Protection against Experimental Cerebral Metastases of Murine Melanoma B16 by Active Immunization

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ABSTRACT

Melanoma patients often develop brain metastases despite effective systemic immunotherapy against melanoma. We have attempted to establish a mouse model to develop strategies to combat this problem. Immunization of C57BL/6 (H-2b) mice with a combination of the syngeneic G3.12/BM2 melanoma (a B16 subclone) and the allogeneic Cloudman-S91 melanoma was effective in preventing the growth of 10,000 viable s.c. injected G3.12 cells in 93% of the mice. Irradiated whole tumor cells pretreated with γ-interferon for 2 days were most effective. A nonspecific adjuvant (DETOX) was injected routinely together with the tumor cells. Active immunization with 2 different doses of irradiated melanoma cells (1 × 10^5 or 2.5 × 10^6 cells/injection × 5 injections) protected against intracerebral challenge with 200 live G3.12 cells in 69% of the mice. This challenge caused the death of all control mice within 30 days. T-cell-mediated, tumor-specific cytotoxicity against G3.12 melanoma was demonstrated in the spleen of immunized mice. Histological observations in the brain, 80 days after tumor challenge, indicated complete eradication of the melanoma, but although CD4+ and CD8+ T-cells and macrophages were present, their number was low. Gliosis was present in both immunized and control animals. Thus, in this murine melanoma model syngeneic mice were protected from death by s.c. and intracerebrally inoculated tumor cells if pretreated with a sufficient number of irradiated syngeneic and allogeneic melanoma cells and an immunological adjuvant. Whether this regimen can treat established tumors of the brain, alone or in combination, is uncertain. Yet its success suggests that the "blood-brain barrier" impeding immunity to tumors may not be absolute.

INTRODUCTION

Melanoma in its earliest stage can be cured by surgery, with a 5-year survival rate of 80%. However, when metastases are present, the prognosis is poor, with a median survival of less than 1 year and a 5-year survival rate of less than 10% (1-3). Chemotherapy can evoke clinical tumor responses in 15 to 35% of such patients, usually of short duration (4). We have treated over 100 patients with metastatic melanoma with a therapeutic melanoma vaccine ("theraccine") composed of 2 allogeneic melanoma cell lines and an immunological adjuvant, DETOX (5-8). A 20% objective clinical response has been observed, with 5% complete remissions. In addition, we have treated patients with low-dose cyclophosphamide and low-dose IL-2,4 with a 28% response rate, including remissions of liver metastases (5-7). Unfortunately, neither treatment with melanoma theraccine nor cyclophosphamide plus IL-2 sufficient to cause systemic remissions could consistently prevent the occurrence of brain metastases (8). The finding of asymptomatic CNS metastases of melanoma has long been noted in 50-75% of patients at autopsy (9, 10), but CNS metastases are becoming more of a clinical problem as patients live longer with their disease under control. If some form of immunologically based therapy could be made more effective in the CNS, both the quality of survival and its length would be improved, with the possibility for cure in some patients.

We therefore attempted to establish a syngeneic mouse melanoma model with a subline of B16 melanoma, G3.12, to examine a situation analogous to the clinical setting we have encountered. The features of this model were to be: (a) a syngeneic melanoma of weak immunogenicity that would metastasize to the brain, and to which the animal would usually succumb; (b) a host that was rendered immune systematically against the melanoma and had no systemic tumor; but (c) nevertheless had brain metastases. To mimic the allogeneic human melanoma theraccine, we used a mouse vaccine comprising not only the syngeneic B16 but also an allogeneic melanoma component.

We planned to use regional or systemic injections of cytokines to aid in local eradication of the tumor, because of an expected blood-brain barrier to the immune response. As we will note, however, a sufficient degree of active immunization had significant protective effects against experimentally induced brain metastases.

MATERIALS AND METHODS

Cell Lines and Animals

For vaccination and tumor cell challenge of C57BL/6J mice (H-2b), the syngeneic mouse melanoma B16-subclone G3.12/BM2 (obtained through the courtesy of Dr. C. W. Stackpole, New York Medical College, New York, NY) was chosen, because it was reported to metastasize preferentially to the brain after s.c. injection (11). B16 melanoma is known to be a weakly immunogenic cell line (12-15). As the allogeneic melanoma cell line, we chose Cloudman-S91 (HCC1 53.1; ATCC, Rockville, MD), which is syngeneic to DBA/2 mice (H-2b) (16). For preliminary cerebral metastases experiments, parental B16 melanoma cells (ATCC) were used. Cells were grown in tissue culture flasks with RPMI 1640, 2 mM l-glutamine, and 10% (Cloudman 20%) fetal bovine serum, and passed once weekly after PBS-EDTA incubation for 10 min. For cytotoxicity studies, the murine myeloma cell line YAC-1 (H-2b; ATCC) was used as a target for nonspecifically activated killer cells.

Six- to 8-week-old female C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME) were kept in filter top cages in University of Southern California vivaria facilities under standard food and housing conditions during the experiments. Endpoint of each survival study was death of the mice.

For anesthesia, either inhalation of Metofane (Pitman-Moore, Inc., Mundelein, IL) or i.p. injection of 0.25 mg/mouse xylazine (Ana-Sed; Lloyd Laboratories, Shenandoah, IA) and 1.2 mg/mouse ketamine i.p. (Ketasert; Aveco Co., Inc., Fort Dodge, IA) were used.

Vaccine Preparation

Cell Lysates. With a similar procedure to that which we used in human melanoma for our theraccine preparation (5-8), melanoma cells were counted with trypsin blue, mechanically disrupted with a Polytron stainless-steel high-speed homogenizer (Tekmar Co., Cincinnati, OH), and frozen in aliquots of 5 × 10^6 cell-equivalents at ~70°C. For injection into mice, G3.12 and Cloudman cell lysates were thawed, washed in PBS, and mixed with the adjuvant DETOX. The s.c. injection dose was 500,000 tumor cell equivalents each of G3.12 and Cloudman cells in 100 µl PBS once weekly for 5 weeks.
Irradiated Cells. As a second mode of preparation, melanoma cells were cultured to confluency in 10-chamber cell factories (no. 1-64327; Nunc, Roskilde, Denmark) and washed with PBS. They were incubated for 48 h with 100 IU/ml recombinant murine IFN-α (Genentech, South San Francisco, CA) in serum-free medium to up-regulate H-2 antigens and melanoma-associated antigens. The cells were analyzed by FACS for H-2 antigen expression and frozen in 10% dimethyl sulfoxide, 20% fetal bovine serum, and 70% RPMI. For injection, cells were thawed, washed in PBS, and irradiated with 4000 rad. After mixing with DETOX, both G3.12 and Cloudman cells were injected in a volume of 200 μl, either s.c. (28 mice) in a dose of 1 × 10⁵, 5 × 10⁵, or 2.5 × 10⁶ cells, or i.p. (130 mice) in a dose of 1 × 10⁶ or 2.5 × 10⁶ cells, once weekly for 5 weeks. Three days prior to the first injection, all mice received one injection of 3 μg cyclophosphamide (Sigma, St. Louis, MO) i.p. to decrease the number of T suppressor cells (5, 17, 18).

Immunological Adjuvant

DETOX (Ribi ImmunoChem, Inc., Hamilton, MT), a nonspecific immunological adjuvant that contains detoxified endotoxin (monophosphoryl lipid A) from Salmonella minnesota, cell wall skeletons of Mycobacterium phlei, squalane oil, and emulsifier, was combined with the vaccine, because it stimulates both cell-mediated and humoral immunity (19) and was also used in our human studies (20-23). The concentration used in all experiments was 150 μg cell wall skeletons and 100 μg monophosphoryl lipid A per injection.

s.c. Tumor Cell Inoculation

In a first series of experiments, 250,000 each of viable G3.12 and Cloudman cells were injected s.c. into naive C57BL/6 mice to study tumor growth, pattern of metastasis, and survival of the animals. Tumor cells were injected immediately after thawing and after being for 3 to 4 weeks in tissue culture.

In the second series of experiments, we studied the tumor growth in immunized animals and chose a lower number of tumor cells, in order not to overload the host with a tumor burden that could possibly mask an immune response. One to 3 days after the immunization procedure, 10,000 live melanoma cells (incubated with IFN-γ), were injected into both abdominal flanks s.c. (right side G3.12, left side Cloudman cells) as a challenge, to test whether animals were successfully immunized. This important step in our immunization schema could not be omitted, since we wanted to prove that our murine vaccine was effective. Tumor growth and survival were monitored weekly. Animals that did not develop tumors within at least 50 days after injection were considered to be successfully immunized against syngeneic melanoma and were used for further studies of experimental brain metastases.

i.c. Tumor Cell Inoculation

Despite the reported tendency of G3.12/BM2 to metastasize to the brain (24), spontaneous i.c. metastases after s.c. tumor inoculation never occurred in our preliminary experiments. To mimic cerebral tumor cell metastasis, we decided to use i.c. tumor cell inoculations rather than intracarotid or intracardial injections. We wanted to save the carotid artery [which has to be ligated after injections (25)] for regional injection of cytokines. Localized i.c. injections into the right precentral parietal hemisphere resulted in brain melanomas diffusely growing in both hemispheres.

To study the kinetics of tumor growth after i.c. inoculation, we used naive C57BL/6 mice with various numbers of B16 or G3.12/BM2 melanoma cells (100, 400, 2000, 10,000 or 100,000 cells, n = 6-8 mice/group). In a volume of 20 μl using a Hamilton microliter syringe (Hamilton Corp., Reno, NV) with a 26-gauge needle, cells were injected at a depth of 3 mm in the right precentral parietal cerebral hemisphere as described (26). No deaths related to this procedure occurred, and the animals recovered within minutes without any neurological symptoms. Based on these data, constant i.c. injection doses were chosen for the subsequent immunization experiments.

Successfully immunized animals and PBS-treated controls were injected under inhalation anesthesia with 200 (or 1,000 in the lystase study) live, IFN-γ-pretreated G3.12 cells. The animals were monitored for signs of i.c. tumor growth and for survival.

Cytokines

Recombinant human IL-2 (specific activity, 3 × 10⁶ U/mg; Cetus Corp., Emeryville, CA) and recombinant murine IFN-γ (specific activity 8 × 10⁶ U/mg; Genentech, South San Francisco, CA), each at 10,000 units/injection, were used for i.p. injections in lystate experiments. IFN-γ was also used for injections into the carotid artery and to up-regulate expression of H-2 antigens on tumor cells (100 U/ml for 48 h).

Intracarotid Injection

For a regional delivery of cytokines into mouse brains, internal carotid arteries were exposed, using a microsurgical technique (25). Inhalation anesthesia with Metofane and i.p. anesthesia with Ana-Sed and Ketaset were applied; 10,000 units IFN-γ in 100 μl 0.9% NaCl were injected once, using a 30-gauge glass cannula. Mortality due to injection procedure was significant (2 of 9 mice).

Cell-mediated Cytotoxicity Assay

Spleens from immunized mice and controls were collected 1 week after the last vaccination and weighed. Spleens obtained from 3 animals per group were mechanically disrupted by injecting 3 ml PBS into the spleen capsule. Splenocytes were pooled groupwise and passed over a nylon wool column (Nylon Wool Type 200 L; DuPont Biotechnology, New England Nuclear Products, Boston, MA). The nonadherent cells, which were enriched 2- to 3-fold for T-cells (CD3+), were tested for cytotoxicity in comparison with unseparated spleen cells. Spleen cells were plated at various effector:target cell ratios (ratios of 200, 100, 50, and 25) into 96-well round-bottomed microtiter plates (Corning, Inc., Corning, NY) in RPMI 1640, containing 25 μg N-2-hydroxyethylpiperezine-N'-ethanesulfonic acid with 10% fetal bovine serum, 2 mM L-glutamine, 5 × 10⁻⁵ M 2-mercaptoethanol, 1% nonessential amino acids, 1% Na-pyruvate, 1% vitamins, and 2% penicillin/streptomycin (all Sigma). Target cells were labeled with [³¹Cr]Na₂CrO₄, 40 μCi/10³ cells (Amersham Corp., Arlington Heights, IL) for 45 min at 37°C and washed 3 times; 5,000 target cells were added to each well to a final volume of 200 μl. After 16-h incubation at 5% CO₂ and 37°C, supernatants were collected and radioactivity determined in a gamma-counter (Gamma 5500; Beckman, Fullerton, CA). The data were calculated as:

\[
\text{Specific % cytotoxicity} = \left( \frac{\text{cpm}_{\text{control}} - \text{cpm}_{\text{test}}}{\text{cpm}_{\text{control}}} \right) \times 100
\]

where spont. = spontaneous release.

Proliferation Assay

Spleen cells were collected and separated as described above and incubated for 7 days under stimulation with irradiated Cloudman, G3.12 cells, or medium (control) at various effector:stimulator ratios (ratios of 20, 10, 5, and 2.5) in 96-well round-bottomed microtiter plates (10,000 stimulator cells/well). [³²P]-Thymidine (1 μCi/well; New England Nuclear, Boston, MA) was added 6 h prior to harvest. Cells were harvested on glass fiber filters with a 24-channel cell harvester (PHD Cell Harvester; Cambridge Technology, Cambridge, MA). Radioactivity was determined after adding 2 ml of ScintiVerse E (Fisher Scientific, Fair Lawn, NJ) in a liquid scintillation beta-counter (Beckman LS 8000).

Statistical Analysis

Survival data were tested for significant differences at the 5% error level (P < 0.05) using a log-rank test. Spleen weight, cytotoxicity, and proliferation data were analyzed with a 2-tailed Student t test. Means are listed with SD and number of observations (n), unless otherwise stated.

Flow-Cytometric Analysis

To determine expression of H-2 antigens on tumor cells, 5 × 10⁶ cells were incubated with 50 μl rat-anti-mouse-H-2 antibody (TIB 126 supernatant; ATCC) for 30 min on ice, washed 3 times with cold RPMI 1640/3% fetal bovine serum, and spun at 1500 rpm for 10 min. The pellet was resuspended and incubated with 20 μl FITC-conjugated mouse-anti-rat IgG Fc-fragment (Jackson Immunoresearch Laboratories, Inc., West Grove, PA) for 30 min on ice. Cells were washed and analyzed, using a FACS 440 analyzer (Becton Dickinson, Mountain View, CA).
Growth of Syngeneic and Allogeneic Tumor Cells in Naive Mice

**s.c. Inoculation.** The s.c. injection of G3.12 and Cloudman cells (250,000 each), freshly thawed and less than 4 days in culture, into abdominal flanks of naive C57BL/6 mice generated G3.12 tumors of 10-mm size within 25 ± 4.5 (SD) days. No (allogeneic) Cloudman tumors grew, as shown in Table 1. However, when tumor cells were used after growth in tissue culture for 4 weeks, Cloudman tumors also developed in most of the animals given injections (Table 1). The consistent growth of the syngeneic G3.12 tumor indicated its intrinsic poorly immunogenicity, whereas growth of the allogeneic Cloudman tumor showed that it had become poorly immunogenic during tissue culture.

**RESULTS**

<table>
<thead>
<tr>
<th>Mice injected</th>
<th>Tumor growth</th>
<th>Days to develop a 10-mm tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells after thawing</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G3.12</td>
<td>16/16</td>
<td>100</td>
</tr>
<tr>
<td>Cloudman</td>
<td>0/15</td>
<td>0</td>
</tr>
</tbody>
</table>

| Cells cultured for 4 wk | | |
| G3.12 | 17/17 | 100 | 21 ± 11.4 |
| Cloudman | 11/12 | 92 | 29 ± 7.2 |

Fig. 1. Survival of naive C57BL/6 mice after i.c. injection of 100 to 100,000 B16 melanoma cells, 100,000 G3.12/BM2 melanoma cells, or NaCl. Survival in all groups was shorter than in NaCl group (P < 0.001). Survival was equal in mice given injections of 100,000 G3.12 or 2,000 B16 cells (P > 0.1) and equal in mice given injections of 2,000 B16 or 400 B16 cells (P > 0.05).

**I.c. Inoculation.** Results of i.c. injections of untreated B16 and G3.12 cells into naive C57BL/6 mice, compared to 0.9% NaCl injections, are shown in Fig. 1. All mice that had been given injections of melanoma cells died earlier than NaCl-injected controls, which were sacrificed after 90 days and had no pathological findings (P < 0.001). Survival was equal (median, 20 days) in mice that received injections of 400 or 2,000 B16 cells (P > 0.05), but longer (median 33 days) in mice given injections of only 100 B16 cells (P < 0.01); 100,000 G3.12/BM2 cells were less aggressive than 100,000 B16 cells (P < 0.001) and equal to the injection of 2,000 B16 cells (P > 0.1). Based on these data, 200 G3.12 cells (or 1,000 for the lysate study) were chosen as the inoculum for further studies in immunized mice to permit a sufficiently long survival for subsequent therapy to be administered.

**Attempts to Immunize with Melanoma Cell Lysates and to Treat Cerebral Metastases with Cytokines**

G3.12 and Cloudman cell lysates were mixed to provide a sufficient, partly allogeneic stimulation of the immune system. Allogeneic stimulation was shown in transfection studies of melanoma fibroblasts to increase the host's specific immune response (12). Moreover, our human theraccine contains alloantigens, which may serve the same function (22, 23).

Mice were given weekly s.c. injections of G3.12 and Cloudman melanoma cell lysates (not pretreated with IFN-γ) and DETOX for 5 weeks. Three to 4 days after the last vaccination, mice were challenged i.c. with 1000 IFN-γ-pretreated G3.12 cells. Seven days later, they were randomized and treated in 4 groups: (a) IL-2, 10,000 Cetus units twice daily i.p. for 2 weeks, with injections on 5 consecutive days and 2 days of rest; (b) IL-2 and IFN-γ, each at 10,000 units twice daily i.p. for 2 weeks, with injections on 5 consecutive days and 2 days of rest; (c) NaCl 500 μl i.p. twice daily for 2 weeks; and (d) IFN-γ (10,000 units) injected once into left carotid artery. The survival curves of this experiment are shown in Fig. 2. All mice developed cerebral G3.12 melanoma and died within 33 days after tumor cell injection. Treatment with cytokines, regardless of whether IFN-γ was injected i.p. or into the carotid artery (i.c.), by itself or in combination with IL-2, did not show any benefit compared to NaCl treatment (P > 0.05).

Thus, like the situation in humans, in this experiment lysates were apparently ineffective in immunization against cerebral melanoma. However, we realized that we had no direct proof in this experiment that the animals were immunized and that the tumors themselves were...
TABLE 2 Melanoma growth in naive mice after inoculation with 10,000 or 250,000 melanoma cells after IFN-γ incubation

<table>
<thead>
<tr>
<th>Cell inoculum 250,000 cells s.c.</th>
<th>Tumor growth</th>
<th>Days to develop a 10-mm tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>G3.12</td>
<td>10/10</td>
<td>100</td>
</tr>
<tr>
<td>Cloudman</td>
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* Mean ± SD.

**Up-Regulation of H-2 Antigen Expression on Tumor Cells**

When Cloudman melanoma cells, which had been in culture for at least 4 weeks, were incubated with IFN-γ (100 units/ml) for 2 days and were injected s.c. into animals, no growth of the allogeneic tumor occurred, as shown in Table 2. G3.12 tumors grew in all animals receiving injection, but with a slower growth rate compared to untreated melanoma cells. Based on these in vivo observations, we studied the H-2 antigen expression of G3.12 and Cloudman cell lines, after thawing, in culture and during incubation with IFN-γ to establish a reliable procedure for H-2 antigen up-regulation. Fig. 3 shows that both cell lines lost their initial level of H-2 antigen expression (3.8 and 38%, after thawing) and reached within 10 days in culture a nadir of 1.2 ± 0.50% (n = 7, G3.12) and 3.95 ± 2.2% (n = 9, Cloudman). However, incubation with 100 units/ml IFN-γ for 2 days was sufficient to induce maximal expression of H-2 antigens (86.6 ± 12.1% for G3.12 and 51.8 ± 13.3% for Cloudman). We made similar observations when we tested MHC antigens of IFN-γ-treated, s.c. injected G3.12 cells, after tumors had grown to 10-mm size in C57BL/6 mice. Initially these tumors showed low expression of H-2 antigens (Fig. 4).

Incubation with IFN-γ for 2 days, however, induced up-regulation of H-2 antigens.

This indicated that these tumor cells in vivo and in vitro lost MHC antigen expression through a mechanism sensitive to IFN-γ up-regulation. As a result of these observations, we considered in further experiments that a maximal MHC antigen expression of >50% (Cloudman) and >90% (G3.12) was crucial for the immunogenicity of those tumor cells. It may also have facilitated rejection of tumor challenges by cytolytic T-cells.

**Protection against Systemic and I.c. Melanoma with s.c. Injected Irradiated IFN-γ-treated Melanoma Cells**

Groups of 3 mice were immunized s.c. weekly for 5 weeks with 3 different doses of irradiated IFN-γ-treated melanoma cells and challenged s.c. with 20,000 IFN-γ-treated viable G3.12 and Cloudman cells. The survival curves of these mice, compared with a nonimmunized control group, are shown in Fig. 5. No acute side effects (allergic reaction, immobilization, infection) were observed after the injections, but most animals developed a 1–2-mm granuloma at the injection site. None of the animals developed Cloudman tumors in these experiments. Local tumor growth of G3.12 tumors up to 30 mm was observed, before animals died from pulmonary or abdominal immunogenic. Therefore, we decided to include a s.c. challenge with 10,000 viable G3.12 cells as an in vivo test for immunity in all subsequent experiments. Moreover, we analyzed the H-2 antigen expression on tumor cells by FACS and tested an immunization schedule with irradiated whole cells, given s.c. or i.p.

Fig. 3. H-2 antigen expression on mouse melanoma cell lines Cloudman and G3.12/BM2. Cell lines were analyzed by FACS after thawing (day −10), after 10 days in culture without IFN-γ (day 0), and at various time points during and after incubation with IFN-γ (100 units/ml) for 5 days. For day 2, when maximal expression of H-2 antigens was reached, means and SD of 7 analyses are shown.

Fig. 4. H-2 antigen expression on G3.12 melanomas grown in C57BL/6 mice, removed as 1-cm tumors, and tested either untreated (left column) or after incubation with IFN-γ (100 units/ml) for 48 h (right column). Bars, means and SD of 7 tumors.
IFN-γ-treated G3.12 and Cloudman cells, together with DETOX, was sufficient to immunize mice against a s.c. challenge with syngeneic or allogeneic melanoma cells. As shown in Fig. 7, 39 of 42 (93%) mice rejected s.c. challenge with 20,000 IFN-γ-pretreated live G3.12 and Cloudman cells. All 14 control animals died from melanoma within 45 days ($P < 0.001$), with a median survival of 43 days. The allogeneic Cloudman tumor grew in none of the mice.

Mice surviving the s.c. challenge were then given i.c. injections of 200 live G3.12 cells. Fig. 8 shows that all control animals died from melanoma brain metastasis within 30 days, with a median survival of 24 days. The median survival in the immunized mice was >80 days and longer than the controls ($P < 0.01$). The survival in both vaccine groups was equal ($P > 0.1$). Taken together, 9 of 13 (69%) immunized mice survived for >80 days and had no signs of i.c. melanoma at autopsy, i.e., the vaccine had a protective effect against i.c. melanoma challenge.
In Vitro

During i.p. vaccination, spleen weight and size of immunized mice increased significantly ($P < 0.001$), compared with control mice (Fig. 9). Hematoxylin-eosin-stained sections of these enlarged spleens showed regions within the red pulp with melanin-containing macrophages and abundant CD4+ and CD8+ T-lymphocytes. Abdominal lymph nodes, especially in the group receiving the highest vaccine dose, had also melanin-containing macrophages, CD4+ and CD8+ T-lymphocytes.

Seven days after the last vaccination, splenocytes were tested for their specific cytotoxicity against G3.12 melanoma and YAC-1 target cells (Fig. 10). All immunized animals showed higher melanoma-directed (G3.12) and natural killer-mediated (YAC-1) cytotoxicity, compared with nonimmunized controls ($P < 0.05$). Unseparated splenocytes that contained 24% (range, 20–29%) T-cells could not generate higher lysis of G3.12 than of YAC-1, whereas the T-cell-enriched fraction (66% T-cells; range, 43–85%, after nylon wool enrichment) could. The highest specific cytotoxicity (43%) against G3.12 was observed with splenocytes from mice immunized with the highest vaccine dose ($P < 0.05$, compared to unseparated splenocytes or to low-dose vaccine group). T-cell-enriched splenocytes seemed to contain less natural killer cells than unseparated splenocytes, because the melanoma-specific cytotoxicity increased while the nonspecific cytotoxicity decreased.

We also tested the proliferative rate of the splenocytes, with and without stimulation by vaccine components (Fig. 11). Insignificant proliferation was seen in splenocytes from nonimmunized control animals, whereas splenocytes from immunized mice proliferated, irrespective of whether they were stimulated with irradiated tumor cells. A correlation between vaccine dose and proliferation was not found. The T-cell-enriched fraction showed higher proliferation rates than unseparated splenocytes only in experiments without in vitro stimulation with tumor cells ($P > 0.05$). This indicates that splenocytes from immunized mice, tested 1 week after the last vaccination, are highly stimulated and proliferate in vitro without further stimulation for at least 7 days.

Histopathology

Brain. In all brain sections, taken from animals that had died from brain melanoma (10 s.c. vaccinated mice and 10 i.p. vaccinated mice), extensive tumor growth with central necrosis and bleeding was obvious. Melanoma cells metastasized into various areas of the cerebrum, the cerebellum, and the meninges, forming mainly perivascular infiltrates. The tumors were usually infiltrated and surrounded by few T-lymphocytes (equally CD4+ and CD8+ cells) and by a somewhat larger number of macrophages, most of them containing melanin. Normal mouse brains ($n = 5$) contained no perivascular T-lymphocytes and very few macrophages. Despite the systemic immunity found in the vaccinated mice, no obvious differences in the cerebral immune response could be seen compared with nonimmunized animals that had received the vaccine, but failed to respond in vivo. The number of macrophages and T-lymphocytes correlated with the tumor load rather than with treatment. Animals that had survived the i.c. tumor challenge showed few macrophages and T-lymphocytes, but no melanoma cells. Two of these brains contained a single extracellular deposit of melanin. Gliosis, defined as glial fibrillary acid protein-positive enlarged astrocytes with perivascular podocytes and with intracellular melanin, was seen close to the developing tumor and in equal amounts in controls and vaccine-treated nonsurvivors. Survivors showed areas of gliosis without melanin, suggesting signs of successful tumor rejection.

Abdomen. In animals that had received i.p. vaccinations, large amounts of melanin were detected in macrophages within the spleen and abdominal lymph nodes. Control mice that had received 200 melanoma cells i.c. without immunization and had died from brain tumors, had splenic macrophages containing significant amounts of...
DISCUSSION

We have previously described active-specific immunotherapy of patients with advanced melanoma, using allogeneic melanoma cell lysates ("theraccine") and DETOX (21). Development of brain metastases in patients successfully treated with immunotherapy was observed by us (8) and other investigators (28, 29). The same problem was found after chemotherapy (30) or radiation (31, 32). One main goal in developing a mouse model was to study melanoma brain metastases in an individual that was immunized against melanoma and had no systemic disease. This was akin to the human clinical situations we had encountered. In contrast to nude mouse (33) or B16 melanoma models (34–36), the model had to be close to the clinical situation in "theraccine"-treated patients, to suggest strategies relevant to our clinical situation.

Unlike the clinical situation, brain metastasis never occurred spontaneously during our experiments with G3.12 cells, although we had chosen a cell line derived from spontaneous B16 melanoma brain metastases after a s.c. injection (24). To preserve the carotid artery for later use in regional immunotherapy, we chose to induce brain metastasis experimentally by i.c. inoculation. That in fact generated the histological picture of a widely metastatic tumor to the brain. We are aware, however, that the i.c. route of injection might have damaged the blood-brain barrier, perhaps permitting local entry of lymphocytes. Against this is the histological finding of a metastasizing tumor throughout all brain regions and uniformly infiltrated with T-cells and macrophages. This is unlikely to occur solely after a localized injury of the brain such as an i.c. injection. We found in further histological studies that there is a lack of inflammatory response in the brain after sterile i.c. PBS injection, at least 1 to 7 days after the injection, indicating a rather minor immune response to the local injury. There is also the observation that large numbers of macrophages and a small number of CD4+ lymphocytes were found in metastatic cerebral melanomas in a murine model where tumor cells were injected via the carotid artery and the blood-brain barrier was proven to be intact (37). Nevertheless, we cannot entirely exclude minor local injury as a contributing factor in the success of the immunization procedure.

The proposed trafficking of tumor cells from a local site to metastatic sites involves intravasation, cell arrest, and extravasation, during which a large number of tumor cells are destroyed mechanically (38). The low number of cells necessary to induce lethal tumors (200 i.c., 10,000 s.c.) in our model compared to other models (11, 25, 26, 39) indicates a highly malignant potency and a poor immunogenicity of the G3.12/BM2 cell line.

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Fig. 11. Proliferation of splenocytes (unseparated fraction, left graphs; T-cell enriched fraction, right graphs) after 7 days without (top graphs), with allogeneic (middle graphs), or with syngeneic (bottom graphs) stimulation. Control group splenocytes (○) show less (P < 0.05) proliferation than vaccine group splenocytes (high-dose vaccine, △; low-dose vaccine, ×). T-cell enriched fraction shows more (P < 0.05) proliferation than unseparated fraction in the "no stimulation" group and equal proliferation to unseparated splenocytes in the stimulation group (P > 0.05). For maximal proliferation, splenocytes of immunized mice did not need stimulator cells, /./m', means of triplicates with SD.
We anticipated that local therapy, such as systemic or regional administration of cytokines, might be required in addition to systemic immunization. Our first experiments with a murine melanoma lysate similar to the human theraccine met with failure. Lysates were not sufficient to prevent brain metastases, either alone, or in combination with systemically or regionally delivered cytokines. A possible explanation for this result is that murine T-cells may prefer tumor-associated antigens presented on whole cells rather than lysates, such as irradiated tumor cells or coated beads (40). In any event, we found that irradiated whole tumor cells could best be made to generate specific immunity; s.c. tumor cell challenge was an important step in our immunization procedure that could not be omitted because we had to prove the effectiveness of our vaccine before i.c. tumor cell challenge. Both s.c. and i.p. routes of vaccine injection were equally effective, as measured in vitro and by in vivo challenge. The effect of s.c. administered active-specific immunotherapy has been described in various B16 melanoma models (13, 34, 35, 41), whereas i.p. vaccine injections are less common (12, 42, 43). An allogeneic component (Cloudborn) was included to mimic the human theraccine, which contained alloantigens. Allostimulation might conceivably add to the immunostimulatory effects of a vaccine, by generating additional cytokines from other T-helper cells in the same milieu. Recent support for this view has come from studies with allogeneic mouse fibroblasts transfected with allogeneic melanoma antigens. These were highly effective immunogens (12). Whether the allogeneic component of our vaccine is necessary for most effective syngeneic immunization is being studied in follow-up investigations, stimulated by the effectiveness of our described immunization regimen.

To cause successful immunization and consequent protection of the mice against s.c. and i.c. challenges with viable tumor cells, it was required to pretreat the irradiated melanoma cells with IFN-γ. The expression of H-2 antigens was significantly increased by the maneuver. This probably contributed to the enhanced immunogenicity of the weakly immunogenic B16 (G3.12) cells (44, 45). Poor expression of H-2 antigens has been associated with loss of immunogenicity, and facilitation of tumor growth and metastasis (46–48). Tumor-associated antigens (49, 50) and accessory molecules (51) on tumor cells are also increased in their expression by IFN-γ, which further augmented the immunogenicity of the pretreated melanoma cells. Up-regulation of H-2 molecules, tumor-associated antigens, and accessory molecules on autologous tumor cells in vivo, such as by IFN-γ, all can contribute to increased sensitivity of the tumor to destruction by class I-restricted cytolytic T-cells.

Histological observations on brain metastases from 6 melanoma patients whose disease was otherwise controlled by systemic immunotherapy (8) indicated that impaired movement of lymphocytes into the brain could not fully explain why metastases had not been rejected. The “blood–brain barrier” to the immune response was incomplete at its best, since lymphocytes and macrophages were present. Although the melanoma cells appeared intact and viable, macrophages and astrocytes, which are antigen-presenting cells in the central nervous system (52–54), were both “unactivated” appearing.

It was thus possible that local production and/or movement of systemic lymphokines into the brain was also involved in the functional “blood–brain barrier.” Failure of “activation” of antigen-presenting cells involving the lack of MHC class II molecules may in part explain why the central nervous system seems to be an immunological sanctuary for metastases, as has been observed earlier (55). Activated T-cells pass freely into the brain from the systemic circulation, but leave equally readily (52). The function of MHC class II-bearing antigen-presenting cells, such as astrocytes and macrophages, in the CNS, may be to reintroduce such cells to the specific antigen against which they were originally sensitized and thus cause them to remain (52). Cytokines may be required for the adequate treatment of existing brain metastases. Those that act upon T-cells and macrophages, such as IFN-γ, IL-1, IL-2, and tumor necrosis factor-α, may be the best candidates in this regard.

In any event, we found that active specific immunization alone led to protection against a subsequent i.c. tumor challenge. Immunohistology showed that the melanoma was completely rejected in the immunized mice, but the precise effect of T-cells and macrophages in the process was impossible to discern by that means. Although T-cells and macrophages were found in the lesions, the number of infiltrating cells was low in the vaccinated mice. Gliosis was present in both vaccinated and nonvaccinated mice. These findings (except for gliosis) are very similar to those made with s.c. nodules of melanoma patients who rejected those lesions after active specific immunotherapy (22). Since we have not done weekly serial analyses with immunohistology, it is possible that earlier lesions might have shown a greater infiltration with immune cells than those we have examined later (20 to 90 days after i.c. injection) in the course. Tumor-specific T-cells were present in abundance in the spleen after immunization. Regardless of histology, rejection as a result of active immunization indicates that a sufficient number of sensitized T-cells (helper and/or cytotoxic) must have entered the brain to cause eradication of the tumor.

In conclusion, a vaccine composed of irradiated IFN-γ-pretreated G3.12 (B16 subclone) and Cloudman cells, and the unspecific adjuvant DETOX, was sufficient of itself to generate systemic immunity against melanoma. It improved survival after i.c. melanoma challenge, compared to controls, and could prevent brain metastasis in 69% of the mice. This implies that sufficient numbers of immune cells are capable of crossing the blood–brain barrier and remain there to cause tumor rejection. Whether this strategy will prove successful by itself with established brain metastases remains to be seen. It may be necessary to add other modalities as radiosurgery (32), chemotherapy (56), or regionally administered cytokines to bring about rejection of such established lesions.

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