Treatment of Artificial Metastases of Methylcholanthrene-induced Rat Sarcomas by Autoimmunization of the Autochthonous Hosts

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SUMMARY
Attempts were made to demonstrate the possibility of immunotherapy for tumor metastases in the autochthonous host. Transplantation immunity against autochthonous methylcholanthrene-induced sarcomas in the rat was induced by a ligation and release method. The induced immunity varied in intensity, which suggested that the tumors varied in antigenicity even when these were induced in the same strain of rat and by the same carcinogen.

Based on the results, the tumors were classified into tumors of high, moderate, and low antigenicity (the HML classification of Takeda). Subcutaneous artificial metastases (autotransplantation) of methylcholanthrene-induced sarcomas of rats could be suppressed or rejected by intensive immunization against the primary autochthonous sarcomas. The degree of the effect was related to the immunologic characteristics of the tumor strains. With highly antigenic tumors, the most effective suppression was demonstrated in the artificial tumor metastases in the primary autochthonous host or in isoinnune rats. Moderately antigenic tumors showed moderate suppression and tumors of low antigenicity showed the least effect.

The earlier the time of immunization (ligation and release of the primary tumor) after implantation of artificial metastases, the more marked was the suppression of the metastasized tumor. The model experiments in the isologous tumor-host system showed similar results as in the autologous system. These results may suggest the possibility of the immunotherapy of human cancer, since the experiments were made in an autologous system.

INTRODUCTION
Our previous studies (13, 14) demonstrated that resistance to transplantation of methylcholanthrene-induced sarcomas can be induced in primary autochthonous hosts by a particular immunization procedure, the ligation and release method. Since the implantation of its tumor in the autochthonous host may be regarded as the establishment of an artificial metastasis, these studies concern the immunologic prevention of metastasis.

The next problem we investigated was whether the same autotransplantation immunity would be effective in causing the regression or rejection of an existing autologous tumor graft established before the immunization. This could be considered an attempt at immunotherapy of artificial metastases. Strictly speaking, metastases of cancer are the spontaneous or natural collection of cancer cells settled in organs or tissues and spread via blood or lymph from the primary tumor. The difficulties in obtaining and studying spontaneous or natural autochthonous metastases in tumors induced in experimental animals forces us to use artificial metastases, such as subcutaneous tumor grafts in primary autochthonous hosts.

It is very likely that there are some biologic differences between spontaneous and artificial metastases. However, the immunotherapy of artificial metastases may well be meaningful for the possible immunotherapy of naturally occurring metastases.

In the literature, except for some preliminary trials in human or experimental cancer (2, 3, 5, 11, 12), studies in this field have not been systematically undertaken because the presence of cancer-specific antigens in animal systems has been demonstrated only in recent years (2, 3, 7, 9, 10, 13, 14, 17).

MATERIALS AND METHODS
Animals used were male and female adult inbred rats of the Wistar/Mk and Fisher (line 344) strains, weighing 150 to 200 gm. Isoantigenic homogeneity of these strains was confirmed by reciprocal skin grafting and by testing histocompatibility factors (1) in the respective strains. Tumors were induced in 210 rats by the subcutaneous injection in the dorsal area with a pellet of 5 to 10 mg of 3-methylcholanthrene dissolved in cholesterol. Methylcholanthrene-induced sarcomas developed in 150 rats, which were used to study induction of resistance to transplantation of autochthonous tumors. The other 60 rats died of causes other than cancer or did not have tumors when experiments began.

The method of immunization was previously described in detail (14) together with the method of preparing single-cell tumor suspensions. Briefly, the methylcholanthrene-induced tumors grown to an appropriate size (20 to 30 mm in diameter) were ligated with a rubber band which was released 24 hours later. Through this procedure most of the primary tumor regressed and was gradually absorbed as a presumed antigenic stimulus over a period of 2 to 3 weeks.

The antigenicity of each tumor used in this study was determined as described in other reports (7, 14). To determine the antigenicity of the tumor, induced transplanted immunity in the autochthonous host was compared with that in the isologous host on the basis of the minimum requirement of tumor cells for a positive take. Antigenicity was graded according to the ratios of the minimum number of tumor cells for a positive...
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more, subcutaneous artificial metastases were established without exception in the autochthonous tumor-bearing hosts. However, following the immunization procedure on the primary tumor, the growth rate of metastases, as Charts 2 and 3 indicate, exhibited 3 different patterns.

In one group of tumors (A), the growth rate of the metastasis was as high as the control, despite the ligation and release of the primary autochthonous tumor. Six tumors out of twenty-three were classified in this group. In another group (B) the growth rate was moderately suppressed. Eleven tumors were classified in this group. In a third group (C), six tumors were apparently suppressed for a long period. Tumors of Groups A, B, and C were proved later to correspond to tumors of low, moderate, and high antigenicity respectively.

Utilizing this immunization procedure with a delay between implantation of the metastases and the ligation of the tumor, the suppressive effect on growth of the metastases was correlated with the degree of antigenicity of the primary tumor. Tumor growth in animals of Group A (LAT) was not only rapid, but also infiltrative. Most of them died of tumor in spite of excision or ligation of the tumor. Growth rate of tumors in immunized animals of Group B (MAT) were lower than those of controls. The immunized animals of Group C (HAT) showed strikingly inhibited growth suggesting prolonged survivals. The life span, however, could not exactly compare with that of controls because the implanted tumors were excised or ligated and the animals were used for antibody analysis (16). Although

RESULTS

Autologous System with Immunization

Host Resistance against Artificial Metastases Implanted 1–2 Weeks before Immunization. With a dose of $10^8$ cells or

![Chart 1](chart1.png)

Chart 1. Method of artificial metastasis and immunization in the autochthonous host. MC, methylcholanganthrene.

![Chart 2](chart2.png)

Chart 2. Schematic presentation of size of the artificial metastases. Artificial metastases were implanted 1–2 weeks before immunization. P, primary tumor growth; A, noninhibited growth of metastases (6 lines of tumor); B, relatively inhibited growth (11 lines of tumor); C, inhibited growth (6 lines of tumor). Growth pattern of control isograft is almost the same as A (see Chart 3).
Immunologic Treatment of Autochthonous Tumors

Chart 3. Growth curve of the artificial metastases implanted in autochthonous hosts 1–2 weeks before immunization. In the graphs of the autografts, each line is a representation of weekly measurement of tumor in each autochthonous host, and in graphs of the isografts, each line shows the average of growth of tumors implanted in one to three nonimmunized isologous rats. Animals died of tumor at the end of the period represented in the lines except in the middle and lower graphs of autografts (see text). LAT, tumor of low antigenicity; MAT, moderately antigenic tumor; HAT, highly antigenic tumor.

The marked decrease of growth of metastases in Group C was observed, the complete cure of the artificial metastases was confirmed in only one.

Immunoization Simultaneously with Implantation of Metastases. The results are summarized, in Charts 4 and 5, following the classification of tumors as of high, moderate, and low antigenicity (the HML classification) as previously described (14, 16).

In 10 animals in which low antigenicity was demonstrated, there was no inhibition of the growth rate by the ligation and release of the primary autochthonous tumor. The growth pattern of metastases was almost the same as that of the control implants of the corresponding tumor in the isologous nonimmunized host.

Of the 14 moderately antigenic tumors tested, one demonstrated no host resistance, and 6 cases produced some degree of host resistance, as indicated by slower growth of the artificial metastases. In 3 of the 7 remaining cases, the growth of artificial metastases was more decisively inhibited for a prolonged period. In the four remaining tumors, despite the fact that the metastatic implants apparently grew to 4–10 mm in diameter in 1–2 weeks, they regressed rapidly and completely thereafter.

The control implants in isologous nonimmunized hosts formed tumors with 20-mm diameters in 2 weeks without exception.

In the case of the 12 tumors of high antigenicity, growth of metastases was markedly inhibited in two cases for about 20 days. In the 10 remaining cases, the growth of the artificial metastases was restricted to 5 mm or less, followed by complete regression within two weeks from the time of immunization by the ligation and release of the primary tumor in autochthonous host. In some cases no macroscopic growth of the metastatic implants was observed from the beginning of implantation in autochthonous hosts. Repeated further attempts were made to implant subcutaneous, intraperitoneal, and intravenous (10⁶ tumor cells) artificial metastases in these highly immune autochthonous animals with no growth observed.

It was possible in all cases to implant these highly antigenic tumors in isologous animals if no ligation procedure was carried out. The implanted tumors in nonimmunized isologous animals showed steady growth, no regression, and killed the animals 20 to 50 days after implantation.

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Chart 5. Growth curve of artificial metastases implanted in the autochthonous hosts simultaneously with immunization (ligation and release of the primary induced tumor). In curves of the autografts, one line shows growth of one tumor in one autochthonous host, and in curves of the isografts, one line shows the average of growth of tumors implanted in one to two nonimmunized isologous rats. Each line is a representation of weekly measurement of the tumor of each animal. Each of the lines of control isografts means death of an animal with tumor. However, most of the autografted tumors were either ligated or excised or completely rejected at the end of the line. LAT, tumor of low antigenicity; MAT, moderately antigenic tumor; HAT, highly antigenic tumor.

Immunization in an Isologous System

Following the results of the foregoing experiments in an autologous system, the effects of immunization on artificial metastases were investigated in detail in an isologous system using three methylcholanthrene-induced sarcomas with various HML antigenicities at their 2nd to 10th generations.

WMT 69 cells, which are highly antigenic, were implanted to establish a tumor in isologous rats as a model of primary tumor with a trocar implant. When this first implant grew to 20-30 mm in diameter, it was treated by the immunization, ligation, and release procedure. Subcutaneous artificial metastases (10⁵ cells) were established simultaneously with the first implantation in 2 animals (A) and 4 days before the start of immunization in 5 animals (B). As presented in Chart 6, the two artificial metastases in Group A showed slow growth for about 14 days and then regressed completely within 30 days after the beginning of ligation and release of the first implant tumor. In Group B, the host resistance induced by immunization was effective in producing rapid regression of the artificial metastases. With simultaneous implantation of tumors (C), out of 9 animals, 8 showed a complete regression of metastases which had grown to 7 mm in diameter. In the other case, the inhibitory effect was not sufficient to produce a complete regression of the artificial metastases, but still reduced the growth rate. The implants set up after the start of immunization (D) did not form any visible tumors in any of the four animals tested. The control implants were made in 4 animals in each experiment and showed constant rapid growth without regression, followed by death of the hosts.

WMT 32, a moderately antigenic tumor, and WMT 56, a tumor of low antigenicity, were investigated in the same way as the above.

The results obtained with WMT 32 are shown in Chart 6. Growth of artificial metastases in 2 animals established simultaneously with the first implantation (A) and in 11 animals established 4 days before the start of immunization (B) did
not differ from the growth of the implants in nonimmunized isologous control rats. In contrast, when the artificial metastases in 6 animals were implanted simultaneously with the start of immunization (C), the tumors in 5 animals showed a sudden regression and complete cure after a period of 7 days of progressive growth. The implants in 4 animals after the immunization (D, E) did not form any tumor growth. Some of the animals previously immunized with WMT 69 were injected intravenously or intraperitoneally with approximately 10^8 cells of WMT 69 with no growth observed.

Chart 6 presents the results obtained with WMT 56, a tumor of low antigenicity. The artificial metastases in 9 animals established simultaneously with the first implants (A) showed almost the same pattern of growth as that of the control implants. Essentially the same growth pattern was observed in the artificial metastases in 3 animals implanted 13 days before the start of immunization (B) and in 10 animals implanted simultaneously with the start of immunization (C). However, the artificial metastases in 3 animals established some days after the start of immunization (D) showed slow growth for a long period when compared with control animals. There was a complete regression of the metastasis only in one case.

DISCUSSION

Experimental immunologic treatment of the primary autochthonous tumors has been rarely reported. Haddow and Alexander (5) reported the effect of active immunization with irradiated autochthonous tumor tissue on the primary tumor. Alexander (2) reported the arrest of the growth of primary chemically-induced sarcoma in the rat by transfer of sensitized thoracic duct lymphocytes.

Our results have shown that to a certain extent it is possible to treat metastases of the primary autochthonous tumors in rats with immunotherapy. Further, the efficacy of such treatment was directly correlated with the antigenicity of the tumor tested. In the case of tumors of low antigenicity, the autoimmunity induced by the immunization procedure of ligation and release did not markedly affect the artificial metastases. The moderately antigenic tumors induced host resistance which was capable of producing inhibition or complete regression of existing artificial metastases in the autochthonous host if the immunization procedure was carried out simultaneously with or immediately after the establishment of metastases. The host resistance induced by highly antigenic tumors caused a complete regression or failure of take of the artificial metastases in the autochthonous host when the immunization procedure was carried out at the same time or just after the establishment of metastases. Once the tumor is well established in the host, even in case of highly antigenic tumor, it is difficult to cause a complete regression, although the growth rate is decreased by immunotherapy. The earlier the time of immunization after implantation of artificial metastases, the more striking was the suppression of the metastasized tumor.

In the isologous system, the results enable us to conclude that the degree of host resistance induced by ligation and release of the first tumor graft is dependent upon the degree of antigenicity of the tumor utilized, as was shown in the experiments in an autochthonous system. Significant differences between treated and control animals were observed if the metastasis was established shortly before immunization.

Since this experiment was designed to use an autologous tumor-host system, one might draw an analogy between animals used in the experiment and human cancer patients. Although no antigenicity specific to cancer has really been proved in human patients, suggestive results have been shown in some clinical experiments (Southam et al. 11, 12). Everson and Cole (4) also reported spontaneous regressions of cancer which might be caused by the immunologic resistance in cancer patients.

The results of the present paper may suggest that the possibility of immunologic treatment of metastases of human cancer exists, if we can choose patients with "highly antigenic" cancers and use some suitable immunization procedure.

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