Immune-mediated Arrest and Reversal of Established Visceral Metastases in Athymic Mice

Robert H. Wiltrout, Philip Frost, Murdoch K. Morrison, and Robert S. Kerbel

Department of Immunology and Microbiology, Wayne State University School of Medicine, Detroit, Michigan 48201

ABSTRACT

The metastasizing MDAY-D2 tumor of DBA/2 mice disseminates in BALB/c allogeneic athymic nude (nu/nu) mice in a manner identical to that observed in the syngeneic host. Both the kinetics and organ distribution pattern of metastases from s.c. implants of MDAY-D2 are routinely predictable at any given tumor dose. BALB/c heterozygote (nu/+ ) litter-mates reject MDAY-D2 grafts on the basis of the multiple minor histocompatibility differences that exist between DBA/2 and BALB/c mice. The in vitro cell-mediated cytotoxic response detected in tumor-bearing BALB/c nu/+ mice is "low grade" (isotope release is ~40 to 50% by 24-hr 111-indium-8-hydroxyquinoline assay and ~6 to 8% by 6-hr 51Cr assay) and yet correlates directly with tumor rejection. BALB/c nu/nu mice can be protected against MDAY-D2 by previous reconstitution with lymphoid cells from normal or MDAY-D2-sensitized BALB/c nu/+ mice. In addition, surgically documented, established visceral metastases in BALB/c nu/nu mice can be arrested and regressed by the adoptive transfer of MDAY-D2-sensitized BALB/c nu/+ spleen cells. This represents one of the few models where established metastases have been immunotherapeutically regressed. As such, the MDAY-D2 BALB/c nu/nu mouse model offers unique advantages for studying the role of the immune system in regulating the metastatic process.

INTRODUCTION

The role of the immune response in the inhibition and/or facilitation of tumor growth has been extensively studied (17, 26, 30). Most of these studies have been concerned with effects of the immune response on experimentally induced primary tumors. By comparison, relatively little has been done to evaluate the interaction of the immune system with either actively disseminating tumors or secondary tumor growths that are anatomically distant from the site of the primary tumor. Aside from the questions surrounding the origin of neoplasms, the phenomenon of metastasis is perhaps the most fundamental problem in contemporary oncology. Recent evidence from a number of laboratories has indicated that the immune response may play a role in determining whether metastases occur. This subject was recently discussed by Fidler (9) and by Sugarbaker and Ketcham (33). However, as Fidler et al. (10) have noted, while there appears to be a relationship between the host immune response and the dissemination of metastases, the nature of this interaction is currently unclear.

A major problem in the study of metastases has been the scarcity of good experimental models with which to study the metastatic process. Unlike their human counterparts, tumors of experimental animals often do not metastasize, metastasize only to local draining PLN, metastasize only to selective organs (usually the lung), or metastasize relatively late, when the animal is already in danger of succumbing to the primary tumor growth (9, 21, 23, 33). Most studies of metastasis in mice have been performed using one of 2 available artificial metastasis models, the B-16 melanoma and Lewis lung carcinoma of C57BL/6 mice. The B-16 melanoma system has contributed significantly to our understanding of the mechanisms of blood-borne tumor metastases (6-8) and of the predilection that some tumors have for selective metastasis to specific organs (11). The Lewis lung carcinoma (34) metastasizes to the lungs and has been used to evaluate the effects of various drugs on metastasis. A variety of tumors which metastasize primarily to draining lymph nodes, to isolated visceral organs, or only after i.v. injection have been described (9, 10, 23, 33). Recently, Kerbel et al. (18) have described a tumor in DBA/2 mice which offers advantages over other metastatic tumor models. This tumor, designated MDAY-D2, metastasizes from s.c., i.p., or i.d. sites to most visceral organs in a strikingly consistent manner. The reproducibility of the dissemination of this tumor offers a unique opportunity to study the kinetics of host responses as they relate to metastasis.

Congenitally athymic nude (nu/nu) mice have proven to be valuable research tools in tumor immunology. However, their usefulness in the study of metastasis has been limited because most tumors, which normally metastasize in their syngeneic host, grow but either fail to metastasize or metastasize poorly in nu/nu mice. This has been documented in syngeneic (2, 10, 31), allogeneic (10), and xenogeneic (13, 14, 29) tumor graft models. Even the highly metastatic variant lines of B-16 melanoma (8), when injected i.v. into nu/nu mice, metastasize poorly, if at all (10).

In this paper, we show that MDAY-D2 has the same kinetics and distribution of metastasis in BALB/c nu/nu mice as it has in the syngeneic DBA/2 host. The ability of MDAY-D2 to metastasize in nu/nu mice makes this model useful in evaluating potential applications of immunotherapy to metastasis. This report describes the use of the MDAY-D2 nu/nu mouse model to demonstrate that a specific immune response will

1 This work was supported in part by NIH Grant CA 16426 and the Harper Hospital Medical Staff Trust Fund.
2 To whom requests for reprints should be addressed, at Department of Immunology and Microbiology, Wayne State University School of Medicine, 540 East Canfield Avenue, Detroit, Mich. 48201.
3 Current address: Department of Medicine, Veteran's Administration Hospital, 5901 E. Seventh Street, Long Beach, Calif. 90822.
4 Department of Pathology, Queen's University, Kingston, Ontario, Canada.

Received July 5, 1979; accepted July 10, 1979.

The abbreviations used are: PLN, peripheral lymph nodes; i.d., intradermal; OMCI, cell-mediated cytotoxicity; "111InOx; 111-indium-8-hydroxyquinoline (oxine) complex; RPMI-1640 medium, Roswell Park Memorial Institute Tissue Culture Medium 1640; FCS, fetal calf serum; PS, penicillin (100 units/ml) and streptomycin (100 µg/ml); Con A, concanavalin A; SR, specific release; TAA, tumor-associated antigen; i.i., intrasplenic.
immunocompetent allogeneic mice (18). In short, MDAY-D2 exhibits no unusual H-2 or non-H-2 antigenic properties and behaves as other transplantable murine tumors, with the notable additional attribute of enhanced metastatic potential.

Other tumors used in these studies include the P815 mastocytoma of DBA/2 mice, the EL-4 and RBL-5 tumors of C57Bl/6 mice, and the SS-5 methylcholanthrene-induced tumor of BALB/c mice which was developed in our laboratory. The A strain tumor, YAC-1, was kindly supplied by Dr. Ronald Herberman (NIH, Bethesda, Md.). All tumors were maintained in ascitic form in vivo or by in vitro culture. The viability of cell suspensions was determined by trypan blue dye exclusion.

Radioisotopes. $^{111}$InCl$_3$ (specific activity, 2400 mCi/mg) was purchased from Medi-Physics, Inc., Emeryville, Calif.

Labeling Procedures. The procedure for the labeling of tumor cells with $^{111}$In has been reported previously (12, 36). Prior to use, $^{111}$In was complexed with 8-hydroxyquinoline in a brief chelation procedure to form the $^{111}$InOx complex (35). We have modified this procedure as follows. The $^{111}$InCl$_3$ (3 mCi in 1.5 ml) was transferred into a 16- x 125-mm glass tube, to which an equal volume of sterile distilled water was then added. At this point, 0.2 ml sodium acetate buffer, pH 5.0, was added to the tube, followed by the addition of 15 µl 8-hydroxyquinoline (10 mg/ml in absolute ethanol). The tube was vortexed vigorously and allowed to stand for 15 min at room temperature; 3 ml of chloroform were then added, and the contents of the tube were thoroughly mixed. The water layer was removed, and the chloroform was evaporated to dryness in a boiling water bath.

The $^{111}$InOx was dissolved by the addition of 100 µl absolute ethanol, followed by 1.5 ml 0.9% NaCl solution and then by 10 µl of 1 N HCl. This mixture was warmed, mixed well, and allowed to cool. The activity of the $^{111}$InOx was calculated by comparing the activity of a 10-µl sample of the finished $^{111}$InOx with the activity in a 10-µl aliquot of the original $^{111}$InCl$_3$. Chelation of the $^{111}$InOx required 30 to 45 min, and the $^{111}$InOx was routinely used over a 2-week period. Once the $^{111}$InOx was prepared, cell labeling was extremely simple and rapid. Cells were labeled with 20 to 30 µCi $^{111}$InOx at a concentration of 10$^4$ viable cells per 0.5 ml of RPMI-1640 medium supplemented with 10% FCS and PS for 15 min at 37°. After labeling, the cells were washed 3 times in 15 ml of medium and resuspended to an appropriate concentration.

Mitomycin C Treatment. All tumor cells used as inhibitors of CMC were treated with mitomycin C by incubating 10$^7$ cells with 100 µg mitomycin C per ml in 2 ml RPMI-1640 medium plus 20% FCS for 30 min at 37°. After treatment, the cells were washed 3 times and resuspended to the appropriate volume. Mitomycin C treatment was performed to eliminate differing cell cycle lengths of the various inhibiting cells as an experimental factor.

Con A-induced Lymphoblasts. Lymphoblasts were derived from DBA/2 spleen cells by incubating 2.5 x 10$^6$ cells/ml with Con A (3 µg/ml; Calbiochem-Behring Corp., La Jolla, Calif.) in RPMI-1640 medium plus 10% FCS plus PS for 72 hr at 37° and 5% CO$_2$. Cultures were performed in upright 25-ss cm tissue culture flasks (Falcon Plastics, Oxnard, Calif.). After incubation, the cells were harvested and washed 3 times prior to use as inhibitors. Lymphoblasts were selected as the normal cell control because their size closely approximates the size of
the tumor cells used as inhibitors of CMC and eliminates cell size as an experimental variable.

**Assessment of Metastasis.** The presence of metastases in visceral organs was confirmed in 2 ways at different times after s.c. injection of $10^5$ MDAY-D2 tumor cells into nu/nu mice. First, visceral organs and PLN were examined for grossly visual metastatic foci. Second, livers and spleens which exhibited no macroscopic tumor foci were examined microscopically for histological evidence of tumor cells.

**Microcytotoxicity Assay for CMC.** For studies of CMC by BALB/c mice in response to MDAY-D2, we injected $5 \times 10^5$ tumor cells s.c. and assayed for CMC at various times. Both spleens and pooled ipsilateral, axillary, and brachial PLN were assayed for CMC. Since both the BALB/c and the DBA/2 are $H-2$ identical ($H-2^d$) and since MDAY-D2 retains its $H-2^d$ antigens alone, the CMC observed in this system is directed against minor histocompatibility antigens.

CMC was measured by a modification of the isotope release method described by Brunner et al. (3) and involved the use of $^{111}$InOx rather than $^{51}$Cr as the releasable isotopic label (36). $^{111}$InOx has been shown to offer several advantages over $^{51}$Cr in long-term cytotoxicity assays (12, 36). Since we were attempting to demonstrate reactivity (i.e., CMC) against minor histocompatibility antigens, relatively long incubation periods were required to achieve significant levels of lysis.

Effector cells for the cytolytic assay were prepared by excising spleens and draining PLN from either immunized or normal BALB/c mice. In some instances, nu/+ littermates of the BALB/c nu/nu mice were used as a source of immunized or normal cells. There were no apparent differences in the CMC responses of BALB/c or BALB/c nu/+ mice in any of our studies. Single cell suspensions were prepared by teasing the spleens or PLN in RPMI-1640 medium. Lymphocytes were washed 3 times in medium and resuspended to a concentration of $10^7$ viable cells/ml in RPMI-1640 medium plus 10% FCS plus PS. MDAY-D2 target cells were obtained by aspiration of ascites from the peritoneal cavity of tumor-bearing DBA/2 mice, washed twice in medium, and resuspended to the appropriate volume for $^{111}$InOx labeling.

The cytolytic assay was performed in round-bottomed microplates (Falcon Plastics). Lymphocytes and $^{111}$InOx-labeled MDAY-D2 target cells were incubated together in triplcate in a total volume of 0.2 ml RPMI-1640 medium plus 10% FCS plus PS for 24 hr in 5% CO$_2$ at 37°C. Effector:target cell ratios of 100:1 were used, with target cells used at $5 \times 10^3$ $^{111}$InOx-labeled MDAY-D2 cells per microwell. Immediately prior to incubation, the microplates were centrifuged at 300 rpm for 3 min.

After incubation, assays were terminated by centrifugation at 1200 rpm for 8 min at room temperature. At this point, 100-µl aliquots of the supernatant were removed from each microwell for counting in a Beckman Model 300 gamma counter. Correction for ongoing radioactive decay was made by counting control tubes first, with the result that all data are slightly conservative.

The percentage of SR of $^{111}$InOx was calculated by the following formula,

$$SR = \frac{RSL - RNL}{0.8 \times TC} \times 100$$

where RSL is isotope release in the presence of sensitized lymphoid cells, RNL is isotope release in the presence of normal lymphoid cells, and TC is total incorporated counts/minute. The releasable radioactivity for $^{111}$InOx has been shown to be 80% of the incorporated radioactivity (36).

**Inhibition of CMC Method.** Studies on the specificity of the CMC reaction were performed by using a modification of the inhibition of cytotoxicity assay described previously by Ortiz de Landazuri and Herberman (24). Spleen cells from BALB/c mice sensitized 14 days previously were preincubated with different, unlabeled tumor cells for 15 min at 37°C in 100 µl of RPMI-1640 medium plus 10% FCS plus PS. After this preincubation, the $^{111}$InOx-labeled MDAY-D2 cells were added to each microwell, and a standard 24-hr $^{111}$InOx release assay was performed. The effector:target cell ratio was 100:1, and the inhibitor:target cell ratio was 25:1.

**Statistical Analyses.** Statistically significant differences in in vitro CMC specificity experiments were detected by performing a one-way analysis of variance. Levels of significance between individual experimental groups were calculated by application of Duncan's multiple-range test (5).

**Adoptive Transfer System.** Various doses of thymic and splenic lymphocytes from both normal and MDAY-D2-immunized BALB/c nu/+ mice were injected i.v. into nu/nu mice. At varying times after reconstitution, these animals were challenged s.c. with $10^4$ MDAY-D2 cells and monitored for survival.

**Experimental Design for Demonstrating the Immune Regression of Established Metastases.** BALB/c nu/nu mice were given s.c. injections of $10^4$ viable MDAY-D2 cells. Twelve to 15 days later, tumor-bearing mice were reconstituted by i.v. injections of $5 \times 10^5$ splenocytes. The donor lymphocytes were obtained from BALB/c nu/+ mice which had rejected an s.c. injection of $5 \times 10^5$ MDAY-D2 cells given 14 days prior to lymphocyte transfer. All tumor-bearing nu/nu mice were examined under anesthesia (40 mg sodium pentobarbital per kg i.p.) for the presence of established, macroscopic hepatic metastases on Day 16 post-tumor injection. The presence of metastatic foci was ascertained by making a 1- to 2-cm transverse incision in the abdominal wall. The ventral surface of the liver was then viewed through the intact peritoneal membrane, and the presence or absence of hepatic metastases was noted. The incision was closed, and all mice were then monitored for survival. Survivors were sacrificed and autopsied on Day 45 post-tumor challenge.

**RESULTS**

**Kinetics of MDAY-D2 Metastasis in Athymic Mice.** BALB/c nu/nu mice were given s.c. injections of $10^4$ MDAY-D2 cells. At intervals after injection, groups of mice were sacrificed and examined for the presence of gross metastatic foci in the liver, spleen, lymph nodes, kidney, and lung. In addition, liver sections were examined microscopically to determine when metastatic tumor microfoci become established in the liver. Table 1 demonstrates the progression of metastasis to PLN and visceral organs. Quite often, microscopic metastases appear first in the draining ipsilateral lymph nodes. The first visceral organ to show evidence of gross metastases is almost always the liver. Liver metastases generally appeared in all mice by Day 13 and progressed from a few discrete small foci on Day...
was assumed that the death of the mice resulted primarily from discrete large nodules, while lung metastases appeared as hemorrhagic foci. Metastases in the kidney were usually observed as a few microscopic lesions, while lung metastases appeared as hemorrhagic foci. Histological preparations of liver sections revealed that microfoci of tumor are evident as early as 3 days after s.c. tumor injection. The peak specific isotope release was 40 to 50% (Chart 1), while the 4 to 6 hr 51Cr assays (performed with the same effector cells) detected a specific isotope release of 6 to 8%. Since BALB/c mice have the same major H-2 as DBA/2 (H-2b), mice from which MDAY-D2 originated, it was assumed that rejection of the tumor was based on numerous minor histocompatibility differences between BALB/c and DBA/2 mice. The CMC reaction was completely abrogated by treatment of effector spleen cells with anti-γ serum and complement.

Specificity of BALB/c Anti-MDAY-D2 CMC Response. To support the idea that the specificity of the BALB/c CMC response was directed at DBA/2 minor histocompatibility antigens, we performed an experiment for inhibition of cytotoxicity (Table 2). The only cells to inhibit the BALB/c anti-MDAY-D2 CMC response were MDAY-D2, the syngeneic DBA/2 P815 mastocytoma, and Con A-induced DBA/2 lymphoblasts. All of these cells express DBA/2 minor histocompatibility antigens. The minimal inhibition of cytotoxicity observed with the allogeneic C57BL/6 inhibitor cells, EL-4 and RBL-5, was attributed to a dilutional effect, since the inhibitor:target cell ratio was 25:1. This level of inhibition is considered background. The BALB/c tumor, SS-5, did not inhibit above background, even though it bears the same H-2 as the MDAY-D2, supporting the conclusion that the specificity of the BALB/c MDAY-D2 CMC response was directed at DBA/2 minor histocompatibility antigens. We cannot absolutely rule out CMC reactivity to TAA; however, it is unlikely that 2 DBA/2 tumors of highly different etiology, P815 and MDAY-D2, share the same or cross-reactive TAA. Additionally, successful inhibition of the CMC response by normal DBA/2 cells (Con A blasts) provides strong evidence that the antigenic specificity of the CMC response is to normal DBA/2 minor histocompatibility antigens and not to TAA.

### Table 1

<table>
<thead>
<tr>
<th>Metastasis evaluation (days post-tumor injection)</th>
<th>Gross</th>
<th>Microscopic (liver)</th>
<th>Tumor wt (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>Spleen</td>
<td>PLN</td>
<td>Kidney</td>
</tr>
<tr>
<td>5</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
</tr>
<tr>
<td>7</td>
<td>0/5</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td>11</td>
<td>0/4</td>
<td>0/4</td>
<td>1/4</td>
</tr>
<tr>
<td>13</td>
<td>5/5</td>
<td>2/5</td>
<td>2/5</td>
</tr>
<tr>
<td>15</td>
<td>4/4</td>
<td>4/4</td>
<td>3/4</td>
</tr>
<tr>
<td>18</td>
<td>4/4</td>
<td>4/4</td>
<td>3/4</td>
</tr>
<tr>
<td>20</td>
<td>3/3</td>
<td>3/3</td>
<td>3/3</td>
</tr>
</tbody>
</table>

^2 Groups of animals were sacrificed at increasing periods of time after tumor injection. Gross metastasis was observed macroscopically. Sections of liver were prepared for microscopic analysis. Results expressed as number of animals per total number of animals in each group.

^1 Mean ± S.D.

Kinetics of the Anti-MDAY-D2 CMC Response in BALB/c Mice. The s.c. injection of MDAY-D2 tumor cells into BALB/c or BALB/c nu/nu mice resulted in tumor growth which reached maximum size at Day 8, only to be completely rejected by Day 18. The CMC response, as measured by isotope release, was relatively weak. In the 111InOx 24-hr CMC assay, the peak specific isotope release was 40 to 50% (Chart 1), while the 4 to 6 hr 51Cr assays (performed with the same cytolytic cells) detected a specific isotope release of 6 to 8%. Since BALB/c mice have the same major H-2 as DBA/2 (H-2b), mice from which MDAY-D2 originated, it was assumed that rejection of the tumor was based on numerous minor histocompatibility differences between BALB/c and DBA/2 mice. The CMC reaction was completely abrogated by treatment of effector spleen cells with anti-γ serum and complement.

Specificity of BALB/c Anti-MDAY-D2 CMC Response. To support the idea that the specificity of the BALB/c CMC response was directed at DBA/2 minor histocompatibility antigens, we performed an experiment for inhibition of cytotoxicity (Table 2). The only cells to inhibit the BALB/c anti-MDAY-D2 CMC response were MDAY-D2, the syngeneic DBA/2 P815 mastocytoma, and Con A-induced DBA/2 lymphoblasts. All of these cells express DBA/2 minor histocompatibility antigens.
A second point to note from Table 2 is that the A strain tumor, YAC-1, which is H-2<sup>dr</sup> does not inhibit the CMC response. The lack of inhibition by an H-2<sup>dr</sup>-bearing tumor supports the contention that MDAY-D2 bears no detectable H-2<sup>dr</sup> antigen (18).

Immune Reconstitution of Nude Mice and Their Protection from an MDAY-D2 Challenge. Experiments were performed to determine whether BALB/c nu/nu mice could be protected from MDAY-D2 challenge by reconstitution with BALB/c nu/nu/+ lymphocytes from various sources (Table 3). Nu/nu mice reconstituted with either 10<sup>5</sup> or 5 x 10<sup>7</sup> lymphocytes from the spleen or thymus of sensitized or untreated BALB/c mice were challenged s.c. 10 days later with 10<sup>4</sup> MDAY-D2 cells. The most effective adoptive immunization occurred after the transfer of sensitized BALB/c nu/+ spleen cells, which completely protected the nu/nu recipients at a dose as low as 10<sup>7</sup> spleen cells/recipient. The protective effect of sensitized BALB/c spleen cells was expected, since previous experiments had documented a cytotoxic response in BALB/c mice during the rejection of MDAY-D2 (Chart 1). Of additional interest were mice receiving normal or sensitized thymic cells. Most mice adoptively immunized with either 5 x 10<sup>7</sup> normal thymus cells or 10<sup>7</sup> sensitized thymus cells developed large tumors within 10 days of tumor challenge. However, 67% of these mice eventually rejected these large tumors and survived. The peak size of tumor growth in these 2 groups was 10 times the size at which unreconstituted nu/nu mice develop metastases and die (6). The protection afforded by sensitized thymic cells was greater than that achieved by normal thymus cells. While this was not a striking difference, it could have been due to the transfer of small lymph nodes along with the thymus during reconstitution.

Immune Regression of Gross Visceral Metastases. To investigate directly whether the immune system could reject widely disseminated neoplastic disease, we performed the experiments described in Table 4. On Days 12, 13, and 15 after receiving s.c. injections of 10<sup>4</sup> MDAY-D2 cells, mice were adoptively immunized by the i.v. injection of 5 x 10<sup>7</sup> BALB/c nu/+ spleen cells from mice immunized 14 days earlier with 5 x 10<sup>7</sup> MDAY-D2 cells (Experiment 1). As each group of mice reached Day 16 post-tumor injection, they were anesthetized and surgically examined for the presence of tumor foci on the ventral surface of the liver. In this manner, we were able to follow individual mice with known liver metastases. Table 4 demonstrates that all control nu/nu mice died by Day 21.

### Table 2 Antigenic specificity of the BALB/c anti-MDAY-D2 CMC response

<table>
<thead>
<tr>
<th>Inhibiting cell</th>
<th>H-2 haplotype</th>
<th>% of &lt;sup&gt;111&lt;/sup&gt;InO&lt;sub&gt;4&lt;/sub&gt; SR</th>
<th>% of inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>H-2&lt;sup&gt;dr&lt;/sup&gt;</td>
<td>11.6 ± 2.0</td>
<td>69.0&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>MDAY-D2</td>
<td>H-2&lt;sup&gt;dr&lt;/sup&gt;</td>
<td>9.2 ± 0.3</td>
<td>75.5&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>PB15</td>
<td>H-2&lt;sup&gt;dr&lt;/sup&gt;</td>
<td>32.3 ± 2.3</td>
<td>13.9</td>
</tr>
<tr>
<td>EL4</td>
<td>H-2&lt;sup&gt;dr&lt;/sup&gt;</td>
<td>33.7 ± 2.4</td>
<td>10.1</td>
</tr>
<tr>
<td>RBL-5</td>
<td>H-2&lt;sup&gt;dr&lt;/sup&gt;</td>
<td>30.3 ± 2.2</td>
<td>19.2</td>
</tr>
<tr>
<td>SS-5</td>
<td>H-2&lt;sup&gt;dr&lt;/sup&gt;</td>
<td>32.3 ± 2.1</td>
<td>11.5</td>
</tr>
<tr>
<td>YAC-1</td>
<td>H-2&lt;sup&gt;dr&lt;/sup&gt;</td>
<td>13.1 ± 2.1</td>
<td>65.1&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> All inhibiting cells were treated with mitomycin C. DBA/2 lymphoblasts were obtained by Con A stimulation.

<sup>b</sup> Mean ± S.D.

<sup>c</sup> Significantly greater than all other inhibitors of non-DBA/2 origin (p < 0.01) but not significantly different from other cells of DBA/2 origin. Calculated by Duncan's multiple-range F test.

### Table 3 Survival of reconstituted nu/nu mice after injection of 10<sup>4</sup> MDAY-D2 cells

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Treatment</th>
<th>No. of cells</th>
<th>Day 18</th>
<th>Day 21</th>
<th>Day 29</th>
<th>Day 35</th>
</tr>
</thead>
<tbody>
<tr>
<td>nu/nu</td>
<td>None</td>
<td>6/6</td>
<td>3/6</td>
<td>0/6</td>
<td>0/6</td>
<td></td>
</tr>
<tr>
<td>nu/nu</td>
<td>NT&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10/7</td>
<td>6/6</td>
<td>4/6</td>
<td>1/6</td>
<td>0/6</td>
</tr>
<tr>
<td>nu/nu</td>
<td>ST</td>
<td>10/7</td>
<td>6/6</td>
<td>5/6</td>
<td>4/6</td>
<td>4/6</td>
</tr>
<tr>
<td>nu/nu</td>
<td>NS</td>
<td>10/7</td>
<td>5/5</td>
<td>3/5</td>
<td>3/5</td>
<td>3/5</td>
</tr>
<tr>
<td>nu/nu</td>
<td>SS</td>
<td>10/7</td>
<td>6/6</td>
<td>6/6</td>
<td>6/6</td>
<td>6/6</td>
</tr>
<tr>
<td>nu/+</td>
<td>None</td>
<td>10/7</td>
<td>8/8</td>
<td>8/8</td>
<td>8/8</td>
<td>8/8</td>
</tr>
<tr>
<td>nu/nu</td>
<td>NT</td>
<td>5 x 10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>6/6</td>
<td>6/6</td>
<td>4/6</td>
<td>4/6</td>
</tr>
<tr>
<td>nu/nu</td>
<td>ST</td>
<td>5 x 10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>6/6</td>
<td>4/6</td>
<td>4/6</td>
<td>4/6</td>
</tr>
<tr>
<td>nu/nu</td>
<td>NS</td>
<td>5 x 10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>5/5</td>
<td>5/5</td>
<td>5/5</td>
<td>5/5</td>
</tr>
</tbody>
</table>

<sup>a</sup> Number of animals surviving per total number of animals in each group.

<sup>b</sup> NT, normal thymus; ST, sensitized thymus; NS, normal spleen; SS, sensitized spleen.

Table 4 Regression of gross visceral metastases in BALB/c nu/nu mice by adoptive transfer of sensitized nu/+ spleen cells

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Adoptive immunization</th>
<th>Experimental group&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Macroscopic liver metastasis&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Survival&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Survival Time (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 12</td>
<td>Control</td>
<td>Reconstituted (S)</td>
<td>3/3</td>
<td>0/3</td>
<td>19.3 ± 0.6</td>
</tr>
<tr>
<td>Day 13</td>
<td>Control</td>
<td>Reconstituted (S)</td>
<td>3/4</td>
<td>0/4</td>
<td>19.5 ± 1.0</td>
</tr>
<tr>
<td>Day 14</td>
<td>Control</td>
<td>Reconstituted (S)</td>
<td>3/3</td>
<td>0/3</td>
<td>19.3 ± 0.6</td>
</tr>
<tr>
<td>Day 15</td>
<td>Control</td>
<td>Reconstituted (S)</td>
<td>3/4</td>
<td>0/4</td>
<td>19.3 ± 0.5</td>
</tr>
</tbody>
</table>

<sup>a</sup> N, reconstituted with normal nu/+ spleen cells; S, reconstituted with sensitized nu/+ spleen cells.

The incidence of gross liver metastases was assessed 16 days post-tumor injection for all groups. Mice were anesthetized with 40 mg sodium pentobarbitol per kg, an incision through the abdominal skin was made, and the ventral surface of the liver was then viewed through the intact peritoneum for the presence or absence of metastatic foci. The incision was then sutured, and the mice were allowed to recover from anesthesia.

<sup>b</sup> Number of animals surviving per total number of animals in each group. All groups were monitored for survival. On Day 45, all surviving animals were sacrificed and autopsied for the presence of metastases.

<sup>c</sup> Mean ± S.D.
surgically documented liver metastases on Day 16. Sacrifice and autopsy of these apparently healthy mice on Day 45 revealed no evidence of metastatic disease, indicating that the previously observed metastases had been rejected. Mice which had received their adoptive immunization 14 and 15 days post-tumor injection showed a survival of 40 and 57%, respectively. This was in marked contrast to the failure of any control mice to survive. Survivors of these groups were also sacrificed on Day 45 and showed no gross or histological evidence of tumor growth, either at the initial site of injection or at sites of preexisting liver metastases.

A second related experiment (Experiment 2) was performed to determine whether sensitization of the transferred cells was required for the regression of metastatic nodules. Quite clearly, transferred normal spleen cells were incapable of mediating this regression, while sensitized lymphocytes regressed the metastases in 100% of the tumor-bearing mice.

Further experiments have shown that the immune regression of metastatic nodules by sensitized cells is mediated by T-cells and not by sensitized B-cells or purified antibody.

DISCUSSION

These experiments demonstrate that the immune system can reject large tumor loads, despite the metastatic spread of tumor to visceral organs. BALB/c nu/nu mice bearing the metastasizing MDAY-D2 tumor can be adoptively immunized with spleen cells sensitized to minor histocompatibility antigens present on MDAY-D2 tumor cells of DBA/2 origin. The adoptive immunization can be performed 12 to 15 days after initial tumor challenge when visceral metastases are already established.

The use of MDAY-D2 as a model for metastasis has recently been described by Kerbel et al. (18). This tumor spontaneously metastasizes to most major visceral organs from s.c., i.p., and i.d. sites. Metastases are demonstrable within 3 days of tumor implantation (18), a fact confirmed in this report. MDAY-D2 also has the demonstrated propensity to metastasize in nu/nu mice, a property which separates it from most other mouse and human metastatic tumors (2, 13, 29, 31). Metastasis of MDAY-D2 in nu/nu mice proceeds at the same rate and with the same organ distribution as that observed in the syngeneic DBA/2 host. The s.c. injection of 10⁴ MDAY-D2 cells results in the formation of a small primary tumor which metastasizes rapidly. The liver, draining lymph nodes, spleen, kidneys, and lungs are affected in that order. The pattern and timing of metastasis in nu/nu mice is consistently reproducible with macroscopic liver metastases routinely observed by Day 13. Metastasis proceeds in this manner regardless of whether the tumor inoculum is a suspension or a solid implant. An s.c. injection dose of 10⁴ MDAY-D2 cells consistently kills nu/nu mice within 19 to 21 days.

With the exception of the syngeneic DBA/2 and allogeneic C3H nu/nu or BALB/c nu/nu mice, most mouse strains reject grafts of MDAY-D2 (18). Our experiments show that normal BALB/c or BALB/c nu/+ mice reject the DBA/2 tumor, MDAY-D2, on the basis of numerous minor histocompatibility differences. The minor loci at which BALB/c and DBA/2 differ include H-1, H-3, H-7, H-13, Ly-1, and Ly-2, among others (32). Since minor histocompatibility differences have been shown to be cumulative in stimulating graft rejection (4, 20, 22), it is not surprising that the peak of CMC occurred as early as Days 11 (PLN) and 14 (spleen). This observation is supported by experiments on the inhibition of CMC, in which only cells sharing minor histocompatibility antigens with MDAY-D2 could inhibit the BALB/c anti-MDAY-D2 CMC reaction. Thus, only cells containing a complement of DBA/2 minor histocompatibility antigens, MDAY-D2, P815, and DBA/2 lymphoblasts, inhibit. Allogeneic cells, including those of BALB/c origin (H-2d), did not inhibit the CMC reaction. Allogeneic C57BL/6 and A strain cells do not share with DBA/2 mice most of the minor histocompatibility antigens which separate the DBA/2 antigenically from the BALB/c (32). The lack of CMC inhibition by allogeneic cells lends support to the hypothesis that BALB/c lymphocytes lyse MDAY-D2 on the basis of recognition of DBA/2 minor histocompatibility antigens. This conclusion is further supported by the observed inhibition of CMC by Con A DBA/2 blasts.

In addition to demonstrating the specificity of the BALB/c anti-MDAY-D2 response, these experiments support the work of Kerbel et al. (18). A strain mice possess only the K end of the H-2 haplotype and are, in fact, H-2(k/d) recombinant mice. Kerbel et al. (18) have serologically demonstrated that MDAY-D2 possesses no A strain (H-2k) antigens (18). This is confirmed by studies demonstrating that MDAY-D2 bears only the private specificities associated with the H-2d, but not the H-2(k/d), haplotype (31,4 versus 23,4). Therefore, MDAY-D2 is presumed to be of DBA/2 origin. In our experiments, the A strain tumor, YAC-1, failed to inhibit the CMC response. Allogeneic and syngeneic CMC responses are also not inhibited by H-2k tumor cells.

Rajan et al. (37) have recently shown that CMC may be more sensitive than serological methods for the detection of H-2 antigens. Therefore, our results more definitively illustrate that MDAY-D2, whatever its etiology, does not contain H-2k antigens. Therefore, the cumulative minor histocompatibility differences alluded to previously, rather than a major H-2 difference, most probably provide the basis for the rejection of MDAY-D2 by BALB/c in the short period of time observed.

Adoptive transfer of BALB/c nu/+ lymphocytes to BALB/c nu/nu mice, 10 days prior to MDAY-D2 challenge, affords varying levels of protection, depending on the number and source of the transferred cells. Not surprisingly, sensitized spleen cells protected most efficiently, followed by normal spleen cells. Normal and sensitized thymus never afforded full protection, even at high doses, perhaps because too few mature T-cells were present; therefore, the rapid expression of a completely effective immune response was not possible. Presumably, if the time between lymphocyte reconstitution and tumor cell challenge was increased, the degree of protection afforded by thymus cells would increase by virtue of increased T-cell differentiation. However, some animals given thymus cells under the conditions utilized in this study did survive, often in spite of developing unusually large primary tumors. In some cases, these tumors reached 5 to 10 times the size of tumors which metastasized and routinely killed unreconstituted nude mice. Given the demonstrated metastatic potential of MDAY-D2 in nu/nu mice, we believe that the eventual complete rejection of these large tumors indicated immune control at some stage of the metastatic process prior to arresting and eliminating the primary tumor growth. To investigate this question directly, we reconstituted MDAY-D2-bearing nu/nu mice.
with specifically sensitized BALB/c nu/+ spleen cells 12 to 15 days after tumor injection. Virtually all mice exhibited surgically documented metastases on the ventral surface of the liver by Day 16. In spite of the presence of the grossly visual hepatic tumor foci, 100% of the mice reconstituted 12 or 13 days after tumor injection survived along with approximately 50% of those reconstituted by Day 14 or 15. Normal spleen cells were unable to mediate this regression even when transferred on Day 12. These experiments demonstrate that an appropriately activated immune system can reject large tumor loads, including established visceral metastases. Equally interesting is the fact that this rejection is meditated by adoptively transferred sensitized spleen cells which manifest only very low levels of in vitro CMC (40 to 50% SR in a 24-hr 111InOx assay and 6 to 8% SR in a 4 to 6 hr 51Cr assay). Further studies have shown that the mechanism of immune regression is by cytolytic T-cells, while sensitized B-cells and purified antibody are ineffective. These studies have indicated that a correlation exists between low levels of in vitro CMC and dramatic regression of metastases in vivo. The low levels of CMC (5 to 20% SR) routinely reported for T-cell cytotoxicity in syngeneic tumor models or in studies of natural killer activity are often dismissed as being biologically insignificant. Our data support the idea that low grade in vitro CMC responses may be of biological significance, since these cells are highly efficient in rejecting disseminated tumor. The death of some animals which were reconstituted 14 or 15 days after tumor injection is not surprising, since there must be a point after which the immune system is incapable of reversing the neoplastic sequence of events. This may be due to irreversible hepatic or renal failure. Sacrifice and autopsy of all surviving, apparently healthy mice on Day 45 confirmed the absence of any discernible tumor growth, either at the primary site or at known sites of previously existing liver metastases. Microscopic examination of histological sections made from the livers of these mice revealed no evidence of residual tumor growth but showed an extensive lymphocyte infiltrate, implying that the etiology of regression of metastatic foci was indeed immunological.

We are aware of only a few studies which demonstrate the immune rejection of metastases (15, 16, 37). These studies deal only with immunotherapy of several limited forms of metastasis. The i.l. injection of Bacillus Calmette-Guérin alone or in conjunction with dead tumor cells has been shown to initiate regression of some distant metastases and also some lymph node metastases often associated with a guinea pig hepatocarcinoma (15, 16, 37). Attempts at Bacillus Calmette-Guérin immunotherapy of human cancer have not been encouraging (28).

Our experiments, in which minor histocompatibility antigens provide the antigenic stimulus, demonstrate that an appropriately activated immune response can specifically arrest and eradicate widely disseminated, late-stage neoplastic disease. While the problems of specific syngeneic or autochthonous immune activation remain largely unsolved, we believe that these data portend a brighter potential for specific immunotherapy than has been indicated to date.

ACKNOWLEDGMENTS

We thank Julian Smith for his skilful technical contributions and Karen Duncan for her excellent secretarial assistance.


Immune-mediated Arrest and Reversal of Established Visceral Metastases in Athymic Mice


Updated version Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/39/10/4034