Cancer Immunol Immunother (1995) 41: 227–235

ORIGINAL ARTICLE

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Monocyte chemoattractant protein-1 (MCP-1) gene transduction: an effective tumor vaccine strategy for non-intracranial tumors

Received: 14 July 1995 / Accepted: 22 August 1995

Abstract Recently, there has been renewed interest in the concept of tumor vaccines using genetically engineered tumor cells expressing a variety of cytokines to increase their immunogenicity. Human MCP-1 (JE) is a potent chemoattractant and activator of monocytes and T lymphocytes and thus a good candidate gene for a tumor vaccine. We therefore evaluated the efficacy of vaccines consisting of irradiated tumor cells transduced with the murine MCP-1 gene in the syngeneic 9L gliosarcoma brain tumor model. 9L cell lines stably expressing murine MCP-1 (9L-JE) and control cell lines expressing neomycin 3′ phosphotransferase (9L-Neo) were generated by infection with a Moloney murine leukemia retroviral vector. Fisher 344 rats were immunized with intradermal injections of 5×10^5 or 2×10^6 irradiated (5000 cGy) 9L-JE, 9L-Neo, and wild-type 9L (9L-WT) cells. Two weeks later immunized and non-immunized animals were challenged with varyious doses of intradermal (5×10^6–5×10^7) or intracerebral (2×10^4–5×10^5) 9L-WT cells. Intradermal tumors grew in all non-immunized animals. No tumors grew in animals immunized with irradiated 9L-JE or 9L-Neo cells and challenged with inocula of fewer than 5×10^5 9L-WT cells. With higher inocula up to 10^7 cells, tumors appeared in all the animals, but subsequently regressed in the immunized animals. Tumors in animals immunized with 9L-JE were always smaller than tumors in the other groups. In addition, only the 9L-JE vaccine protected against tumor inocula of 5×10^7 cells. Thus vaccination with MCP-1-expressing cells was able to protect animals against at least a 100-fold larger number of challenge tumor cells than vaccination with control cells. In contrast to studies with intradermal tumors, immunization with 9L-JE and 9L-Neo produced only minimal protection against intracerebral tumors. There was no significant difference between the 9L-JE and 9L-Neo vaccines in intracerebral challenge. This study suggests that tumor vaccines expressing cytokine genes such as MCP-1 can increase the antitumor response. However, the protective effect of these vaccines appears to be largely limited to intradermal tumors rather than intracerebral tumors.

Key words Tumor vaccine · MCP-1 · Malignant glioma · 9L gliosarcoma model

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Introduction

Despite optimal treatment with surgery, radiotherapy, and chemotherapy, the prognosis for patients with malignant gliomas remains poor with a median survival of 9–12 months for patients with glioblastoma and 24–36 months for patients with anaplastic astrocytomas [29]. The disappointing results of current therapy, together with progress in gene transfer techniques, has led to renewed interest in immunotherapy for the treatment of patients with malignant gliomas.

Early studies using autologous tumor cells as vaccines to augment antitumor immunity showed little benefit [4, 18], possibly because tumor antigens either do not exist or evoke poor immune responses in the host [31, 33, 37]. Strategies to improve the efficacy of these vaccines, including the use of non-specific immunostimulants such as bacillus Calmette-Guerin, levamisole and *Corynebacterium parvum*, were also ineffective [22, 45]. Recently, there has been renewed interest in the concept of tumor vaccines using genetically engineered tumor cells expressing a variety of cytokines to increase their immunogenicity [10, 32, 33, 37, 40]. Tumor cells transduced with genes for

interleukin-2 (IL-2) [11, 16], IL-4 [17, 41], IL-6 [34], IL-7 [1], interferon γ [34, 44], and granulocyte/macrophage-colony-stimulating factor [13] have been shown to protect immunized animals against subsequent challenge with parental tumor cells and occasionally to reduce tumor progression and cure animals of pre-established tumors [37, 40, 43]. These encouraging results in animal studies have led to the establishment of clinical trials using genetically engineered tumor vaccines for the treatment of a variety of human cancers [37, 40].

Monocyte chemoattractant protein-1 (MCP-1), a member of the chemokine-β (or C-C) family of cytokines [14, 36, 38], is a potent chemoattractant and activator of monocytes [23] and a chemoattractant of T lymphocytes [7]. MCP-1 is produced by malignant glioma cells in vitro and in vivo and may play an important role in promoting monocyte/macrophage infiltration of these tumors [12, 26]. A vaccine consisting of tumor cells expressing MCP-1 has the potential to increase local recruitment and activation of antigen-presenting mononuclear cells, resulting in a more effective priming of the antitumor response. In this study we evaluated the efficacy of a vaccine consisting of tumor cells expressing murine MCP-1 [14] in the syngeneic 9L gliosarcoma rat brain tumor model.

Materials and methods

Tumor cell lines and animals

The 9L gliosarcoma line was kindly provided by Dr. Peter Black (Brigham and Women's Hospital, Boston, Mass.), and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. MCP-1-producing 10A-2 Chinese hamster ovary (CHO) cells and non-expressing 0A-2 CHO cells [35] were grown in the α modification of minimal essential medium (MEM-α), without ribonucleosides and deoxyribonucleosides, supplemented with 10% bovine calf serum and 10 μg/ml each of adenosine, deoxyadenosine, and thymidine.

Adult male Fischer 344 (CDF) rats (150–175 g) were purchased from Charles River Laboratories (Wilmington, Mass.).

Expression vector construction

The pMV7 vector was kindly provided by Dr. Gerard M. Housey (Columbia University, New York, N.Y.). A 608-bp *Eco*RI insert of mouse JE (the original designation for MCP-1) cDNA from pc-JE1 [36] was cloned into the *Eco*RI site of pMV7. This plasmid was designated as pMV7-mJE. In pMV7-mJE the MCP-1 cDNA is located just 3′, and transcribed from the murine Moloney leukemia virus long terminal repeat. The neomycin 3′ phosphotransferase gene is transcribed from a thymidine kinase promoter (tk).

Retroviral transfection

The PA317 retrovirus packaging cell lines was obtained from ATCC (Rockville, Md.) and grown in DMEM with 4.5 g/l glucose supplemented with 10% fetal bovine serum. Before transfection, PA 317 cells were selected in HAT medium (30 μM hypoxanthine/1 μM aminopterin/20 μM thymidine) for 5 days, and then in 30 μM hypoxanthine/20 μM thymidine for 4 days. The cells were transfected with the plasmids pMV7 or pMV7-mJE using standard calcium phosphate coprecipitation. After incubation for 72 h, the culture supernatant was filtered (0.45 μm). A 1-ml sample of this virion containing medium (a titer of 10^4 geneticin^R cfu/ml) was added to each dish of 9L target cells (10^3 cells/dish) and incubated for 24 h. The retrofected 9L cells were then selected for 2 weeks in the presence of 400 μg/ml (active drug) geneticin sulfate (Gibco BRL, Gaithersburg, Md.). Polyclonal populations of 9L-Neo (transfected by pMV7) and 9L-JE (pMV7-mJE) cells were characterized by Northern blot analysis.

Northern blot analysis

Total cellular RNA was purified by the guanidine isothiocyanate/cesium chloride technique [8], and analyzed (20 μg) by electrophoresis through 1% agarose/formaldehyde gels and then transfered to nitrocellulose filters and hybridized to the horseradish-peroxidase-conjugated DNA probes. The following probes were used: (a) a 608-bp *Eco*RI fragment of the mJE cDNA from the pc-JE1 plasmid, (b) a 1311-bp *Hin*d III fragment sequence of the neomycin 3′ phosphotransferase cDNA from p1 Aneo plasmid (kindly provided by Dr. Earl Ruley, Massachusetts Institute of Technology, Mass.), (c) a 2.0-kb *Pst*I insert of a chicken β-actin gene purified from the pA1 plasmid [9]. Hybridizations were performed at 42 °C for 6 h and signals were detected using ECL direct nucleic acid labeling and detection systems (Amersham, Arlington Heights, Ill.).

Western blot analysis

After 9L, 9L-Neo, and 9L-JE cells had been grown in serum-free medium for 12 h, the culture medium was removed and proteins were concentrated up to 20 times using Centriprep-10 columns (Amicon Inc., Beverly, Mass.). Western blots were performed 1 h after semi-dry transfer of 15% sodium dodecyl sulfate/polyacrylamide gels to nitrocellulose (S&S NC BA85, Keen, N.H.). Blots were blocked with phosphate-buffered saline (PBS) and 5% powdered milk for 1 h at room temperature and then incubated for 2 h with the primary antibody (rabbit serum against murine MCP-1) at a dilution of 1:500 in PBS/Tween (0.05% Tween 20). After several washings in PBS/Tween, the blots were incubated for 1 h with horseradish-peroxidase-conjugated goat anti-(rabbit IgG) antibody (Sigma, St. Louis, Mo.) at a 1:1000 dilution in PBS/Tween with 5% powdered milk, then developed with the ECL Western blotting analysis system (Amersham).

Monocyte chemotaxis assay

Confluent monolayers of 9L, 9L-JE, and 9L-Neo were incubated in serum-free DMEM for 24 h. The media were collected and centrifuged to remove cells and debris. Supernatants were used for assay and the remaining adherent cells were trypsinized and counted. In vitro migration of monocytes was assayed by the micropore filter technique as described previously [6] using a 48-well microchamber. Briefly, human peripheral blood mononuclear cells (PBMC) were obtained from heparin-treated blood on Ficoll-Hypaque. Cells were adjusted to a concentration of 4×10^6 PBMC/ml in cold Gay's balanced salt solution (GBSS) (Gibco BRL) with 0.2% bovine serum albumin (BSA) (Sigma). Samples of 27 μl of varyious dilutions of culture medium were placed in the bottom compartment of the Boyden chamber, and 50 μl PBMC suspension in the upper compartment. A N-formyl-Met-Lev-Phe filter with 5-μm pores was used to separate the two chambers. The chamber was incubated for 90 min at 37 °C in 5% CO_2. In each experiment, 0.2% BSA in GBSS was used as the negative control and 10 nM FMLP (Sigma) was used as the positive standard. The filter was fixed by methanol and stained using a Diff-Quick staining set (Baxter). The amount of MCP-1 protein was calculated by comparison to standard medium from 10A-2 CHO cells expressing MCP-1 [35].

Tumor vaccine

Freshly prepared cell suspensions were used for tumor vaccines. Tumor cells were trypsinized, washed once with culture medium, twice with PBS and then suspended in PBS. The cells were irradiated with 50 Gy using a Gamma Cell 1000 (Atomic Energy of Canada Ltd., Ontario, Canada) with a ^{137}Cs source emitting a fixed dose rate of 14.3 Gy. A dose of 50 Gy was determined from preliminary studies to be the lowest dose of radiation necessary to prevent tumor growth. Fisher 344 rats were immunized with irradiated tumor cells in 100 µl PBS injected intradermally into the right flank. Because the 9L gliosarcoma model is immunogenic, a problem common to virtually all brain tumor models, the protective effect of a vaccine consisting of 9L-JE cells was compared to that of vaccines consisting of 9L cells expressing the intracellular selectable marker neomycin 3′ phosphotransferase (9L-Neo) and wild-type 9L cells (9L-WT). Two weeks later, the immunized animals, together with an additional group of non-immunized rats, were challenged (on the opposite side to the vaccine injection with single intradermal injections of varyious numbers of 9L-WT cells. Tumor size was measured at regular intervals by the same observer who was blinded to the treatment. Tumor volume was calculated by multiplying the values of three perpendicular diameters. Statistical significance between different groups was determined using the two-sample *t*-test.

Implantation of intracerebral tumors

9L tumors were stereotaxically implanted into the right caudate nucleus of Fischer 344 rats using a modification of the method of Kobayashi [24]. In brief, male Fischer 344 rats (150–175 g) were anesthetized with an intraperitoneal injection of pentobarbital (40 mg/kg) and placed in a small-animal stereotaxis frame (Kopf Instruments, Tujunga, Calif.). A sagittal incision was made through the scalp to expose the skull and a small burr hole made 1.5 mm posterior and 4 mm to the right of the bregma. 9L cells were prepared for injection into animals by harvesting cells from tissue-culture dishes, followed by centrifugation with aspiration of media/serum from cells, followed by repetitive washing of cells with PBS to remove any remaining serum. A sample of 2×10^4 tumor cells, suspended in 10 µl PBS, was injected with a 701 Hamilton syringe over 30 s to a depth of 4.5 mm. The needle was left in place for 1 min and then withdrawn slowly. The hole in the skull was plugged with bone wax and the incision closed with surgical clips (Ethicon Plus). This method resulted in a 100% yield of intracerebral tumors with relatively little leptomeningeal or intraventricular spread.

Tissue preparation and immunohistochemistry

After the animals had been sacrificed, the brains and intradermal tumors were removed and snap-frozen in isopentane cooled by liquid nitrogen. Sections of 8 µm were cut, placed onto poly-D-lysine-coated slides and fixed in absolute methanol (2 min at –20 °C). Immunocytochemistry was carried out using the monoclonal antibodies YS-RTMC341 (rat monocytes/macrophages), MAS-1131 (rat CD4+ T cells), and ACL-004 (rat CD8+ T cells and natural killer cells) (Accurate, N.Y.). These antibodies were used in the form of ascites fluid. Monoclonal antibodies were detected using peroxidase-conjugated rabbit anti-(mouse IgG) and FAST DAB (Sigma). In brief, after inactivation of internal peroxidase by methanol/hydrogen peroxide, the sections were incubated serially with 5% BSA/TRIS (30 min), primary antibody (2 h at room temperature), 5% BSA/TRIS (30 min), secondary antibody (30 min), and 0.05% diaminobenzidine tetrahydrochloride (DAB) with 0.01% hydrogen peroxide (5 min). After thorough washing in TRIS buffer, the sections were counter-stained in hematoxylin, dehydrated and mounted in Permount (Fisher Scientific, Pittsburgh, Pa.). The sections were examined using a Nikon Optiphot microscope equipped with differential interference contrast ("Nomarski") optics.

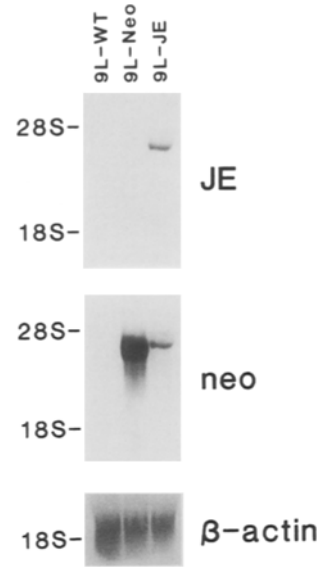

Fig. 1 Expression of the MCP-1/JE gene in 9L cells infected with pMV7-JE (*9L-JE*). 9L cells were selected in geneticin sulfate and analyzed for MCP-1/JE expression by Northern blotting. Wild-type 9L cells (*9L-WT*) and 9L cells expressing neomycin 3′ phosphotransferase (*9L-Neo*) were used as controls. Total cellular RNA (20 µg) from each clone was hybridized to the MCP-1/JE (*JE*) and neomycin 3′ phosphotransferase (*neo*) probes. Hybridization to the β-actin probe demonstrated equal loading of the lanes

Results

Engineered expression of JE/MCP-1 in 9L cells

The pMV7-mJE plasmid or the control pMV7 plasmid were transfected into the murine amphotropic retroviral packaging cell line PA317, and the virus-containing supernatants were used to infect 9L cells. Polyclonal geneticin selection was performed and the selected population of cells were designated 9L-JE or 9L-Neo. Northern blots showed that parental 9L (9L-WT) and 9L-Neo cells expressed only low or undetectable levels of mMCP-1 (Fig. 1). In contrast, the cells retrofected with MCP-1 cDNA expressed high levels of MCP-1 transcripts. The size of the MCP-1 is larger than that of the endogenous MCP-1 RNA since the gene is transcribed/translated from the full-length "genomic" viral transcript. The neomycin 3′ phosphotransferase gene was expressed in both the 9L-Neo and 9L-JE clones.

In Western blots, a protein with a molecular mass of 27–39 kDa was detected in culture supernatant of the 9L-JE cell line (Fig. 2). This pattern of reactivity was similar to that obtained with natural MCP-1 protein and the protein expressed in COS cell expression systems [36]. The wide range of molecular mass of this protein results from different patterns of glycosylation [14].

The monocyte chemotaxis assay was used to evaluate the activity of the secreted protein. Comparison of the numbers of monocytes attracted by proteins secreted by the 9L-JE cells (10^7 cells, 24 h) revealed an activity

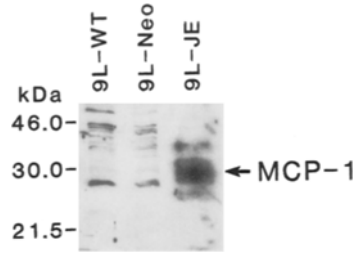

Fig. 2 Detection of secreted MCP-1 in concentrated culture media of 9L-JE cells by Western blot analysis. After 15% sodium dodecyl sulfate/polyacrylamide gel electrophoresis and Western transfer, nitrocellulose paper was incubated with rabbit serum against MCP-1 protein and developed using the ECL system

equivalent to culture medium containing 50 ng/ml MCP-1 protein. The amount of MCP-1 secreted by the retrofected 9L-JE cells, in which the MCP-1 gene is driven by the murine Moloney leukemia virus long terminal repeat, compared favorably to that secreted by mJE 9L transfectants driven by a variety of different promoters, including the simian virus 40 early gene promotor, the cytomegalovirus early gene promotor, the glial fibrillary acid protein promotor and the myelin basic protein promotor (data not shown).

Growth of 9L-MCP-1 cells in vivo

Although in some tumor models MCP-1 had a direct antitumor effect [2, 35], there was no significant difference in the rate of growth of 9L-JE and 9L-Neo cells in the skin of Fisher 344 rats (data not shown). Similarly, there was no difference in survival of Fisher 344 rats implanted intracerebrally with either 9L-JE or 9L-Neo cells (2×10^4 cells/rat; ten rats per group). These studies suggest that high-level expression of the MCP-1 gene does not itself result in rejection of live 9L tumor cells in either the skin or the brain.

To determine whether the failure of expression of the MCP-1/JE gene to suppress 9L cell growth was due to inadequate secretion of MCP-1, we performed a co-injection experiment in which 9L cells were mixed with MCP-1-producing 10A-2 CHO cells. 10A-2 CHO cells had been shown previously to prevent tumor formation when mixed with wild-type CHO cells in nude mice [36]. MCP-1-expressing 10A-2 cells and non-expressing 0A-2 [35] were co-injected with 9L-WT cells (8×10^5 9L-WT cells and 1.1×10^6 CHO cells) and the growth of the tumors determined. Although the tumors with 10A-2 cells had a significantly greater mononuclear cell infiltration than the control tumors, there was no significant difference in the size of intradermal tumors in the two groups after 1 month.

Since the above experiment is confounded by the variables of xenograft rejection of the CHO cells and the

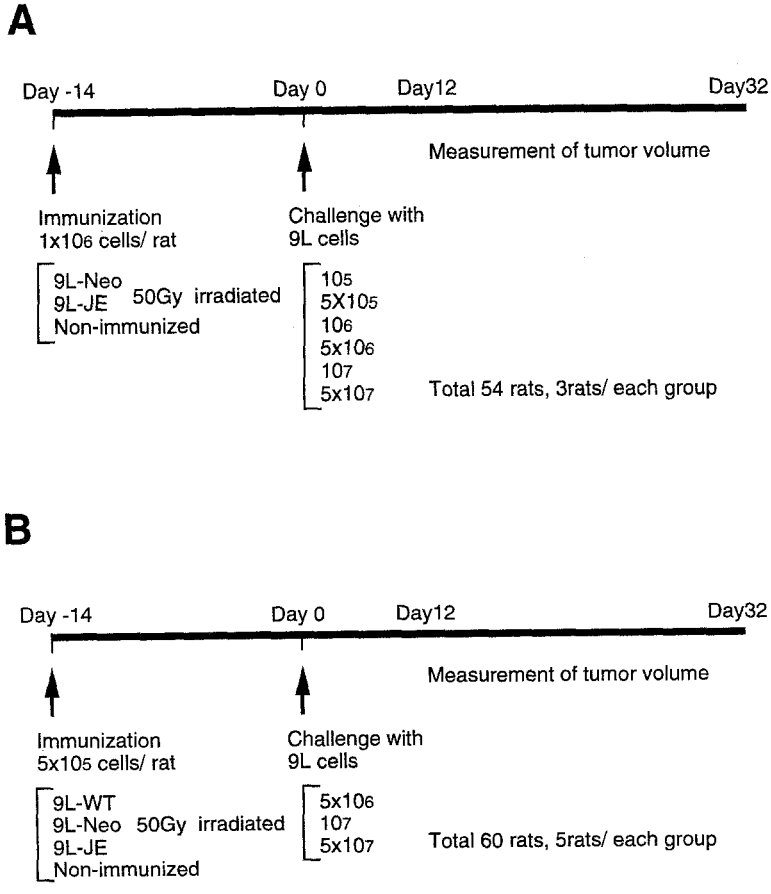

Fig. 3 **A** Vaccination and tumor challenge schedule for the initial set of intradermal tumor experiments. In this experiment, animals were immunized with 106 irradiated 9L-JE, 9L-Neo and 9L-WT cells and challenged with 9L-WT tumor cells. **B** Vaccination and tumor challenge schedule for the second set of intradermal tumor experiments. In this experiment, animals were immunized with a smaller number of irradiated 9L-JE, 9L-Neo and 9L-WT tumor cells (5×10^5) and challenged with larger numbers of 9L-WT tumor cells

Table 1 Average volumes of intradermal tumors on days 14 and 21 following different inocula of wild-type 9L challenge tumor cells in animals previously immunized with 9L-Neo, 9L-JE vaccines (10^6 irradiated cells) and in non-immunized animals

Immunization	Challenged cell number	Tumor volume (mm^3)	
		Day 14	Day 21
9L-Neo	10^5	0±0	0±0
	5×10^5	73±59	0±0
	10^6	131±106	0±0
	5×10^6	364±98	177±80
	10^7	639±88	594±178
	5×10^7	892±1004	2874±468
9L-JE	10^5	0±0	0±0
	5×10^5	0±0	0±0
	10^6	36±62	0±0
	5×10^6	16±23	0±0
	10^7	279±201	140±87
	5×10^7	197±117	85±55
Non-immunized	10^5	268±10	1003±238
	5×10^5	401±45	768±201
	10^6	421±66	1024±290
	5×10^6	704±31	1704±659
	10^7	1428±390	4968±465
	5×10^7	1428±119	5394±547

potential limited duration of survival of the xenotropic CHO cells in the subdermal tissues, we repeated the mixing experiment in rat brain. Previous work has documented extended survival of xenotropic grafts when placed within the context of the immunoprivileged CNS. Twelve rats were co-injected intracerebrally with 2×10^4 9L-WT cells and either 4×10^4 10A-2 or 0A-2 cells. Although a clear mononuclear infiltrate was seen in the tumors of these rats, there was no difference in survival between the two groups (data not shown).

Protective effect of 9L-JE vaccine against intradermal tumors

There is increasing evidence that irradiated tumor cells engineered to secrete a variety of cytokines stimulate long-lasting antitumor immunity [13, 21, 41]. Since MCP-1 is a potent chemoattractant and activator of monocytes and T lymphocytes, we hypothesized that vaccination using tumor cells expressing MCP-1 may increase local recruitment and activation of antigen-presenting mononuclear cells, resulting in effective priming of the antitumor response. 9L tumors are thought to be immunogenic to syngeneic rats since they are often associated with an inflammatory infiltrate and are occasionally spontaneously rejected [39]. Such immunogenic tumor models require careful titration of the number of cells used for vaccination and rechallenge in order to assess accurately the ability of any manipulation to augment tumor immunity beyond that attributable to the tumor cell itself [40]. In the first set of experiments, animals were immunized with 10^6 irradiated 9L-JE, 9L-Neo and 9L-WT cells and challenged with varyious numbers of 9L-WT tumor cells (10^5–5×10^7 cells) (Fig. 3A). Intradermal 9L tumors grew in all non-immunized animals (Table 1). With inocula of up to 5×10^5 cells, tumors did not grow in animals immunized with irradiated 9L-JE or 9L-Neo cells. Using inocula of 10^6, 5×10^6, and 10^7 cells, tumors appeared in all immunized animals, but subsequently regressed, with tumor rejection occurring most quickly in the 9L-JE-vaccinated animals. When animals were challenged with 5×10^7 cells, however, only animals vaccinated with the 9L-JE cells demonstrated tumor rejection.

In the second set of experiments, we used fewer cells for vaccination (5×10^5 cells) to determine if the 9L-JE vaccine could provide protection against a larger tumor challenge than the 9L-Neo vaccine (Fig. 3B). An additional control group, consisting of animals immunized with irradiated 9L-WT cells, was included to determine whether the protective effect of 9L-Neo cells was due to the inherent immunogenicity of 9L cells or to the presence of a foreign gene (neomycin 3′ phosphotransferase). The results obtained were similar to those of the first experiment (Fig. 4). With inocula of 5×10^6 and 10^7 cells, tumors on day 12 were smaller in animals vaccinated with 9L-JE ($P < 0.05$) (Fig. 4A). Subsequently, tumors began to regress in all vaccinated animals, while they continued to grow in the non-vaccinated group. When animals were challenged with a tenfold larger number of tumor cells, smaller tumors were consistently observed in the animals vaccinated with 9L-JE compared to all other groups on days 12 ($P < 0.02$) and 23 ($P < 0.001$) (Fig. 4B). By day 32 all non-immunized and 9L-WT-immunized animals were dead from their tumors, and the 9L-Neo-vaccinated animals had rapidly growing tumors. By contrast, all 9L-JE-vaccinated animals had total regression of their tumors ($P < 0.02$). By day 40, all non-immunized, and 9L-WT- and 9L-Neo-vaccinated animals were dead from tumor, whereas all 9L-JE-vaccinated animals were well and without visible tumor. Long-term observation of these animals (for more than 360 days) revealed no tumor recurrence.

Characterization of the inflammatory infiltrate

Hematoxylin and eosin (H & E) staining of skin immunized with intradermal irradiated 9L-JE and 9L-Neo both showed tumor cells surrounded by inflammatory cells. The numbers of inflammatory cells were significantly greater in the skin sections immunized with 9L-JE compared with 9L-Neo. Immunocytochemical analysis of the 9L-JE inflammatory infiltrate showed that the predominant cells were monocytes/macrophages (Fig. 5), with only small numbers of $CD4^+$ and $CD8^+$ T lymphocytes. Though irradiated 9L-JE cells attracted many more monocytes macrophages than did 9L-Neo cells, there was no qualitative difference between the two groups in the numbers of $CD4^+$ (MAS1131) and $CD8^+$ (ACL004) T lymphocytes infiltrating.

H & E staining of 9L-WT tumors implanted into rats previously immunized with either 9L-JE or 9L-Neo showed an inflammatory infiltrate composed predominantly of

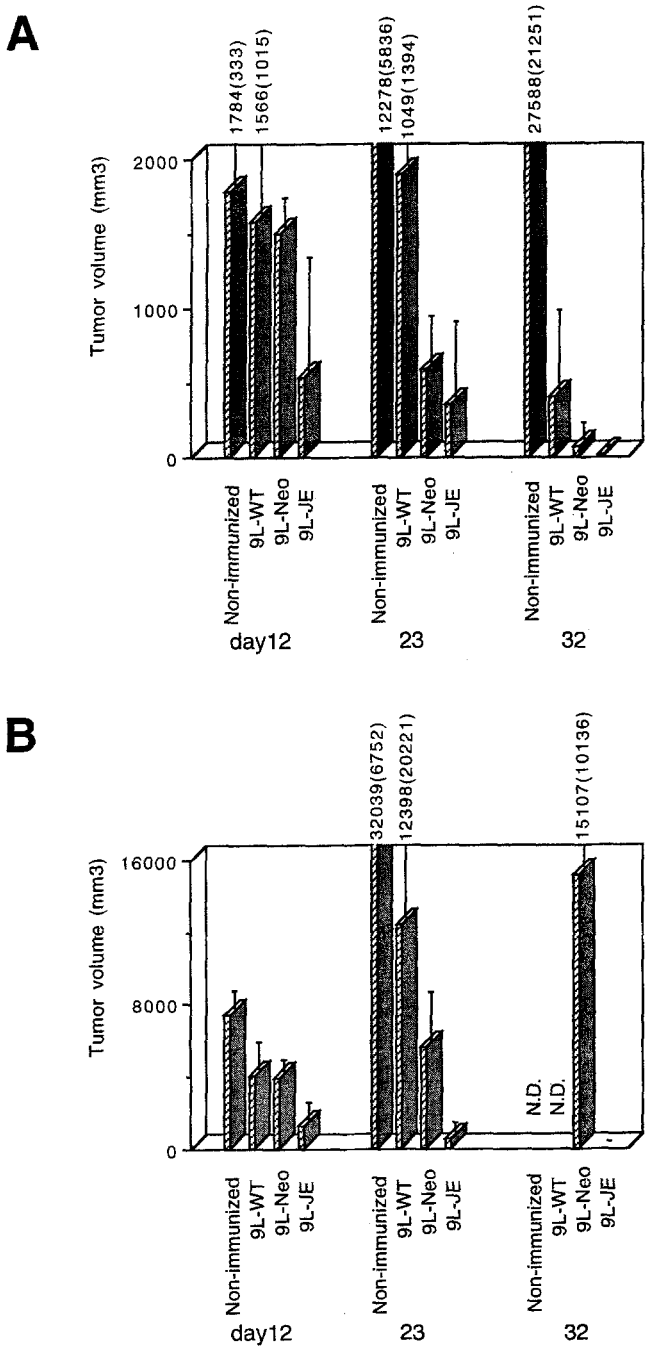

Fig. 4A, B Growth of intradermal 9L-WT cells in rats immunized with irradiated 9L-WT, 9L-Neo, and 9L-JE cells and non-immunized animals. **A** Rats challenged with 5×10^6 9L-WT cells. On day 12, tumors were smaller in animals vaccinated with 9L-JE than any other group ($P < 0.05$). By days 23 and 32, all vaccinated animals had smaller tumors than did the non-vaccinated group ($P < 0.05$). **B** Rats challenged with 5×10^7 cells. At all assay times tumors were smaller in the 9L-JE-vaccinated animals than in any of the other groups ($P < 0.02$). By day 40, all non-immunized animals and 9L-WT- and 9L-Neo-vaccinated animals were dead, while all 9L-JE-vaccinated animals were cured of their tumors (alive without tumors for more than 360 days). Tumor volumes shown represent the average for five rats. Bars or parentheses represent standard error of mean

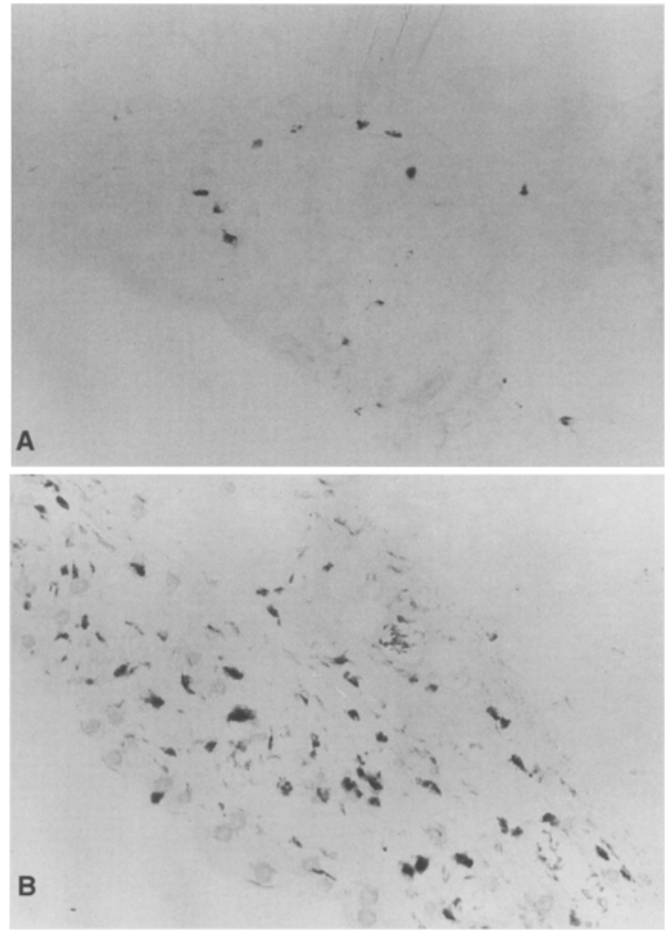

Fig. 5A, B Immunohistochemical staining of the immunization sites in the skin using the monoclonal antibody YS-RTMC34 against rat monocytes/macrophages. The skin had been inoculated 24 h previously with either irradiated 9L-Neo (**A**) or 9L-JE cells (**B**). 9L-JE vaccine attracted significantly more monocytes/macrophages than 9L-Neo vaccine. Magnification × 200

T lymphocytes, with a smaller number of monocytes. There was no difference in the nature of the tumor infiltrate in animals immunized by either 9L-JE or 9L-Neo.

Effect of 9L-JE vaccine on intracerebral tumors

To evaluate the protective effect of the 9L-JE vaccine against an intracerebral tumor challenge, Fischer 344 rats were immunized with intradermal injections of 5×10^5 irradiated 9L-JE and 9L-Neo tumor cells (Fig. 6A). Two weeks later the immunized animals, together with an additional group of non-immunized rats, were challenged with intracerebral injections of 9L-WT cells (2×10^5, 10^5, 5×10^5). As shown in Fig. 6B, although there were a few long-term survivors in the immunized group, the median survival of immunized animals was not significantly different from that of non-immunized animals. These intracerebral challenge experiments were repeated several times with similar results.

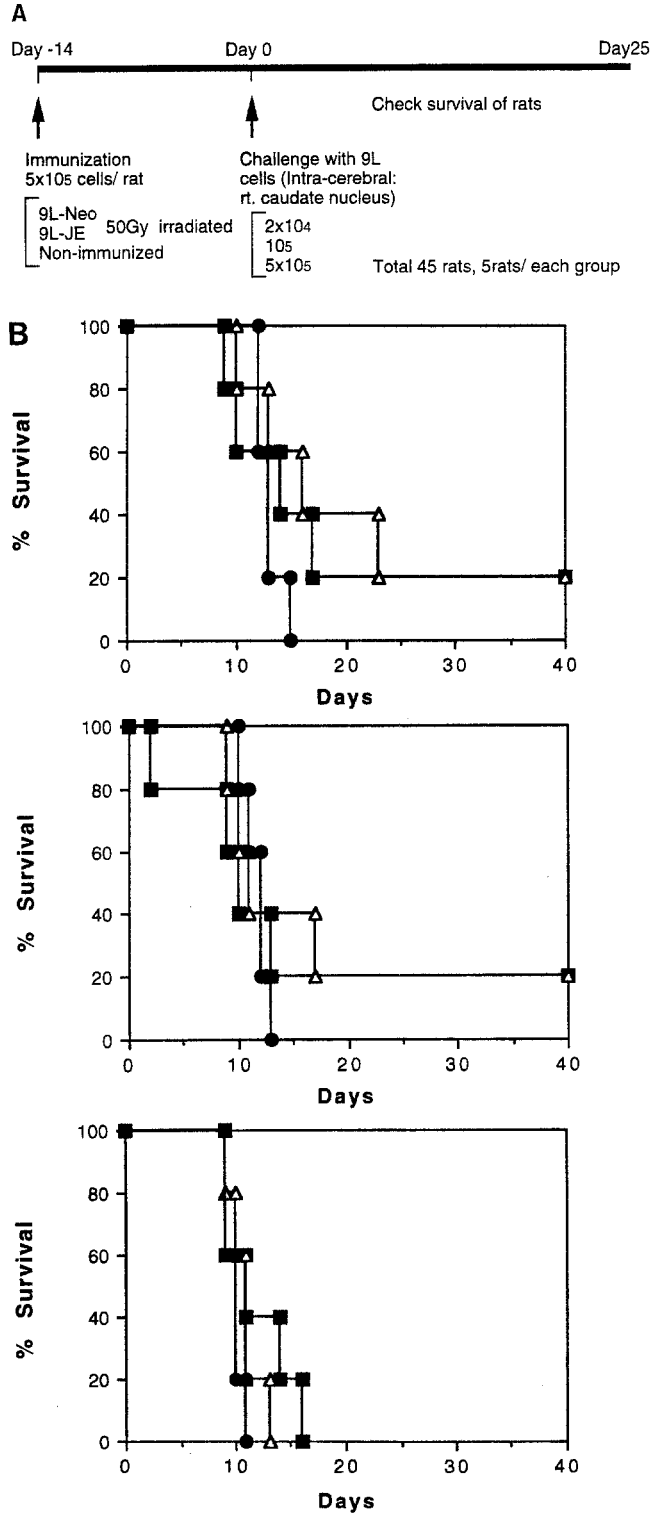

Fig. 6 A Vaccination and challenge schedule for intracerebral 9L tumors. Varyious numbers of 9L cells were stereotactically injected into the brains of non-immunized rats and rats immunized 14 days previously with either 2×10^5 irradiated 9L-Neo or 9L-JE cells. **B** Kaplan Meier graphs showing survival of rats with intracerebral 9L tumors. Group A received an intracerebral tumor challenge of 2×10^4 cells (*upper*), group B received 10^5 cells (*middle*), and group C received 5×10^5 cells (*lower*). There were a few long-term survivors (for more than 360 days) in the vaccinated animals; however, median survival was not significantly different in any of the groups. ● Non-immunized, ■ 9L-neo-immunized, Δ 9L-JE-immunized

Discussion

There has been long-standing interest in the use of tumor vaccines for the treatment of patients with malignant gliomas. Bloom and co-workers first attempted to induce an immune response in a patient with a malignant glioma by implanting autologous tumor cells in the thigh [4]. Unfortunately, no antitumor response was noted and tumor cells grew locally in the thigh and in draining lymph nodes. A year later, a similar study by Grace et al. yielded similar results, although a delayed hypersensitivity response to tumor extracts was detected in two of six patients [18]. More recent studies, using tumor vaccines consisting of irradiated autologous tumor cells alone [3] or in combination with Freund's adjuvant, BCG, and levamisole [30], have also been ineffective, although some studies have shown serological evidence of an immune response to the immunizing cell line [30]. The disappointing results with active immunotherapy may be due in part to the fact that tumor antigens generally evoke a very poor immune response in the tumor-bearing host [31, 33, 37]. The problem is compounded in glioma patients by the fact that these tumors are growing in the brain, an immunologically privileged environment [20, 28], and the tumor cells themselves produce immunosuppressive factors such as transforming growth factors (TGF) β2 [5], interleukin-1, and prostaglandins [25, 27, 45].

Recently there has been increasing evidence that the effectiveness of tumor vaccines can be significantly increased by genetically engineering tumor cells to express a variety of cytokines [10, 37, 40]. The precise mechanisms involved have yet to be clearly elucidated, and probably vary with on the cytokine. Most cytokines produce a nonspecific inflammatory response at the vaccine site, which eventually leads to augmentation of specific immunity dependent on $CD8^+$ T lymphocytes [19]. In this study we demonstrated that transfection of 9L cells with the MCP-1 gene did not lead to rejection of the genetically modified cells by their syngeneic hosts. This contrasts with the results in other tumor systems, such as CHO cells, in which transfection of the MCP-1 gene completely suppressed their ability to form tumors in nude mice [35]. It is impossible to explain the difference in sensitivity to MCP-1 between the different cell lines since the mechanism of MCP-1-induced tumor inhibition has not been elucidated. The simplest explanation is that the glioma cells were producing insufficient levels of MCP-1 to elicit tumor rejection. Although possible, this seems unlikely, given that the level of MCP-1 produced from the 9L JE cell lines (50 μg/ml) was sufficient to cause rejection of CHO cells. We also attempted to rule out insufficient MCP-1 production as a possible cause by performing co-injection of the 9L-JE cells with CHO cells that produce large amounts of MCP-1. The inability to slow tumor growth using this approach suggests that the level of MCP-1 expression was not the problem, although it could be argued that the survival of the CHO cells in the immunocompetent rats was too short to allow an antitumor effect. We suspect a

more plausible explanation for the ineffectiveness of 9L tumor growth inhibition in this system is the result of an intrinsic resistance to MCP-1-mediated tumor killing. The mechanism of this resistance remains unclear but could include inappropriate presentation of tumor antigens for MCP-1-mediated cell killing (although we know the appropriate antigens for lymphocyte-mediated cell killing are present; see below), intrinsic resistance to monocyte or possible natural killer (NK) cell mechanisms of cell killing (i.e. perforin), and/or secretion by glioma cells of substances that inhibit MCP-1-mediated cell killing (i.e. TGFβ).

While MCP-1 was unable to mediate a direct anti-9L tumor effect, we have demonstrated that a tumor vaccine, consisting of irradiated 9L cells retrofected with the MCP-1 gene, provides significant protection against subsequent challenge of wild-type intradermal tumor cells. Although the mechanism of this protective effect has yet to be elucidated, the immunohistochemistry was informative. Specifically, histological examination of the MCP-1-expressing vaccination site showed an increased inflammatory infiltrate consisting predominantly of monocytes and macrophages. This is in contrast to the histological picture seen in the regressing tumors, where the mononuclear cell infiltrate was predominantly of T cell origin. We believe this picture is consistent with the hypothesis that local secretion of MCP-1 by the tumor cells leads to recruitment and activation of antigen-presenting monocytes/macrophages [7]. This might allow for more efficient priming of a T-cell-mediated antitumor response than can be achieved following vaccination with irradiated wild-type cells.

Another important observation in these studies was that, despite the significant protective effect of the 9L-JE vaccine against intradermal challenge with wild-type tumor cells, we were unable to demonstrate a similar protective effect against intracerebral tumor challenge. Several factors may have contributed to this discrepancy. One simple explanation may be that the antigens presented on the 9L cell surface in the skin differ from those in the brain. It also is possible that the relative immunological privilege of the brain may have reduced the effectiveness of the antitumor immune response stimulated by the tumor vaccine. The microenvironment of the brain suppresses immune reactivity because of the relative absence of major histocompatibility complex (MHC) expression and the presence of the blood/brain barrier, which prevents conventional lymphocytic recirculation [15, 28]. Nevertheless, under certain circumstances, a significant inflammatory response can occur in the brain [15, 46]. Monocytes, in particular, appear to have the ability to enter the CNS easily in a number of pathological conditions such as experimental allergic encephalitis and multiple sclerosis. This was the rationale for initially evaluating the effectiveness of MCP-1 in a brain tumor model. Despite this potential ability of inflammatory cells to invade the CNS, the response may be attenuated enough to reduce the effectiveness of a tumor vaccine. Other factors contributing to the discrepant results of intradermal and intracerebral tumors may be the limitations of the 9L gliosarcoma model itself. Because of the restricted intracranial volume of rats, a therapeutic approach that depends on inducing an antitumor inflammatory response may lead to increased peritumoral edema, resulting in elevation of intracranial pressure and death of the animals from herniation before the tumors themselves can be effectively eradicated. A similar problem does not exist for skin tumors, which can reach a significant size before being gradually eliminated by the antitumor immune response. Another factor may be the immunogenicity of the 9L model itself [39].

Since a vaccine of 9L-Neo cells alone confers significant protection against subsequent tumor challenge, it may not be possible to demonstrate enhancement of immunogenicity by the 9L-JE vaccine without using much larger numbers of animals and many more subgroups. Unfortunately, the lack of non-immunogenic syngeneic brain tumor models (the other commonly utilized models such as C6 and RT-2 are equally immunogenic) complicates our ability to evaluate accurately the therapeutic potential of tumor vaccines and other forms of immunotherapy for brain tumors.

In summary, we have demonstrated that tumor vaccines genetically engineered to express MCP-1 provide significant protection against a subsequent tumor challenge for intradermal tumors, and warrant further evaluation. However, we were unable to demonstrate a similar protective effect against intracerebral tumors. This study also points out the necessity of using not only wild-type cells as a control in animal tumor vaccine studies, but also of using a cell line that expresses a non-specific, but potentially immunogenic gene like neo^R. In addition, these experiments demonstrate that strategies that generate effective systemic tumor immunity may not be protective against tumor development within the central nervous system. Nevertheless, further studies of vaccines expressing MCP-1 for the treatment of systemic tumors are warranted, including combining MCP-1 with other cytokines (e.g. interferon γ), immunomodulatory factors (e.g. T cell costimulators such as B7) [42], or cytotoxic therapies.

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