, have been discussed previously (26) and are still under discussion.

However, the simplest explanation is that the tumor has insufficient specific antigenic stimulus to induce an immune response capable of rejecting the tumor. It has been shown that antigenically modified tumor cells are able to induce a stronger immune response than unmodified tumor cells (1, 8, 18, 23, 25). Different approaches to experimental immunotherapy utilizing the modification of tumor antigenicity result in either a tumor-specific effect (15) or a modifying agent-specific effect (3, 4) on the inhibition of tumor growth.

An approach that would induce both tumor-specific and modifying agent-specific effects simultaneously might inhibit tumor growth even more effectively. However, such an approach has not been reported. Recently, we have shown that it is possible to utilize PhM as a hapten and produce 3 distinct antibodies that are independently specific to PhM, to tumor, and to the PhM-tumor complex in Wistar strain rats immunized with PhM-conjugated extract of Walker 256 carcinosarcoma (2). Utilizing this antiserum, we have evaluated the effects of the simultaneous use of tumor-specific antibody and modifying agent-specific antibody on the antigenically modified tumor in situ.

MATERIALS AND METHODS

Modifying Agent, Experimental Animals, and Tumor

PhM was chosen as the modifying agent because this alkylating agent possesses haptenic activity (2, 4). It also reportedly accumulates in the area of the tumor tissue (28) and appears to create a modified protein by cross-linkage after breaking the hydrogen bands of DNA (7, 29). It is important to emphasize that the expected role of PhM in this study was not its direct cytotoxic effect but its modifying effect on tumor antigenicity. Inbred, male Wistar rats (Huntingdon Farms, West Conshohoken, Pa.) weighing approximately 250 g were used throughout the study. The Walker 256 carcinosarcoma was obtained from the National Cancer Institute. This tumor occurred spontaneously in another strain of rat (11), and we confirmed in a pilot study that it grows continuously in the Wistar rat, with a uniformly fatal outcome. Although a spontaneous tumor developed in inbred Wistar rats would be preferable, the animal-tumor system we used should yield reliable data if appropriate controls are studied simultaneously.
Preparation of PhM-conjugated Tumor Antigen and Antiserum

The Walker 256 tumor was transplanted i.m. to Wistar rats. The tumor, maintained by serial transplantation in this rat strain, was removed, and carrier protein was prepared and conjugated with PhM in vitro. The PhM-conjugated tumor protein was dialyzed, concentrated (6 mg dry weight per ml), and mixed with the same volume of complete Freund's adjuvant for the immunization of Wistar rats (2). Antisera obtained from 30 pools were pooled and used in this study. It has been confirmed (2) that this antiserum possesses specificities against PhM, Walker 256 tumor, and PhM-tumor conjugate by agar gel immunodiffusion.

Preparation of PhM Solution for Injection

One hundred mg of PhM were mixed in 2 ml of 1/10 N HCL and dissolved by heating. After the solution cooled to room temperature (25°), 3 ml of 0.9% NaCl solution were added. This solution was adjusted to pH 6.6 with 0.15 M phosphate buffer, pH 8.0. The resultant solution (5 mg/ml) was used for i.m. or i.t. injection. The PhM solution was prepared immediately before use.

Experimental Methods

General Procedure

Walker 256 tumor tissue obtained from a Wistar rat was carefully trimmed (2 cu mm) and was inoculated with a trocar into a superficial site in the thigh muscle of a Wistar rat. The tumor was allowed to grow, and on the day of treatment it was measured 3 dimensionally with a Vernier caliper in the following manner: (a) maximum diameter perpendicular to the femur, (b) maximum diameter parallel to the femur, and (c) maximum depth perpendicular to the skin surface. All measurements were performed by 1 person to eliminate individual variation. Rats were preselected to minimize the variation in starting tumor volume. They received complete Freund's adjuvant for the immunization of Wistar rats (2).

Charts 1 to 7. Tumor growth curves and survival times (from day of tumor transplantation) of individual rats in each group in regular-sized tumor series. All cured rats were sacrificed 90 days after transplantation. Numerals, survival times of rats surviving more than 16 days. *, complete regression of tumor; †, death occurring before termination of daily measurements; saline, 0.9% NaCl solution. Chart 1. Untreated group. Chart 2. The i.m. PhM group. Chart 3. PhM group. Chart 4. PhM-antiserum group. Chart 5. Antiserum group. Chart 6. PhM-hydrocortisone group. Chart 7. PhM-antiserum-hydrocortisone group.

"Regular-sized"-Tumor Series

Sixty-five rats divided into 7 groups were treated on the 8th day after tumor transplantation. On this day there were no significant differences (p > 0.05) between the groups with respect to tumor diameter (mean of all diameter values for all rats) or volume, and the mean body weights of the groups were almost uniform. The experimental groups were as follows. 3

The i.m. PhM Group. Ten rats with a mean tumor volume of 4.7 ± 0.5 cu cm received i.m. injections of PhM solution at a site 2 cm from the edge of the tumor tissue, the dose being based on the tumor volume (0.03 ml/cu cm).

PhM Group. Twelve rats with a mean tumor volume of 5.3 ± 0.4 cu cm received PhM (i.t.) followed by 0.9% NaCl solution (i.v.).

PhM-Antiserum Group. Eight rats with a mean tumor volume of 5.6 ± 0.6 cu cm received PhM (i.t.) followed by antiserum (i.v.).

Antiserum Group. Nine rats with a mean tumor volume of 4.9 ± 0.7 cu cm received 0.9% NaCl solution, (i.t.) followed by antiserum (i.v.).

PhM-Hydrocortisone Group. Seven rats with a mean tumor volume of 4.5 ± 0.5 cu cm were given PhM (i.t.) followed by 0.9% NaCl solution (i.v.) and in addition received an i.m. injection of 20 mg of hydrocortisone 3 hr before the PhM injection, plus a daily dose of 10 mg for 9 days.

PhM-Antiserum-Hydrocortisone Group. Ten rats with a mean tumor volume of 6.2 ± 1.0 cu cm were given PhM (i.t.) followed by antiserum (i.v.), along with the hydrocortisone treatment outlined above.

Untreated Group. Nine rats with a mean tumor volume of 4.4 ± 0.7 cu cm were observed without treatment.

"Advanced" Tumor Series

Forty-four rats with large tumors (approximately 4 times the volume of those in the previously described experiments) were treated on the 13th day after tumor transplantation. The mean of all diameter values for all tumors in the series was 2.7 cm. On the day of treatment the rats were divided into 4 groups, which did not differ significantly (p > 0.05) in mean diameter or volume of tumors. The experimental groups were as follows.

PhM Group. Seven rats with a mean tumor volume of 24.0 ± 4.6 cu cm received PhM (i.t.) followed by 0.9% NaCl solution (i.v.).

PhM-Antiserum Group. Fifteen rats with a mean tumor volume of 19.8 ± 1.7 cu cm received PhM (i.t.) followed by antiserum (i.v.).

Antiserum Group. Seven rats with a mean tumor volume of 24.6 ± 3.0 cu cm received 0.9% NaCl solution (i.t.) followed by antiserum (i.v.).

Untreated Group. Fifteen rats with a mean tumor volume of 19.7 ± 1.9 cu cm were observed without treatment.

* Values are expressed as mean ± S.E.
RESULTS

**Regular-sized-Tumor Series**

**Untreated Group** (Chart 1). All 9 untreated rats showed continuous tumor growth and died between 21 and 48 days after transplantation (mean, 32.1 ± 2.7 days). One tumor showed a temporary reduction in volume, but it rapidly resumed its former growth pattern, and the survival time of the rat was not prolonged.

**The i.m. PhM Group** (Chart 2). None of the rats showed any tissue reaction at the injection site. All tumors grew rapidly and continuously, and all rats died between 24 and 35 days after transplantation, with a mean survival time of 31.1 ± 1.1 days. This mean survival time was not significantly different (p > 0.05) from that of the untreated group. However, the mean tumor volume was significantly larger (p < 0.05) than that of the untreated group 8 to 10 days after treatment.

**PhM Group** (Chart 3). Most of the tumors showed swelling during the 1st and 2nd days after treatment. Complete tumor regression was noted in 6 of the 12 rats (in 1 rat on the 9th day, in 3 rats on the 16th day, and in 2 rats on the 24th day after treatment). These rats survived without recurrence of tumor until they were sacrificed on the 90th day after transplantation. Four rats that had a transient decrease in tumor size followed by a resumption of growth died between Days 22 and 32 after transplantation. The remaining 2 rats died on Days 22 and 25, respectively, despite a reduction in tumor size. The mean survival time of the group, 58.8 ± 9.5 days, was significantly longer (p < 0.05) than that of the untreated group or the group treated with PhM i.m. The 6 rats without complete tumor regression had no prolongation of survival time.

**PhM-Antiserum Group** (Chart 4). All 8 rats showed swelling and increased warmth in the area of the tumor on the 1st and 2nd days after treatment. By the 3rd day, all tumors were reduced to less than the pretreatment volume, and this reduction continued. Seven of the tumors disappeared completely (1 on the 5th day, 2 on the 8th day, 2 on the 9th day, and 1 on the 24th day after treatment). The rats with complete regression survived without recurrence of tumor until they were sacrificed on the 90th day after transplantation. The remaining rat showed a maximum reduction of tumor volume (78%) on the 5th day after treatment, but the tumor gradually resumed growth, and the rat died on the 64th day after transplantation. The mean survival time of the group, 86.8 ± 3.3 days, was significantly longer (p < 0.05) than that of the PhM group. The rate of complete regression was higher and occurred earlier than in the PhM group. Furthermore, the survival time of the rat with the tumor that failed to regress completely was also remarkably prolonged.

**Antiserum Group** (Chart 5). Treatment with antiserum alone did not inhibit tumor growth, and all of the rats died between 23 and 47 days after transplantation. In 3 rats, tumor growth appeared to be somewhat accelerated. The mean survival time (30.1 ± 2.4 days) and the rate of growth were not significantly different (p > 0.05) from those of the untreated group.

**PhM-Hydrocortisone Group** (Chart 6). All 7 tumors showed a temporary reduction in volume but resumed growth on the 4th to 5th day after the initiation of treatment. All of the rats died with rapidly growing tumors by the 20th day after transplantation (mean survival time, 17.7 ± 1.1 days).

**PhM-Antiserum-Hydrocortisone Group** (Chart 7). All 10 tumors were reduced in volume by the 2nd day after the initiation of treatment and reached a maximum reduction (mean percentage change, −73 ± 2.7) on the 4th day. However, all tumors began to grow rapidly between the 5th and 7th days, and the mean survival time was 18.3 ± 1.1 days after transplantation. Although the change in tumor volume was similar to that of the PhM-hydrocortisone group, the mean survival time (30.1 ± 2.4 days) was significantly longer (p < 0.05) in this group. The short survival times of the PhM-hydrocortisone and PhM-antiserum-hydrocortisone groups may be related to hydrocortisone administration.

**Advanced Tumor Series**

**Untreated Group** (Chart 8). All 15 rats showed continuous tumor growth and died between 21 and 48 days after transplantation, with a mean survival time of 32.9 ± 2.0 days.

**PhM Group** (Chart 9). Three of the 7 rats showed temporary regression of tumor, but all died from their growing tumor between 26 and 34 days after transplantation. The mean survival time, 29.9 ± 1.2 days, was not significantly different (p > 0.05) from that of the untreated group.

**PhM-Antiserum Group** (Chart 10). The growth of most tumors was temporarily suppressed but resumed 4 to 6 days after treatment. Complete disappearance of the tumor without recurrence occurred in only 1 rat (on Day 9). Five rats had a prolonged survival time (54 to 63 days after transplantation). The mean survival time of the group, 46.1 ± 4.6 days, was significantly longer (p < 0.05) than those of the other 3 groups in this series.

**Antiserum Group** (Chart 11). None of the tumors responded to treatment, and all 7 rats died between 22 and 37 days after transplantation. The mean survival time, 27.7 ± 1.7 days, was not significantly different (p > 0.05) from that of the untreated group.

**DISCUSSION**

The results of our experiments showed that the local administration of PhM followed by the systemic administra-

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Charts 8 to 11. Tumor growth curves and survival times (from day of tumor transplantation) of individual rats in each group in advanced tumor series. All cured rats were sacrificed 90 days after transplantation. Numerals, survival times in days; ○, complete regression of tumor; saline, 0.9% NaCl solution. Chart 8. Untreated group. Chart 9. PhM group. Chart 10. PhM-antiserum group. Chart 11. Antiserum group.
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tion of anti-PhM-tumor serum is able to induce complete regression of an established tumor.

Although the Walker 256 carcinosarcoma is allogeneic with the rat strain used, the growth pattern of this tumor in untreated animals indicates that the observed high incidence of regression was not due to histoincompatibility. It is also obvious that tumor regression was not caused by physical pressure due to i.t. injection of the solution, since the injection of 0.9% NaCl solution did not cause any regression in the group treated with 0.9% NaCl solution plus antiserum.

The enhancement of tumor growth noted in the i.m. PhM group suggests that the drug has an immunosuppressive potential, as has been reported recently (6, 16). The alkylating agents are most effective in blocking the inductive phase of antibody production (6). Such an effect might block the autoimmune mechanism initiated by the tumor (5, 14), thereby preventing the immunological defense mechanisms of the host from controlling tumor growth. However, PhM injected directly into tumors initiated regression rather than enhancement of tumor growth. The i.t. injection of PhM seems to prevent the immunosuppressive manifestation of the drug, possibly because the localization of PhM in the tumor tissue (28) and its rapid conjugation with this tissue prevent the occurrence of free PhM in both the tumor and the systemic circulation.

The effect of i.t. injection of the same amount of PhM per cu cm of tumor tissue was variable in the PhM group, indicating that there was no dose relationship between PhM and the regressive effect. In addition, the i.m. injection of PhM did not produce any observable tissue destruction at the injection site. These observations indicate that the tumor regression observed after i.t. PhM injection was not due merely to the destruction of tumor cells by the direct cyotoxic activity of PhM.

The tumor-regressive effect of i.t. PhM injection was clearly blocked by the addition of hydrocortisone, which is known to possess immunosuppressive activity. This result suggests that immunological mechanisms may have played a role in the tumor regression. Martin (19) observed that the therapeutic effect of another derivative of nitrogen mustard, cyclophosphamide, on cancer was also eliminated by the additional administration of cortisone, and he suggested that the effect of a chemotherapeutic agent may depend on the responsiveness of the host's immunological defense mechanisms. Thus it would seem reasonable to speculate that the i.t. injection of PhM, even without subsequent antisem administration, will initiate tumor regression through immunological mechanisms. The PhM-conjugated tumor protein will induce humoral antibodies and cellular immunity. These antibodies will be specific to the PhM, to the tumor protein, and to the PhM-tumor complex, as demonstrated in our previous studies (2), and will be able to react with the PhM-conjugated tumor proteins remaining in the tumor tissue.

The administration of antiserum in addition to i.t. PhM treatment enhanced the regressive effect remarkably in both the regular-sized and advanced tumor series. The antiserum alone had no effect. Therefore, the tumor regression observed in the PhM-antiserum group can be attributed neither to PhM alone nor to the antiserum alone, but rather to an immunological reaction between the PhM-treated tumor tissue and the passively introduced antiserum. The PhM-antiserum treatment inhibited the early resumption of tumor growth (regular-sized tumors) and also accelerated the onset of tumor regression (advanced tumors), compared with the PhM group. Thus the added antiserum appears to play a role at the early stages of tumor regression. The immune reaction occurring between the PhM-treated tumor and the passively introduced humoral antibodies might be the Arthus reaction and/or the "immunologic tissue injury" proposed by Dixon (9). The experimental conditions used in the PhM-antiserum therapy are essentially sufficient to induce a passive Arthus reaction at the tumor site. In fact, in a pilot study we observed that necrosis or hemorrhage in the tumor (or both) accompanied tumor regression in all cases. Therefore, the Arthus reaction cannot be ruled out as a key mechanism of tumor regression. Folkman (12) recently proposed that neoplastic growth may be inhibited by preventing neovascularization. Induction of the Arthus reaction at the tumor site would obstruct the blood supply to the tumor and would not only inhibit tumor growth but would also destroy established tumor tissue.

The effect of PhM-antiserum treatment was palliative in the advanced tumor series rather than curative as in the regular-sized tumor series. This result might be due to a lack of PhM conjugation with a significant portion of the tumor, since we limited the injection to 3 sites, regardless of tumor size, in order to avoid injuring the tissue by the injection itself. Perhaps regional arterial infusion of PhM followed by the antiserum injection would be more effective with large tumors.

Vaccination with antigenically modified tumor cells renders the host resistant to a subsequent challenge with isogenic tumor cells, and the induced resistance has been noted to be tumor specific (1, 25). Hamburg and Svet-Moldavsky (15) and Burke et al. (4) reported that the immune response induced against a virus or chemical can be utilized to inhibit tumor growth, although it is not tumor specific. The work of Sato and Ichimura (24) and Maltezeau et al. (18) indicates that the continued presence of antigenically altered tumor is necessary for the induction of adequate resistance. All of these responses may play a role in the regression of tumor treated with PhM and specific antiserum.

Immunotherapy with antiserum is known to have the potential danger of enhancing tumor growth (22, 27). However, no enhancement was observed with the PhM-antiserum therapy. In addition, in the same animal-tumor system, we observed that the PhM-antiserum treatment caused complete regression not only of the treated tumor but also of an untreated tumor at a distant site (unpublished data).

The results of our study indicate that an alkylating anticancer agent is capable of playing an immunological role in tumor regression. Confirmation of these findings would necessitate a change in conventional in vitro screening techniques for chemotherapeutic agents and also would alter the basic concepts underlying regional perfusion therapy. A
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combined therapy comprising the direct modification of tumor antigenicity and the administration of antiserum specific to both the modifying agent and the tumor deserves further investigation.

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