Haptenic Activity of L-Phenylalanine Mustard

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

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

SUMMARY

The immunological activity of an alkylating anticancer agent, L-phenylalanine mustard (PhM), was studied by hemagglutination, gel immunodiffusion, and counterimmunoelectrophoresis techniques. Wistar rats immunized with PhM-conjugated syngeneic normal tissue protein produced PhM-specific humoral antibodies. Rats of the same strain immunized with PhM-conjugated extract of Walker 256 carcinosarcoma produced carrier protein-, hapten-, and conjugate-specific antibodies. In comparative studies of Wistar and Fischer rats and New Zealand White rabbits immunized with PhM-conjugated extracts of Walker 256 or human tumors, a common PhM-specific antibody was detected in sera from the animals, regardless of the strain or species and regardless of the origin of the carrier protein. Intratumoral or intradermal injection of PhM that had not been conjugated with a protein moiety induced a PhM-specific antibody in Wistar rats with and without tumor. These results indicate that PhM may act against tumor by immunological as well as cytochemical mechanisms.

INTRODUCTION

Alkylating anticancer agents have been widely used in the treatment of cancer, both systemically and by local perfusion. It has been suggested that these agents have a cytotoxic effect caused by interference with DNA replication and RNA transcription (6, 18). Some clinicians have noted that regional chemotherapy of a primary tumor with an alkylating anticancer agent has been followed not only by destruction of the treated tumor but also by the disappearance of distal metastases (17). This phenomenon suggests a generalized reaction, which might be accounted for by immunological pathways. Martin (10) reported that the curative effect of the alkylating agent cyclophosphamide in tumor-bearing mice was nullified by the addition of the immunosuppressant cortisone. Further evidence supporting this concept was presented by Sato and Ichimura (12), who demonstrated the need to retain a treated tumor in situ following local infusion by nitrogen mustard N-oxide in order to obtain a curative effect and prevent distal metastases. The haptenic activity of mustard gas was discovered by Berenblum and Wormald (1) in 1939. More recently, PhM, a nitrogen mustard derivative, exhibited haptenic activity when conjugated with a heterologous carrier protein and administered with Freund’s adjuvant (2–4). These reports suggest to us that the beneficial effects of local perfusion of tumor with an alkylating anticancer agent may result not only from its direct cytotoxic effect but also from a modification of tumor antigenicity due to its haptenic activity and a subsequent augmentation of the host rejection mechanisms. If such an immunological mechanism exists, it may become necessary to reconsider the methodology of local perfusion therapy with alkylating agents. As an initial step in investigating the clinical significance of this immunological approach to chemotherapy, we must prove that an alkylating agent such as PhM possesses haptenic properties when conjugated with an autologous carrier protein. The effects of conjugating PhM with tumors and normal tissue in inbred animals were studied and compared with homologous and heterologous models.

MATERIALS AND METHODS

Alkylating Agent, Animals and Tumor

PhM was chosen as the alkylating agent, since it accumulates in tumor tissue (15) and exhibits haptenic activity when conjugated with a heterologous carrier protein (3). Inbred male Wistar and Fischer rats (Huntingdon Farms, West Conshohoken, Pa.) weighing approximately 250 g and male New Zealand White rabbits (from Ralston Purina, St. Louis, Mo.) weighing from 1.5 to 2 kg were used in these experiments. The Walker 256 carcinosarcoma was obtained from the National Cancer Institute.

Preparation of Carrier Proteins

The following sources of carrier protein were used: (a) pooled normal tissues (liver, spleen, heart, and muscle) of Wistar rats; (b) pooled normal serum of Wistar rats; (c) Walker 256 carcinosarcoma maintained in Wistar rats; (d) Walker 256 carcinosarcoma maintained in Fischer rats; (e) human esophageal squamous cell carcinoma; and (f) human colonic adenocarcinoma.

Walker 256 carcinosarcoma maintained in 3 successive rats of the same strain (either Wistar or Fischer) and surgically excised human tumor tissues were utilized. After necrotic parts were removed, the tissues were washed with

1 The abbreviation used is: PhM, L-phenylalanine mustard.
distilled water, minced, and homogenized in a high-speed homogenizer (VirTis 45; VirTis Co., Gardiner, N. Y.) at 40,000 rpm for 5 min. The homogenizer was continuously cooled with ice, and the material was allowed to stand for 1 min at the mid-point to prevent the generation of heat. The homogenate was added to 3 volumes of distilled water, stirred with a Teflon-coated magnetic stirrer at 4° overnight, and centrifuged at 2000 x g for 10 min at 4°. The supernatant was lyophilized and stored at −20°. Bacterial contamination was monitored. Pooled normal serum from Wistar rats was lyophilized directly.

Conjugation of Carrier Proteins with PhM

The lyophilized tissue extracts were conjugated with PhM by the method of Burke et al. (3). The PhM solution was prepared immediately before use. The PhM-conjugated extracts were dialyzed against phosphate buffer at 4° for 48 hr. lyophilized, and stored at −20°.

Preparation of PhM Solution for Injection

In the experiment to determine the immunological effects of PhM injection without prior protein conjugation, a more concentrated PhM solution was used. One hundred mg of PhM were dissolved in 2 ml of 0.1 N HCl, and 3 ml of 0.9% NaCl solution were added. The solution was adjusted to pH 6.6 and to a final concentration of 5 mg/ml with 0.15 M phosphate buffer, pH 8.0.

Production of Antisera

The PhM-conjugated tissue extracts, reconstituted with distilled water to a concentration of 6 mg/ml, were used as antigens. PhM-conjugated normal tissue and Walker tumor extracts derived from Wistar rat were used in syngeneic rats. A mixture of 1 ml of antigen and the same volume of complete Freund's adjuvant was injected i.m. in each gluteal region of the rat. One month later, a booster dose of 1 ml of antigen was given i.p. Blood samples were drawn by cardiac puncture 2 weeks after the last immunization. In another group of 6 Wistar rats, a blood sample was obtained 7 to 14 days after transplantation of the Walker tumor for determination of the tumor-specific antibodies as a reference to the carrier specificity of the antisera induced by PhM-Walker tumor extract. For determination of the influence of the strain of the antibody-producing animal on antibody specificity, a group of Fischer rats were immunized with PhM-Walker tumor extract derived from Fischer rat by the method described above. So that the influence of the species of the antibody-producing animal on antibody specificity could be determined, New Zealand White rabbits were given i.m. injections of a mixture of 2 ml of PhM-Walker tumor extract from Wistar rat and 2 ml of complete Freund's adjuvant. One month later, 1 ml of the antigen was given i.v. The antisera was collected 2 weeks after this injection. For a comparison of the specificity of the above antisera and that produced by the combination of heterologous animal and PhM-tumor protein, another group of rabbits were immunized in a similar manner with PhM-human tumor extracts (12 mg/ml, dry weight).

Whether PhM without protein moiety or adjuvant would induce the production of antibodies against PhM was determined in normal and tumor-bearing Wistar rats. Six normal rats received intradermal injections in the back with 0.5 ml of PhM solution (5 mg/ml) 3 times at weekly intervals. A blood sample was obtained on the 9th, 14th, 20th, or 30th day after the last injection. Five rats bearing Walker tumor received intratumoral injections of PhM solution (5 mg/ml), 0.03 ml/cm of tumor, 8 days after tumor transplantation. Blood samples were obtained from the tail vein before the injection and again 3 and 7 days afterwards.

Preparation of Antigens andAntisera for in Vitro Studies

Slightly purified extracts of rat origin were used for the in vitro tests. Each PhM-conjugated or unconjugated tissue extract, reconstituted in distilled water, was centrifuged at 3000 rpm for 10 min at room temperature to remove insoluble particles. The supernatant was lyophilized, and the concentration of each antigen was adjusted to 30 mg/ml. Because of the scarcity of material, the human tumor extracts were not subjected to this process.

For confirmation of the specificity of anti-PhM-Walker serum induced in Wistar rat, each ml of antisera was absorbed with 30 mg of lyophilized Walker tumor, normal tissue, PhM-Walker tumor, and PhM-normal tissue antigen, respectively. The mixture of antiserum and antigen was stored at 4° overnight and centrifuged at 10,800 x g for 1 hr at 4°. The supernatant was reabsorbed with 10 mg of the corresponding dried antigen by the same procedure, and the final supernatant was used as absorbed antisera. Anti-PhM-Walker serum induced in rabbit was treated by a similar procedure. Each ml of antiserum was absorbed with 30 mg of dried normal tissue antigen from Wistar rat to remove antirat antibodies. The 2 antisera induced in rabbits by PhM-human tumor extracts were absorbed with equal volumes of normal human group O serum or of the patient's own serum. The mixture of antiserum and human serum was incubated at 37° for 30 min, stored overnight at 4°, and centrifuged at 20,000 x g for 2 hr. The supernatant was re-concentrated by lyophilization to the original volume of the antisera.

In Vitro Determination of Antibody Specificity

Hemagglutination Test. Passive hemagglutination (8) was performed in duplicate for the titration of anti-PhM-Walker serum from Wistar rats. Formalinized and tanned human group O red cells were sensitized with PhM-conjugated or unconjugated extract (30 mg/ml) of Wistar normal tissue or Walker tumor by the method of McKenna (11). The pooled normal serum of Wistar rats was also studied as a control.

Gel Immunodiffusion. The agar gel microimmunodiffusion technique described by Charney and Coriell (5) was modified slightly for the determination of antibody specificity. We used 0.4% agar (Agarose; Sigma Chemical Co.,
Ammonium Sulfate Fractionation of Anti-PhM-Walker Tumor Serum. Anti-PhM-Walker tumor serum induced in Wistar rat was fractionated with 33% ammonium sulfate, pH 6.5 (16). This fraction was designated as the 33% fraction. All supernatants obtained during the above fractionation were pooled and dialyzed against 0.9% NaCl solution (changed daily) for 4 days at 4°C. The dialyzed material was fractionated with 50% ammonium sulfate, pH 6.5. The precipitate was washed with 50% ammonium sulfate and dissolved in 0.9% NaCl solution; this procedure was repeated twice. The resultant precipitate was collected by centrifugation at 1000 x g for 15 min at 4°C, dissolved in 0.9% NaCl solution, dialyzed against 0.9% NaCl solution (changed daily) for 4 days at 4°C, and lyophilized. This 2nd fraction was designated the 50% fraction.

The electrophoretic characteristics of these 2 fractions were evaluated by microzone electrophoresis (Model R-100 Microzone electrophoresis system; Beckman Instruments, Inc., Palo Alto, Calif.). Their specificities were compared by gel immunodiffusion. The concentration of each fraction and antigen was 30 mg/ml (dry weight), and 15 μm of each sample was applied.

RESULTS

Specificity of Antiserum Induced in Wistar Rat by PhM-Normal Tissue or PhM-Walker Tumor Antigen. In the hemagglutination test (Table 1), the antiserum showed the highest titer against PhM-Walker tumor extract, a moderate titer against PhM-Wistar normal tissue extract, and a lower titer against unconjugated Walker tumor extract. There was no definite titer against unconjugated extract of St. Louis, Mo.) in 0.02 M phosphate buffer, pH 7.2, and applied 10 μl samples. The established slides were leached with 0.02 M phosphate buffer, pH 7.2, for 2 hr and then with distilled water for 2 hr. The dried slides were stained with 0.25% Amido black in methanol: water: acetic acid solution (5:5:1) for 2 min and rinsed with 3 changes of 0.5% glycerol in 1% acetic acid.

Counterimmunoelectrophoresis. To correct for false negative results due to the low sensitivity of or the nonoptimal ratio of reactivity between antigen and antibody in the gel diffusion method, each antigen-antibody system was confirmed by the counterimmunoelectrophoresis technique of Gocke and Howe (7) with appropriate controls.

Anti-PhM-normal tissue serum induced in Wistar rat showed 2 common precipitation bands against PhM-Walker tumor, PhM-normal tissue, and PhM-normal serum antigens but showed no detectable reaction with the corresponding unconjugated antigens in gel immunodiffusion. Anti-PhM-Walker tumor serum from Wistar rat also showed 2 PhM-specific bands against these PhM-conjugated antigens. In addition, the anti-PhM-Walker tumor serum produced a common faint band against the unconjugated Walker tumor antigen and the PhM-Walker tumor antigen, which band appeared to differ from the PhM-specific bands (Fig. 1). Since the demonstration of this band was relatively difficult, its specificity was analyzed by counterimmunoelectrophoresis with 2-fold concentration of the antigens and antiserum. The concentrated anti-PhM-Walker tumor serum clearly showed a precipitation band against the Walker tumor antigen, but it did not react with the similarly concentrated normal tissue antigen (Table 2). Thus it appears that the anti-PhM-Walker tumor serum contained not only PhM-specific antibodies but also a weak but definite antibody against Walker 256 carcinosarcoma.

Despite the fact that the rats immunized with PhM-conjugated Walker tumor antigen produced tumor-specific antibody, as described above, the samples obtained from 6 tumor-bearing Wistar rats 7 to 14 days after transplantation showed no tumor-specific antibody in gel immunodiffusion. Figs. 2 and 3 illustrate further results of gel immunodiffusion. Two precipitation bands produced by PhM-Walker tumor antigen against anti-PhM-Walker tumor serum, by PhM-normal tissue antigen against anti-PhM-Walker tumor serum, and by PhM-normal tissue antigen against anti-PhM-normal tissue serum were common to all 3 combinations (Fig. 2). Absorption of anti-PhM-Walker tumor serum by either PhM-Walker tumor antigen or PhM-normal tissue antigen eliminated the precipitation band usually found against PhM-Walker tumor or PhM-normal tissue antigen, whereas absorption by unconjugated Walker tumor or normal tissue antigen had no obvious effect (Fig. 3). These results were confirmed by counterimmunoelectrophoresis.

Influence of Strain and Species on Specificities of Antisera Induced by PhM-conjugated Antigens. Figs. 4 to 8 illustrate the results of gel immunodiffusion performed with homologous and heterologous materials. Anti-PhM-Walker tumor serum from Fischer rat, anti-PhM-Walker tumor serum from Wistar rat, and anti-PhM-normal tissue serum from

### Table 1

<table>
<thead>
<tr>
<th>Type of extract</th>
<th>Anti-PhM-TwW* serum</th>
<th>Pooled normal serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>NW</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PhM-NW</td>
<td>1:64</td>
<td>0</td>
</tr>
<tr>
<td>TwW</td>
<td>1:32</td>
<td>0</td>
</tr>
<tr>
<td>PhM-TwW</td>
<td>1:512</td>
<td>0</td>
</tr>
</tbody>
</table>

* TwW, Walker tumor extract from Wistar rat; NW, normal tissue from Fischer rat.
Wistar rat showed a common specificity against PhM-Walker tumor antigen from Wistar rat (Fig. 4). Anti-PhM-Walker tumor serum induced in New Zealand White rabbit or Wistar rat by PhM-Walker tumor extract from Wistar rat produced a common band against the PhM-Walker tumor antigen. These bands were also common with those produced by anti-PhM-Walker tumor serum and PhM-normal tissue antigen from Wistar rat (Fig. 5). Anti-PhM-Walker tumor serum from Wistar rat showed a common specific band, not only against PhM-Walker tumor antigen and PhM-normal tissue antigen from Wistar rat but also against PhM-conjugated human adenocarcinoma extract. No reaction was evoked by unconjugated human tumor antigen or normal human serum (Fig. 6). Similar results were observed when PhM-conjugated human squamous cell carcinoma antigen was applied in place of the PhM-conjugated adenocarcinoma antigen. Rabbit antiserum induced by PhM-conjugated human adenocarcinoma showed a common specificity against PhM-human tumor antigen, PhM-Walker tumor antigen from Wistar rat, and PhM-normal tissue antigen from Wistar rat. Unconjugated Walker tumor, normal tissue, and normal serum antigens from Wistar rat did not react with the antiserum (Fig. 7). This antiserum, anti-PhM-Walker tumor serum from Wistar rat, and anti-PhM-normal tissue serum from Wistar rat produced a common precipitation band against PhM-conjugated human adenocarcinoma antigen. This precipitation band was identical to that produced between PhM-Walker tumor antigen and anti-PhM-Walker tumor serum from Wistar rat (Fig. 8). When antiserum induced by PhM-conjugated human squamous cell carcinoma was absorbed with normal human group O serum, the results were the same as those shown in Figs. 7 and 8. Absorption of the 2 anti-PhM-human tumor sera with the corresponding patient's own serum instead of normal group O serum did not alter these results.

Antiserum induced by PhM-Walker tumor antigen and PhM-adenocarcinoma antigen also clearly showed PhM specificity by counterimmunoelectrophoresis (Table 2). These results agree with those obtained by the gel immunodiffusion technique.

**Specificity of Antiserum Induced by PhM Alone.** Intradermal administration of PhM without prior protein conjugation produced PhM-specific antibody in 4 of the 6 normal Wistar rats, and its specificity was common with that of the antiserum produced by PhM-Walker tumor administered with complete Freund's adjuvant (Fig. 9). The presence of antibodies showed no relation to the time interval between the last PhM injection and blood sampling (Fig. 9). The antiserum showed common precipitation against PhM-Walker tumor antigen and PhM-normal tissue antigen from Wistar rat (Fig. 10).

The results of counterimmunoelectrophoresis of sera obtained from 5 tumor-bearing Wistar rats given an intratumoral injection of PhM alone are shown in Table 3. A positive reaction against the PhM-conjugated antigens occurred in 3 animals on Day 3 and in 4 animals on Day 7. The results suggest that these sera contain PhM-specific antibodies.

**Analysis of Ammonium Sulfate Fractionation.** The electrophoretic mobilities of anti-PhM-Walker tumor serum from Wistar rat and its 33 and 50% ammonium sulfate

### Table 2

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Adenocarcinoma extract</th>
<th>Walker tumor extract</th>
<th>Wistar rat normal tissue extract</th>
<th>Wistar rat normal serum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unconjugated</td>
<td>PhM-conjugated</td>
<td>Unconjugated</td>
<td>PhM-conjugated</td>
</tr>
<tr>
<td>Rat Anti-PhM-Walker tumor serum</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Rabbit Anti-PhM-Walker tumor serum</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Rabbit Anti-PhM-Walker tumor serum absorbed with Wistar rat normal tissue extract</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Rabbit Anti-PhM-adenocarcinoma serum</td>
<td>+</td>
<td>+</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>Rabbit Anti-PhM-adenocarcinoma serum absorbed with human Group O serum</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*Walker tumor was obtained from Wistar rats.

- - definite negative; +, definite positive; ±, very faint, indefinite positive.

- Positive by 2-fold concentrated antiserum and antigen.
Fractions are shown in Chart 1. Fig. 11 shows the difference in immunological specificity of these fractions in gel immunodiffusion. The 33% fractionation produced 2 precipitation bands against PhM-Walker tumor antigen alone, whereas the 50% fraction obtained from the supernatant of 33% fractionation produced 2 common precipitation bands against both PhM-Walker tumor antigen and PhM-normal tissue antigen. This result demonstrates that the antiserum contained not only hapten-specific antibodies but also antibodies specific to hapten-carrier conjugate itself. The 2 kinds of antibody were not identical, despite the partial sharing of some specificity. Repetition of this procedure 8 times produced an identical result each time.

**DISCUSSION**

Our findings clearly indicate that PhM has haptenic activity and is able to produce humoral antibodies. The role of PhM as a hapten rather than a complete antigen is supported by the following 3 observations. First, on immunodiffusion, PhM alone was unable to produce any precipitation against PhM-specific antisera, whereas PhM conjugated with any carrier protein produced PhM-specific precipitation against these antisera. Second, the conjugation of PhM with normal tissue proteins rendered the proteins antigenic and induced antibodies specific to PhM in syngeneic rats. Finally, analysis of anti-PhM-Walker tumor serum demonstrated the antibody which is independently specific against PhM-tumor conjugate, suggesting that PhM works as an antigen in the form of a conjugate with protein. This haptenic activity was affected by neither the origin of the carrier proteins nor the strain or species of the antibody-producing animal.

PhM was also able to produce specific antibody even without prior in vitro conjugation with a protein moiety and the use of adjuvant (Figs. 9 and 10). This result suggests the possibility of antibody induction by local perfusion with PhM alone as a therapy for cancer patients.

Antibody considered to be PhM specific was detectable as early as 3 days after intratumoral injection. This interval was too short for the induction of the hapten-specific antibody as a primary immune response. However, the tumor-bearing rats may have been primed by tumor-associated antigen before the injection of PhM, in which case the release of PhM-tumor proteins initiated by the intratumoral PhM injection may have acted as a secondary stimulus. Segal et al. (13, 14) demonstrated that the formation of antibodies to the hapten by B-cells depends upon successful prior sensitization of thymus-derived lymphocytes (T-cells) against the specific carrier. Thus the prior recognition of a tumor-associated antigen by T-cells in tumor-bearing rats may shorten the induction period of antibody against secondarily-released PhM-tumor proteins from the PhM-injected tumor tissue. Further studies are required to elucidate this point.

The analysis of anti-PhM-Walker tumor serum from Wistar rat demonstrated that it contained at least 5 different antibodies (1 specific to carrier protein, 2 specific to hapten, and 2 specific to hapten-carrier protein conjugate).

![Chart 1. Electrophoretic migration of original anti-PhM-Walker tumor serum from Wistar rat (——), 33% ammonium sulfate fraction (-----), and 50% ammonium sulfate fraction from supernatant of 33% fractionation (------).](chart1.png)

**Table 3**

| Table 3 | Results of counterimmunoelctrophoresis (specificity of sera from Wistar rats given intratumoral injections of PhM) |
|-----------------|-------------------------------------------------|-------------------------------------|-----------------|-------------------------------------|-----------------|-----------------|-------------------------------------|
| Rat            | Unconjugated | PhM-conjugated | Unconjugated | PhM-conjugated | Unconjugated | PhM-conjugated | Unconjugated |
| 121 | Preinjection | — | — | — | — | — | — |
| 122 | Preinjection | — | — | — | — | — | — |
| 124 | Preinjection | — | — | — | — | — | — |
| 129 | Preinjection | — | — | — | — | — | — |
| 140 | Preinjection | — | — | — | — | — | — |

*a* —, definite negative; +, definite positive; ±, very faint, indefinite positive.

**AUGUST 1973 1909**

The reactivity of carrier-specific antibody seemed to be weaker than that of hapten-specific antibody, and the specificity of antibody against hapten-carrier protein conjugate was not identical to that of hapten- or carrier-specific antibody.

These findings support our concept that an alkylating anticancer agent may act by an immunological as well as a cytotoxic mechanism.

With local infusion or perfusion therapy of a tumor by PhM, the agent will accumulate in the tumor tissue (3, 15), and the conjugation of PhM with the proteins in the tumor probably renders them more antigenic. Subsequently, antibodies specific against PhM, tumor protein, and the complex itself will be induced. All of these antibodies will be able to react with the PhM-treated tumor in situ because of the continued presence of PhM-conjugated tumor protein. In the absence of PhM conjugation of tumor in situ, only a weak antitumor antibody will be available to react at the tumor site.

Whether these induced immune responses will depress or enhance tumor growth is an important question which has not been answered. However, in a pilot study in which 10 inbred rats with the Walker tumor were utilized, we observed that an intratumoral injection of PhM caused partial regression of the tumor. When it was followed by an i.v. injection of anti-PhM-tumor serum, the tumor completely disappeared at a faster rate. A controlled study involving a larger number of animals is now being completed.

Further evidence that an alkylating anticancer agent may attack the tumor through immunological mechanisms has been reported by Sato and Ichimura (12). In rats with Yoshida sarcoma, the tumor-bearing extremity was amputated and the opposite extremity was fused with nitrogen mustard N-oxide. In other rats, the tumor-bearing extremity was perfused with nitrogen mustard N-oxide, and the tumor-bearing thighs were amputated at varying intervals after perfusion. The length of survival was directly related to the time that elapsed before amputation. These findings suggest that the inhibition of tumor growth was due primarily to the chemical-tumor complex remaining in situ. Recently, Maltezeanu et al. (9) reported that the inoculation of PhM-treated Jensen sarcoma cells into Wis- tar rats induced marked resistance to a subsequent challenge with the isogenic tumor cells. These authors suggest a correlation between the duration of the presence of treated cells in the inoculation site and the degree of resistance produced.

On the basis of our experiences and those reported in the literature, we would strongly urge further evaluation of alkylating anticancer agents from an immunological point of view. It is conceivable that a new therapy for cancer can be developed with this old armamentarium.

REFERENCES

Haptenic Activity of PhM

Figs. 1 to 11. Results of gel immunodiffusion.

Fig. 1. Specificity of anti-PhM-Walker tumor (Wistar rat) serum against PhM-conjugated or unconjugated extracts of Walker tumor, normal tissue, and normal serum from Wistar rat. Well 1, PhM-Walker tumor extract; Well 2, PhM-normal tissue extract; Well 3, PhM-normal serum; Well 4, normal serum; Well 5, normal tissue extract; center (C), anti-PhM-Walker tumor serum.

Fig. 2. Comparison of specificity of anti-PhM-Walker tumor serum and anti-PhM-normal tissue serum against PhM-Walker tumor extract or PhM-normal tissue extract. All samples were obtained from Wistar rats. Well 1, anti-PhM-Walker tumor serum; Well 2, anti-PhM-normal tissue serum; Well 3, anti-PhM-Walker tumor serum; Well 4, normal serum; Well 5, anti-PhM-normal tissue serum; Well 6, PhM-Walker tumor extract; center (C), PhM-normal tissue extract.

Fig. 3. Absorption test of anti-PhM-Walker tumor (Wistar rat) serum by PhM-conjugated or unconjugated extracts of Walker tumor and normal tissue from Wistar rat. Well 1, anti-PhM-Walker tumor serum, unabsorbed; Well 2, anti-PhM-Walker tumor serum absorbed with Walker tumor extract; Well 3, anti-PhM-Walker tumor serum absorbed with PhM-Walker tumor extract; Well 4, normal serum; Well 5, anti-PhM-Walker tumor serum absorbed with normal tissue extract; Well 6, anti-PhM-Walker tumor serum absorbed with normal tissue extract; center (C), PhM-Walker tumor extract.

Fig. 4. Specificity of anti-PhM-Walker tumor sera induced in Wistar and Fischer rat compared with anti-PhM-normal tissue serum from Wistar rat. Well 1, anti-PhM-Walker tumor serum (Wistar rat); Well 2, anti-PhM-Walker tumor serum (Fischer rat) induced by PhM-Walker tumor extract from Fischer rat; Well 3, normal serum (Fischer rat); Well 4, no sample applied; Well 5, normal serum (Wistar rat); Well 6, anti-PhM-normal tissue (Wistar rat); center (C), PhM-Walker tumor extract (Wistar rat).

Fig. 5. Comparison of specificity of anti-PhM-Walker serum induced in rabbit (New Zealand White) and in rat (Wistar). Well 1, anti-PhM-Walker tumor serum (Wistar rat); Well 2, anti-PhM-Walker tumor serum (rabbit) absorbed with Wistar rat normal tissue extract; Well 3, rabbit preimmune serum; Well 4, normal tissue extract (Wistar rat); Well 5, no sample applied; Well 6, PhM-normal tissue extract (Wistar rat); center (C), PhM-Walker tumor extract (Wistar rat).

Fig. 6. Specificity of anti-PhM-Walker tumor (rat) serum against PhM-conjugated extracts of human tumor, rat tumor, and rat normal tissue. Well 1, PhM-Walker tumor extract (Wistar rat); Well 2, PhM-normal tissue extract (Wistar rat); Well 3, PhM-adenocarcinoma extract (human); Well 4, adenocarcinoma extract (human); Well 5, normal tissue extract (Wistar rat); Well 6, normal human group O serum; center (C), anti-PhM-Walker tumor serum (Wistar rat).

Fig. 7. Specificity of anti-PhM-human adenocarcinoma (rabbit) serum. Well 1, PhM-adenocarcinoma extract (human); Well 2, PhM-normal tissue extract (Wistar rat); Well 3, PhM-Walker tumor extract (Wistar rat); Well 5, Walker tumor extract (Wistar rat); Well 6, Wistar rat normal serum; center (C), anti-PhM-human adenocarcinoma (rabbit) serum absorbed with pooled normal human serum.

Fig. 8. Comparison of specificity of rabbit anti-PhM-human tumor serum, rat anti-PhM-rat tumor serum, and rat anti-PhM-rat normal tissue serum. Well 1, anti-PhM-Walker tumor serum (Wistar rat); Well 2, PhM-normal tissue extract (Wistar rat); Well 3, anti-PhM-human adenocarcinoma serum (New Zealand White rabbit) absorbed with normal human group O serum; Well 4, normal serum (Wistar rat); Well 5, no sample applied; Well 6, PhM-Walker tumor extract (Wistar rat); center (C), PhM-human adenocarcinoma extract.

Fig. 9. Comparison of specificity of serum from rat given intradermal injection of PhM alone and antiserum induced by PhM-conjugated Walker tumor extract. All samples were obtained from Wistar rats. Well 1, anti-PhM-Walker tumor serum; Well 2, serum from rat given PhM alone, obtained 9 days after the last injection; Well 3, serum from rat given PhM alone, obtained 30 days after the last injection; Well 4, serum from rat given PhM alone, obtained 20 days after the last injection; Well 5, serum from rat given PhM alone obtained 14 days after the last injection; Well 6, normal serum; center (C), PhM-Walker tumor extract.

Fig. 10. Specificity of pooled serum from Wistar rats given injections of PhM alone. All samples were obtained from Wistar rats. Well 1, PhM-Walker tumor extract; Well 2, PhM-normal tissue extract; Well 3, normal serum; Well 4, normal tissue extract; Well 5, Walker tumor extract; Well 6, anti-PhM-Walker tumor serum; center (C), serum from rat given intradermal injections of PhM alone.

Fig. 11. Comparison of specificities of ammonium sulfate fractions from anti-PhM-Walker tumor (Wistar rat) serum. Fractions and extracts were of equal concentration (30 mg/ml), and 15 µl of each sample was applied. Wells 1 to 3, 50% fraction from supernatant of 33% fractionation; Well 4, original anti-PhM-Walker tumor serum; Wells 5 to 7, 33% fraction; C1, Walker tumor extract; C2, PhM-normal tissue extract; C3, PhM-Walker tumor extract.
Haptenic Activity of L-Phenylalanine Mustard

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


Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/33/8/1905