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Results of a phase 1 study utilizing monocyte-derived dendritic cells pulsed with tumor RNA in children and young adults with brain cancer[1]

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We conducted a phase 1 study of 9 pediatric patients with recurrent brain tumors using monocyte-derived dendritic cells pulsed with tumor RNA to produce antitumor vaccine (DC_{RNA}) preparations. The objectives of this study included (1) establishing safety and feasibility and (2) measuring changes in general, antigen-specific, and tumor-specific immune responses after DC_{RNA}. Dendritic cells were derived from freshly isolated monocytes after 7 days of culture with IL-4 and granulocyte-macrophage colony–stimulating factor, pulsed with autologous tumor RNA, and then cryopreserved. Patients received at least 3 vaccines, each consisting of an intravenous and an intradermal administration at biweekly intervals. The study showed that this method for producing and administering DC_{RNA} from a single leukapheresis product was both feasible and safe in this pediatric brain tumor population. Immune function at the time of enrollment into the study was impaired in all patients tested. While humoral responses to recall antigens (diphtheria and tetanus) were intact in all patients, cellular responses to mitogen and recall antigens were below normal. Following DC_{RNA} vaccine, 2 of 7 patients showed stable clinical disease and 1 of 7 showed a partial response. Two of 7 patients who were tested showed a tumor-specific immune response to DC_{RNA}. This study showed that DC_{RNA} vaccines are both safe and feasible in children with tumors of the central nervous system with a single leukapheresis. *Neuro-Oncology 6, 236–246, 2004 (Posted to Neuro-Oncology [serial online], Doc. 03-066, June 4, 2004. URL http://neuro-oncology.mc.duke.edu; DOI: 10.1215/S1152851703000668)*

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[4] Abbreviations used are as follows: DC, dendritic cell; DC_{RNA}, vaccine prepared from dendritic cells pulsed with tumor-derived RNA; DT, diphtheria and tetanus vaccine; EAE, experimental allergic encephalitis; ELISA, enzyme-linked immunosorbent assay; HLA, human leukocyte antigen; HRP, horseradish peroxidase; ID, intradermally; Ig, immunoglobulin; IV, intravenously; MoDC, monocyte-derived dendritic cell; PBMC, peripheral blood mononuclear cell; PBS, phosphate-buffered saline; PHA, phytohemagglutinin; TMB, 3,3',5,5-tetramethylbenzidine.

Immunotherapy approaches for treating brain tumors present unique challenges compared to those for treating other types of cancer: the brain is considered an immune privileged site, and concern exists around the possibility of inducing experimental allergic encephalitis (EAE)[4] when CNS tumor material is used as an antigen source. Despite these challenges, it is clear that the dismal prognosis for patients with relapsed cancers of the CNS necessitates investigation into novel therapies.

The concept of immune privilege for the brain was formulated early in the twentieth century (Murphy and Sturm, 1923; Shirai, 1923). More recently, a variety of contemporary studies show that the immune system has access to the brain but that the qualitative components of effector responses may differ from those of a systemic immune response.

A number of previous animal studies have suggested that immunotherapy approaches to treating brain cancers may be successful. Early tumor vaccine studies in humans used allogeneic brain tumor cell lines as antigens. These studies showed that patients mounted humoral responses to the vaccines, may have had longer survival rates than historical controls and, importantly, showed no signs of EAE (Bullard et al., 1985). More recently, it has been shown that tumor extracts or tumor RNA-pulsed dendritic cell (DC_{RNA}) preparations are effective as an anti–brain tumor vaccine and are able to protect mice from developing CNS tumors (Ashley et al., 1997).

A study utilizing dendritic cells (DCs) pulsed with peptides from autologous glioma cells in adult patients with glioblastoma multiforme and anaplastic astrocytoma yielded encouraging results (Yu et al., 2001). Four out of 7 patients showed induction of T cell cytotoxicity, while 2 of 4 patients who were subjected to additional surgery demonstrated cytotoxic and memory T-cell tumor infiltration (Porgador and Gilboa, 1995). In one study of pediatric patients with solid tumors, DCs were pulsed with autologous tumor lysate or keyhole limpet hemocyanin and then combined for administration to the patients. One of 10 patients showed significant tumor regression, 3 of 7 patients showed tumor-specific IFN-γ production, and 3 of 6 showed delayed-type hypersensitivity reactions to tumor lysate (Geiger et al., 2001).

We have conducted a phase 1 immunotherapy study using monocyte-derived dendritic cells (MoDCs) pulsed with tumor RNA in pediatric patients with recurrent brain tumors. Primary objectives were to evaluate safety, feasibility, and toxicity. Secondary objectives were to examine baseline immune function in children with advanced brain tumors and to assess the effect of DC_{RNA} vaccination on tumor-specific immunity and other immune responses. The study showed that our methods for producing and administering DC_{RNA} vaccines were both safe and feasible. Sufficient amounts of tumor RNA were obtained in 8 of 9 patients, and in no case were symptoms of EAE or other autoimmune responses observed. Three of 7 patients who received vaccines had clinical responses to vaccination with DC_{RNA}: 1 with partial response and 2 with stable disease. Vaccination with DC_{RNA} did not elicit robust, tumor-specific immune responses but did increase cellular responses to other nonspecific stimuli. Our data suggest that children with recurrent cancer of the CNS may have impaired cellular immune responses at baseline, which may prevent the induction of cellular immune responses to "novel" antigens. These results may have major implications for patient selection in future studies utilizing immunotherapy with regard to evaluation of baseline immune function.

Patients and Methods

Study Design

A combined diphtheria and tetanus vaccine (DT) was administered to each patient 1 week prior to the first DC vaccine to assess recall immunity to known antigens. DC_{RNA} vaccines were administered to patients both intradermally (ID) and intravenously (IV) on weeks 0, 2, and 4. Patients with stable disease were eligible for 3 subsequent vaccinations at 3-month intervals if treatments were well tolerated and the available quantity of vaccine allowed. Subject to DC_{RNA} vaccine yields, a 3-tiered dose-escalation strategy using 3 cohorts of patients was planned for the IV route, with low, medium, and high doses of 0.5×10^7, 1.5×10^7, and 5.0×10^7 cells/m^2, respectively. The ID vaccine was delivered at a dose of 0.5×10^7cells/m^2 with a 25-gauge needle on a 1-ml syringe, and the site of administration was rotated for each vaccination. Patients were observed and vital signs monitored before, while, and for 30 min after each vaccine was given, with a 1-h interval between the 2 routes. Toxicity was monitored according to The National Cancer Institute Common Toxicity Criteria version 2.0 (NCI, 1999). Autoimmune serologies were followed to detect any subclinical evidence of immune dysregulation. Feasibility was determined according to whether sufficient vaccines for a patient could be generated to provide the first 3 DC_{RNA} treatments at the low dose (total 3.0×10^7 cells/m^2). Immunological, clinical, and MRI evaluations were performed at baseline, before and after the first 3 vaccinations, after each subsequent vaccination, and 1 month after the final vaccine. Standard response criteria for tumor response were used (Mitchell et al., 2001).

Eligibility Criteria

Protocols were reviewed and approved by the Ethics in Human Research Committee at The Royal Children's Hospital, Parkville, Victoria, Australia. Informed consent was obtained for all patients prior to enrollment. Eligible patients were <25 years with relapsed brain tumors that had failed standard therapies. Patients were required to have adequate performance status (Karnofsky > 50% or Lansky > 50) and organ function and to have recovered from previous treatments. A source of tumor was mandatory to obtain material for histological diagnosis and RNA extraction. Patients received no other treatments while on this study.

RNA Extraction from Tumor Tissue

Tumor tissue was frozen at –80°C immediately following surgical removal. Total RNA was extracted from the tumor tissue onto RNeasy Maxi columns (Qiagen GmbH, Hilden, Germany). Optional DNase treatment of an RNA column was performed by using RNase-free DNase Set (Qiagen) according to manufacturer's instructions. The RNA was eluted and stored in RNase-free water, and the RNA concentration was quantitated by spectroscopy. The quality of RNA was consistently high, with ratios of absorbance A_{260nm}/A_{280nm} between 1.8 and 2.0. RNA was stored at –80°C until required.

Leukapheresis Protocol

A peripheral blood monocyte count of 100 cells/mm^3 or greater was considered sufficient to proceed with leukapheresis. Using the Gambro Spectra automated continuous-flow cell separator (Version 6; Gambro, Lakewood, Col.), the MNC program was selected. For children <25kg, the system was primed with irradiated, filtered, packed red blood cells to maintain hemodynamic stability. Between 2 and 4 blood volumes were processed over approximately 3 to 5 h (calculation based on starting mononuclear cell count). Anticoagulant, acid citrate dextrose at 22 mg/1000 ml, was used at a ratio of 20:1 with blood. Ionized calcium was routinely measured and supplemented with a calcium gluconate IV infusion as required.

DC Generation from Peripheral Blood Monocytes

For generation of clinical-grade DCs, methods previously described by Romani et al. (1996) were used with modifications described by Heiser et al. (2000). Briefly, processing of blood through restricted peripheral blood leukapheresis was performed for each patient, and the product was received in the laboratory at room temperature. Cells were diluted 1:1 with sterile saline and further purified by using density gradient centrifugation over Ficoll-Hypaque Plus reagent (Pharmacia Biotech, Uppsala, Sweden), and cells were resuspended at 6.5×10^6cells/ml in serum-free AIM-V medium (Invitrogen, Carlsbad, Calif.). After 2 h of humidified incubation at 37°C, nonadherent cells were removed and cryopreserved for later assays. Adherent cells were cultured at 37°C in serum-free AIM-V medium containing human rhIL-4 at 25 ng/ml (R&D Systems, Minneapolis, Minn.) and human rhGM-CSF at 800 ng/ml (BD PharMingen, San Diego, Calif.). Following 7 days of culture, nonadherent cells were phenotyped by fluorescence-activated cell sorting analysis using fluorochrome-conjugated monoclonal antibodies to CD14-PE, human leukocyte antigen (HLA) DR-PC5, and CD11c-fluorescein isothiocyanate (Immunotech, Montreal, Canada). Cells were considered suitable for vaccine use if they were >50% HLA-DR+ and <15% CD14+ (Romani et al., 1996).

Pulsing of DCs with Tumor-Derived RNA

Pulsing of autologous DCs with RNA was performed by simple co-incubation (Heiser et al., 2000; Ashley et al., 1997). Briefly, DCs were washed twice with 0.9% NaCl solution (IV infusion-grade saline, Baxter, Deerfield, Ill.) and resuspended at 5×10^6 cells/ml in AIM-V medium (Invitrogen, Carlsbad, Calif.). Cells were co-incubated with 25 μg/ml tumor RNA in a humidified incubator at 37°C for 45 min. The DC_{RNA} cells were washed twice with saline (Baxter) and resuspended in freezing medium containing 80% autologous plasma, 10% saline, and 10% Cryoserv (Edwards Lifesciences, Irvine, Calif.). Aliquots of 10 to 20×10^6 viable cells per milliliter were frozen in a controlled-rate freezer (KYRO 10 series III; Planer Products Ltd., London, U.K.).

Lymphocyte Phenotyping

Lymphocyte subsets were determined by using the Simultest IMK-Lymphocyte Kit (Becton Dickinson, Franklin Lakes, N.J.) performed as a clinical test by the Immunology Laboratory at the Royal Children's Hospital. Briefly, peripheral blood samples were stored at room temperature for no longer than 24 h before being assayed. One hundred microliters of whole blood was used for each test and incubated with fluorochrome-conjugated antibodies against CD19, CD4, CD8, or CD16+CD56 for 30 min in the dark at room temperature. After the red blood cells were lysed with lyse solution (0.8 % NH_4Cl buffer) for 10 min in the dark, the samples were centrifuged for 5 min and washed once with phosphate-buffered saline (PBS). Samples were then analyzed on a Becton Dickinson FacSCAN flow cytometer.

Cellular Proliferative Response

Mitogen-induced cellular proliferation was assessed by standard ^{3}H-thymidine uptake, as previously described (Hosking and Balloch, 1983). Briefly, peripheral blood mononuclear cells (PBMCs; 1×10^5/well) were cultured in AIM-V media supplemented with β-mercaptoethanol (5×10^{-5} M) (Sigma, St. Louis, Mo.) in a 96-well plate for 72 h with (stimulated) or without (unstimulated) phytohemagglutinin (PHA; 30 μg/ml final concentration). Two hours prior to harvesting, 1 μCi ^{3}H-thymidine (Amersham, Buckinghamshire, U.K.) was added to each well. Cells were harvested at 72 h, and radioactivity was measured in a scintillation counter (TopCount-NXT, Packard Biosciences, Meriden, Conn.). Results were expressed as an average number of counts per minute.

Total Immunoglobulin Levels

Antibodies for the immunoglobulin (Ig) classes IgG, IgA, and IgM were analyzed at enrollment to determine if this cohort of patients had normal levels of antibodies prior to vaccination with $DC_{RNA.}$ Patient serum samples were stored at 4°C until total IgG, IgA, and IgM were measured by standard nephelometry (Beckman Array, Beckman

Coulter, Hialeah, Fla.). Normal ranges were established as previously described (Shelton et al., 1974).

Specific Antibodies to Tetanus and Diphtheria

Specific IgG antibodies against tetanus and diphtheria were determined by enzyme-linked immunosorbent assay (ELISA) for each antigen (Simonsen et al., 1986). Tetanus (CSL, Melbourne, Australia) and diphtheria (National Institute for Biological Standards and Control, Hertfordshire, U.K.) toxoids were coated onto 96-well, flat-bottom plates in 100-limit-of-flocculation amounts per milliliter in a volume of 100 μl and incubated overnight at 4°C. The plate was washed 3 times with wash buffer (PBS, 0.05% Tween-20) and then blocked with 150 μl blocking buffer (PBS, 0.05% Tween-20, 1% gelatin) for 1.5 h at 37°C. The plate was washed 3 times with wash buffer, and patient serum samples and controls were added that were diluted with blocking buffer to a final concentration of 1:100 for diphtheria and tetanus in a volume of 100 μl per well. After 1.5 h of incubation at 37°C, the plate was washed 3 times with wash buffer, and 100 μl of horseradish peroxidase (HRP)-labelled sheep anti-human IgG (1:20,000 in blocking buffer) (Silenus, San Francisco, Calif.) was added to each well. After 1.5 h of incubation at 37°C, the plate was washed 3 times with wash buffer and 3 times with deionized water, and 100 μl of 3,3',5,5-tetramethylbenzidine (TMB) was added to each well. The reaction was stopped with 2N H_2SO_4, and the optical density of the samples at 450 nm was determined with reference of 620 nm. Samples were assayed in triplicate with standard deviation of the means less than 5%.

Antitumor Immunoglobulin ELISA

Microtiter plates (Maxisorp plates, Nunc, Roskilde, Denmark) were precoated overnight at 4°C with 100 μg/ml tumor lysate or 100 μg/ml autologous PBMC lysate as control material in separate wells. Plates were then incubated with 1% bovine serum albumin/1% casein solution in PBS (blocking buffer) at 37°C for 1 h. After 1 wash with PBS, patient serum (diluted 1/50 in blocking buffer) was added to each well and incubated at 4°C for 1 h. Plates were washed 3 times with PBS and vortexed on a microplate vortex (orbital shuttle, Rohm Pharma, Piscataway, N.J.) for 15 min at 900 rpm. Wells were incubated with goat anti-human Ig conjugated to horseradish peroxidase (Serotech, Toronto, Ont., Canada) at 4°C for 90 min and washed 3 times with PBS containing 0.5% Tween-20 (wash buffer). The plates were vortexed with wash buffer for 15 min at 900 rpm and washed again. Plates were vortexed with wash buffer for 5 min and wells rinsed 3 times with deionized H_2O. Antibody complexes were detected by development with TMB (Zymed Laboratories, San Francisco, Calif.) at room temperature for 20 min protected from light. Reactions were stopped by using 2N H_2SO_4, and the optical density was measured at 450 nm with a microplate reader (Multiskan Ascent, Labsystems, Helsinki, Finland). Samples were assayed in triplicate and reported as the average ± standard deviation.

IL-10 ELISA Assay

Circulating levels of IL-10 in the serum of patients were determined using Quantikine human immunoassay kit (R&D Systems). Assays were performed according to manufacturer instructions. Samples were stored at –20°C until assayed. Lyophilized recombinant human IL-10 standards were reconstituted in 5 ml calibrator diluent (animal serum). Stock solution was at a final concentration of 2000 pg/ml. Stock solution was doubly diluted with RD6Z. Standards and samples were assayed in duplicate. Fifty microliters of assay diluent RD1W (buffered protein base) was added to each of the murine IL-10 monoclonal antibody-coated wells. Two hundred microliters of standard and samples were added to the IL-10 plate. Plates were incubated at room temperature for 2 h. Plates were washed with 400 μl 1× wash buffer (buffered surfactant) a total of 4 times. Two hundred microliters of IL-10 conjugate (IL-10 polyclonal antibody conjugated to horseradish peroxidase) was added to each well of the assay plate and incubated at room temperature for 1 h. The IL-10 assay plate was again washed 4 times with 400 μl 1× wash buffer. Two hundred microliters of substrate solution (hydrogen peroxide and TMB [v/v]) was added to each well, and samples were incubated at room temperature for 30 min protected from light. Fifty microliters of stop solution (2N H_2SO_4) was added to each well and determination of optical density performed within 30 min by using a microplate reader at 450 nm. Absorbance at 540 nm was determined for wavelength correction. Values calculated at 540 nm were subtracted from 450-nm readings.

Statistical Analysis

Study sample size was determined according to traditional phase 1 dose-level design. Mann-Whitney tests were performed to obtain *P*-values to determine significance. Error bars were generated to represent the standard deviation of the average from triplicate samples.

Results

Patient Characteristics

Patient characteristics are summarized in Table 1. A total of 9 patients were enrolled into this trial. Seven patients went on to receive DC_{RNA} vaccines and were evaluated for toxicity, feasibility, immune status, and tumor response. Two patients were unable to receive DC_{RNA} vaccines, 1 because of inadequate RNA (patient 3) and 1 because of early progressive disease (patient 2). Of the 7 patients receiving DC_{RNA} vaccine, 6 were females and 1 was male, and ages ranged from 9 to 22 years at the time of enrollment on this study. All previous treatment for each patient is shown in Table 1.

Table 1. Patient characteristics and clinical results

Patient No.	Age (years)	Sex	Diagnosis	Previous treatments	Relapse site status at time of vaccination	Vaccines given*	Toxicity	Tumor response† 8w	18w
1	15.5	M	Anaplastic Astrocytoma	S, R, C: CCNU TEMOZ	Nodules in resection margin	5	none	SD	SD
2#	22	F	Medulloblastoma	S, R, C: VCR, ETO, CISP, MEL H, CYC H + PBSC, oETO, CCNU	NA	0	NA	NA	NA
3#	14	F	Pilocytic Astrocytoma	S, R, C: CARB 6TG, PROC, CCNU, VCR	NA	0	NA	NA	NA
4	16.5	F	Glioblastoma Multiforme	S, R	NMD	3	none	PD	PD
5	13.5	F	Glioblastoma Multiforme	S, R, C: CCNU, VCR	Nodules in resection margin	3	none	PD	PD
6	9	F	Ependymoma	S, R, C: CISP, CYC, VCR, oETO	NMD	3	none	SD	PD
7	19.5	F	Ependymoma (spinal)	S, R, C: CYC H, CARB CISP, IRINO	Multiple metastatic nodules	3	none	SD	NA
8	13	F	Pleomorphic Xanthoastrocytoma	S, R, C: CCNU, TEMOZ	Nodules in resection margin	4	none	PR	SD
9	16	F	Ependymoma	S, R	NMD	3	none	SD	NA

Abbreviations: C, chemotherapy; CARB, carboplatin; CISP, cisplatin; CYC, cyclophosphamide; CYC H, cyclophosphamide high-dose therapy; DOX, doxorubicin; ETO, etoposide; 5FU, 5-fluorouracil; IFO, ifosfamide; IRINO, irinotecan; MEL H, melphalan high-dose therapy; NA, not applicable; NMD, no measurable disease; oETO, oral etoposide; +PBSC, peripheral blood stem cell (autologous stem cell support); PD, progressive disease; PR, partial response; PROC, procarbazine; R, radiation; S, surgery; SD, stable disease; TEMOZ temozolamide, 6TG 6-thioguanine, VCR, vincristine.

Patients 2 and 3 did not complete study protocol, and evaluations are incomplete.

* One DC_{RNA} vaccine includes both intradermal and intravenous components.

† Tumor response at 8 and 18 weeks from first DC_{RNA} vaccine.

Feasibility

RNA and DC production feasibility is shown in Table 2. Tumor RNA extraction was adequate for vaccine preparation in all but 1 case (patient 3). An average of $11.8 \times 10^7/m^2$ DC_{RNA} was prepared, with all 8 leukapheresed patients satisfying feasibility criteria (required at least $3 \times 10^7/m^2$ DC_{RNA}).

This study was designed to test the use of a single leukapheresis preparation. A 3-tiered dose escalation strategy using 3 patient cohorts, according to order of enrollment, was planned for the intravenous route, with low, medium, and high doses of $0.5 \times 10^7/m^2$, $1.5 \times 10^7/m^2$, and $0.5 \times 10^8/m^2$, respectively. Table 2 shows DC_{RNA} adjusted for patient body surface area. Patients are listed in order of enrollment, where patients 1 and 2 had high yields of vaccines compared with patients enrolled later in the study. While feasibility criteria were met in all 8 apheresed patients, dose escalation was not achievable as there was insufficient DC_{RNA} in patients enrolled later to provide all 6 planned doses at anything above the low dose level. Dose escalation would have required multiple PBMC collections. Therefore, all patients that received DC_{RNA} vaccines were treated at the low dose.

Toxicity

All vaccinations were safely administered with no measurable toxicity, and none of the patients developed any clinical or biochemical signs of autoimmune disease (Table 1).

Tumor Responses

Clinical evaluations. Tumor response was monitored by clinical and MRI assessments in 7 treated patients (Table 1). Three patients showed clinical responses during treatment with DC_{RNA} vaccine. Patient 1, a 15-year-old boy with an anaplastic astrocytoma who had residual nodular disease in the resection margin at study entry, continues to remain stable after 5 vaccines and 21 months of follow-up. Patient 8, a 13-year-old girl with pleomorphic xanthoastrocytoma who had residual nodular disease in the resection margin at study entry, showed an early partial response and continues to have stable disease after 6 months of follow-up (Fig. 1). Patient 9, a 16-year-old girl with anaplastic ependymoma, continues to have stable disease after 2 months of follow-up. The remaining 4 patients had progressive disease while on study.

Table 2. RNA and DC production feasibility

Patient No.	RNA µg	RNA µg/g tumor	PB monocytes* x 10^6/liter	Blood volumes apheresed	PBMNC x 10^6	DC† PBMNC/m^2 x 10^6	DC† Pre-pulse/ PBMNC %	DC† Prepared DC_{RNA}/m^2 x 10^7
1	984§	1148	300	2.01	13332	6259	6.9	18.2##
2	1200	2000	400	2.21	5627	3370	10.5	23.4##
3#	46.8	49	NA	NA	NA	NA	NA	NA
4	785§	1662	600	2.16	9215	5389	3.3	9.4
5	769§	748	300	2.06	7917	4102	3.0	12.5
6	3571	2976	300	2.29	5669	6162	2.4	9.6
7	801§	**	300	2.19	16294	9585	1.2	7.3##
8	586§	**	200	3.19	9608	6814	2.1	8.7##
9	419	267	600	1.83	14125	9054	6.2	5.3##
Av.	**1018**	**1264**		**2.24**		**6342**	**4.5**	**11.8**
SD	**1583**	**1031**		**0.41**		**2168**	**3.1**	**1.7**

Abbreviations: DC, dendritic cell; NA, not applicable; PB, peripheral blood monocyte; PBMNC, peripheral blood mononuclear cells.

*Peripheral blood monocyte count on the day of leukapheresis.

†Dendritic cell yield prior to incubation with tumor RNA as a percentage of PBMNC and prepared DC_{RNA} per square meter (feasible if at least $3.0 \times 10^7/m^2$).

§RNA obtained from tissue and aspirated material.

#RNA yield for patient 3 was inadequate, and patient did not undergo leukapheresis.

**Unable to weigh tumor material.

##Only a portion of the DC harvest was pulsed with RNA.

Antitumor immune responses. To determine if vaccination with DC pulsed with tumor RNA was able to induce specific antitumor immunity, we used in vitro assays to examine both humoral and cell-mediated immune responses. We were unable to detect statistically significant cell-mediated antitumor responses in either an IFN-γ-producing assay (n = 4) or in a T cell proliferation assay (n = 3) in any patients (Mann-Whitney test).

Humoral antitumor immunity was examined by ELISA for the presence of tumor-specific antibodies in patient serum. Figure 2 shows that patients 4 and 7 had modest increases in specific antitumor antibodies as compared with prevaccine samples. Three other patients (1, 8, and 9) evaluated did not have measurable specific antitumor antibodies.

General Immune Profile

Lymphocyte cell numbers. Quantitative baseline cell counts are shown in Table 3. Lymphocyte phenotyping revealed that all patients had normal subset numbers and distribution with the exception of patient 2, who had a low proportion of CD4 (22%) and a high proportion of CD19 (36%) positive cells. However, this patient never received DC_{RNA} vaccine because of early progression of disease.

Cellular immune responses. We examined lymphocyte proliferation in response to PHA at baseline and serially during DC_{RNA} treatment. Before vaccine was administered, all patients had significantly poorer proliferative responses to stimulation with PHA compared with normal controls ($P = 0.0016$) (Mann-Whitney test) (Fig. 3).

Humoral immune responses. Humoral immune parameters were also investigated. Table 4 shows titers of Ig isotypes at baseline, before treatment with the DC_{RNA} vaccine. All patients had normal levels of IgA molecules. Patients had normal levels of IgM, with the exception of patient 8, who had elevated IgM (2.09 g/liter). Patients had normal amounts of IgG with the exception of patients 2 and 6, who had slightly decreased IgG levels (3.9 and 4.06 g/liter, respectively).

Specific humoral responses to recall antigens diphtheria and tetanus are summarized in Table 4. Antibody titers were determined twice, before and after patients received DT booster vaccine and DC_{RNA} vaccine. DT booster vaccine was given 1 week before the patients received DC_{RNA} vaccine. These results indicate that in this cohort of patients, the humoral responses to recall antigens were generally intact at the time of study enrollment.

IL-10 cytokine profiles. Our DC_{RNA} production method did not include an additional maturation step following antigen uptake. Recent studies have suggested that immature DC may have an immunosuppressive effect via downstream production of IL-10. We performed an IL-10 ELISA assay on serial patient serum samples, as shown in Fig. 4. All patients who were tested had IL-10 levels below detectable limits (<7.8 pg/ml) for all samples, both before and after vaccine therapy.

Discussion

The primary objectives of this study were to determine the toxicity and feasibility of producing monocyte-derived dendritic cells pulsed with autologous tumor RNA and delivering them to pediatric cancer patients with CNS tumors. We were able to fulfill feasibility requirements in 8 out of 9 patients enrolled on this study. Feasibility was defined as producing 3 doses of an IV and

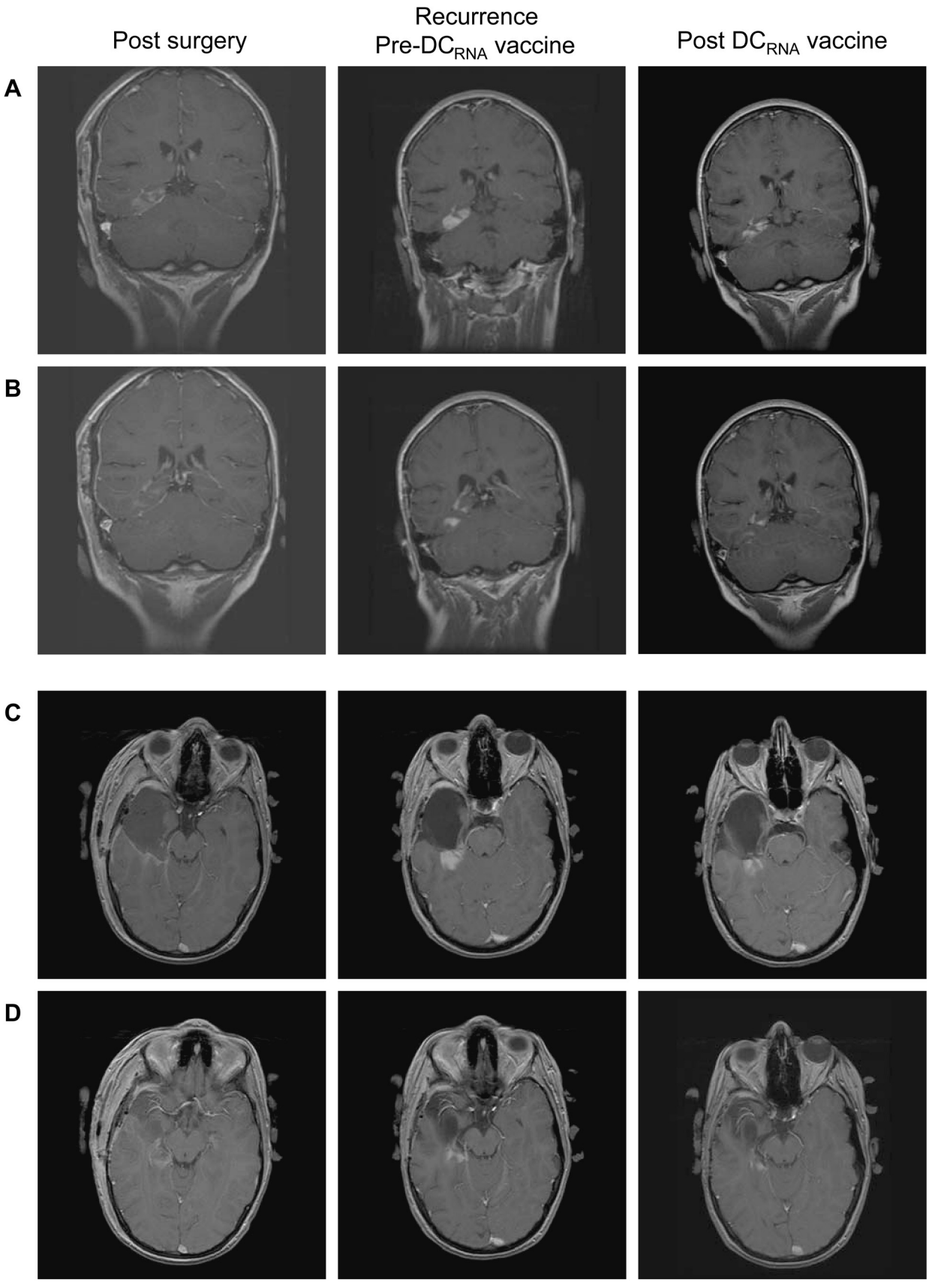

Fig. 1. Magnetic resonance imaging of patient 8. Coronal (A and B) and transverse (C and D) T1-weighted scans were performed at 3 time points as shown, demonstrating a clear reduction in size and degree of enhancement of the tumor.

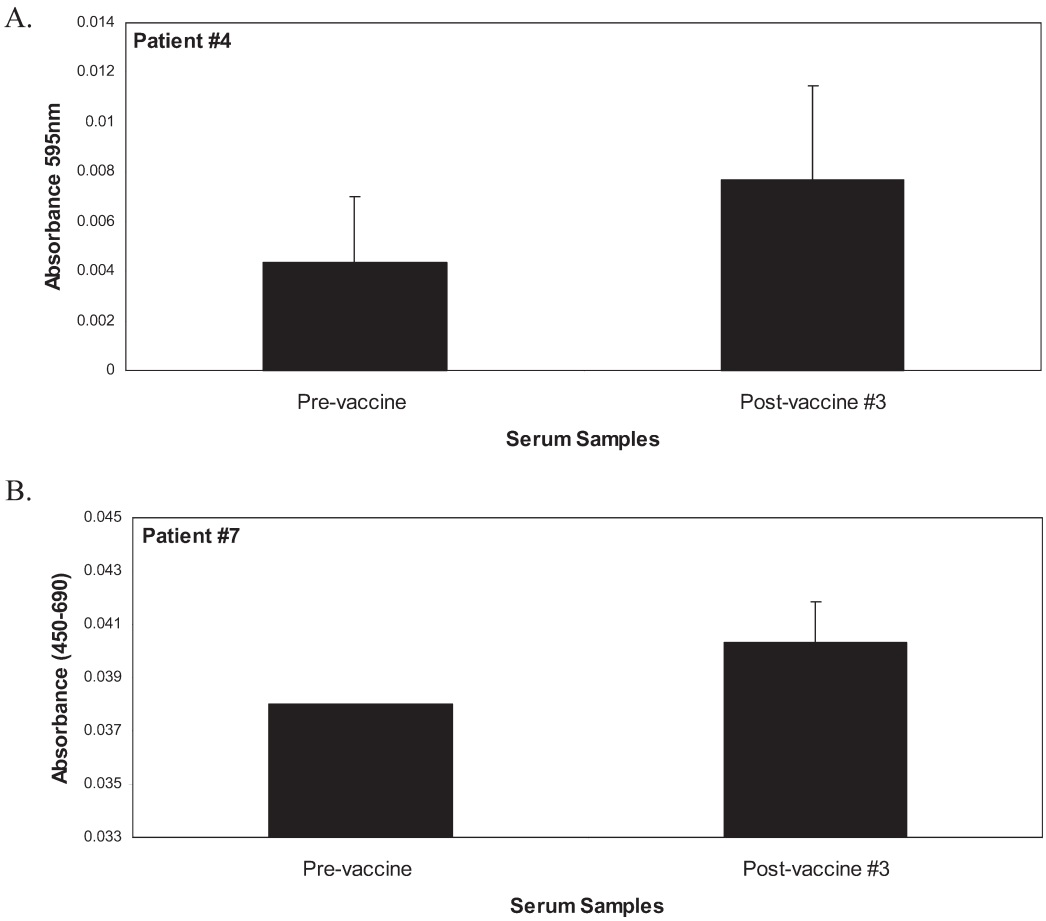

Fig. 2. Antitumor ELISA. Patient serum was diluted in blocking buffer and added to tumor lysate–coated wells and PBMC lysate–coated wells (control for nonspecific binding; data not shown). Panel A shows the results for patient 4, and Panel B shows the results for patient 7. Error bars represent standard deviations of the mean from triplicate samples.

an ID administration at a low dose of 0.5×10^7 cells/m^2 each. In all 8 apheresed subjects, sufficient numbers of cells were obtained from a single apheresis product, processed in vitro, and cryopreserved for later administration. The second component in fulfilling feasibility was to obtain sufficient quantities of tumor RNA. Tumor RNA was extracted in sufficient quantities in 8 of 9 patients. These results indicate that our methods for producing large amounts of clinical-grade DC_{RNA} antitumor vaccines are feasible in children with brain tumors.

All patients tolerated the vaccine well and showed no clinical signs of toxicity. One caveat when using autologous cellular material for reinfusion into a patient is the possibility of producing an autoimmune reaction. Particularly in the case of brain tumors, the possibility exists of inducing EAE, which can be lethal. However, in no case did a patient develop autoantibodies, and there was no clinical evidence of autoimmune disease. Therefore, we demonstrate that it is feasible and safe to produce large numbers of clinical-grade DC_{RNA} from a single apheresis in pediatric cancer patients with recurrent brain tumors.

As secondary objectives of this study, we examined both cellular and humoral immune responses at baseline prior to vaccination with DC_{RNA} to assess overall immunocompetency. Standard clinical quantitative assessments of immunocompetency such as total lymphocyte counts, lymphocyte subset ratios, and total Ig isotype titers suggested that these patients had intact immune systems. As shown in Table 3, no patients were lymphopenic at the time of receiving their first vaccine. Lymphocyte subsets for all except one patient were within normal limits (Table 3), and the majority of patients had normal total Ig levels (Table 4). It therefore appeared that these patients would be immunocompetent to receive active immunotherapy in the form of DC_{RNA} vaccines.

The humoral arm of the immune system appeared to be intact in this cohort of patients at baseline, with the majority of patients demonstrating protective diphtheria and/or tetanus IgG titers at baseline and all patients demonstrating protective tetanus and/or diphtheria IgG titers post–DT immunization. As well, we were able to measure a modest increase in antitumor antibody titer in 2 patients.

In contrast, baseline cellular immune responses were generally compromised before the patients received DC_{RNA} vaccine. In addition, all patients had below-normal cellular proliferative responses before receiving vaccine (Fig 4). This indicates that children with advanced CNS malig-

Table 3. Baseline cellular immune data

Patient No.	Time since chemotherapy[#]	Total count 1200–1300[#]	Lymphocyte Phenotype × 10^6/liter (%)* CD3[†] (61–81)[§]	CD4[†] (32–56)[§]	CD8[†] (23–43)[§]	CD19[†] (7–19)[ß]	CD56[†] (6–25)[ß]
1	3.5 months	1600	1120 (70)	480 (30)	590 (37)	180 (11)	270 (17)
2	14 months	1200	710 (59)	260 (22)	420 (35)	430 (36)	60 (5)
3	11 months	1500	na	na	na	na	na
4	NA	3100	2230 (72)	1300 (42)	960 (31)	530 (17)	190 (6)
5	9 months	2100	1530 (73)	840 (40)	760 (36)	400 (19)	130 (6)
6	8 months	1800	1420 (79)	680 (38)	720 (40)	180 (10)	110 (6)
7	16 months	2800	2040 (73)	1120 (40)	920 (33)	480 (17)	280 (10)
8	2.4 months	1200	970 (81)	460 (38)	490 (41)	130 (11)	70 (6)
9	NA	2000	1500 (75)	860 (43)	580 (29)	320 (16)	60 (3)
Av.	9.1 months	1922	1440 (73)	750 (37)	680 (35)	331 (17)	146 (7.4)

Abbreviations: NA, not applicable; na, not available.

* Lymphocyte count and phenotype 1 week prior to first DC_{RNA} vaccine or at enrollment if not vaccinated.

[#]Time between last chemotherapy and first DC_{RNA} vaccination or enrollment if not vaccinated.

[†]Normal ranges for cell counts.

[§]Normal ranges for lymphocyte populations as a percent.

nancy have significantly impaired cellular immune function, which may not be revealed by quantitative measurements of lymphocyte count or phenotypic assays.

Despite demonstrating clinical responses in 3 of 7 patients treated, this study failed to demonstrate the induction of specific cell-mediated antitumor responses via in vitro immune assays. Animal studies have previously shown that humoral responses are an important component of an anti–CNS tumor response (Sampson et al., 2000), and humoral responses may have played an important role in the patients who responded. As well, it is possible that the cell-mediated assays that were performed in this study were not sensitive or specific enough, despite robust responses from appropriate controls, to detect the immune responses generated in vivo in those patients who achieved clinical responses.

This study differs from previous studies in 2 important ways that may contribute to these findings: (1) using a pediatric cohort of cancer patients with malignancies of the CNS and (2) using a cohort with both undefined tumor-associated antigens and undefined HLA. These variables may also explain the apparent inconsistencies between the in vivo and in vitro responses.

It remains unclear whether this cohort of patients was relatively immunocompromised because of their disease status or because of previous treatment. Little is known

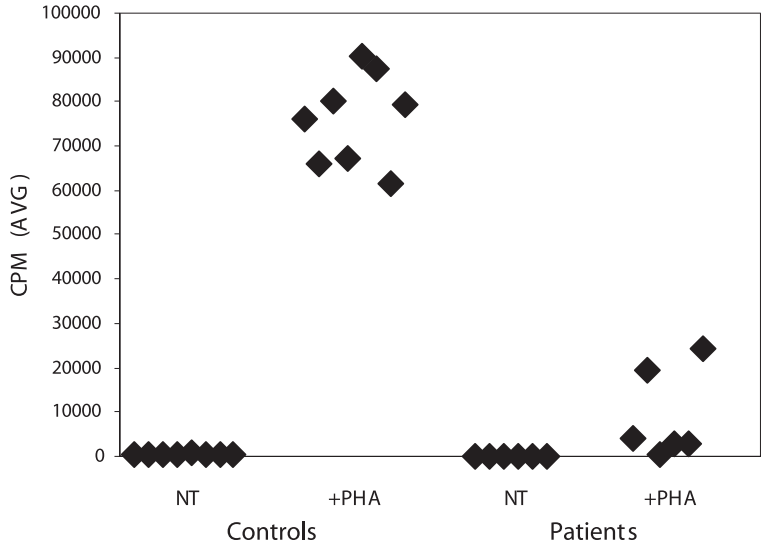

Fig. 3. Lymphocyte proliferation, expressed as an average number of counts per minute (CPM [avg]), in response to stimulation with PHA. PBMCs from normal controls and from patients at baseline prior to DC_{RNA} vaccine were stimulated with PHA or not treated (NT). PHA-induced proliferative responses were significantly lower in patients as compared with controls ($P = 0.0016$).

Table 4. Baseline humoral immune status*

Patient No.	Immunoglobulins, g/liter# IgG (5.18–17.8)	IgA (0.33–2.67)	IgM (0.32–1.35)	Tetanus† IU/ml (0.16) pre	post	Diphtheria† IU/ml (0.10) Pre	post
1	7.49	1.61	0.79	>4	>4	0.42	0.53
2	3.9	0.71	0.59	0.05	NA	0.01	NA
4	6.06	0.90	0.63	1.57	>4	0.15	>0.6
5	9.24	1.05	0.66	0.29	0.36	0.22	0.25
6	4.06	1.46	0.68	0.81	>4	0.06	>0.6
7	6.99	1.25	0.47	0.01	>4	0.02	0.58
8	12.50	1.39	2.09	1.15	>4	0.11	0.24
9	8.08	0.69	1.35	0.2	>4	0.11	>6
Av.	7.29	1.13	0.91	1.01	3.48	0.14	0.49

Abbreviations: Ig, immunoglobulin; NA, not applicable.

*Data for 8 of 9 patients is available. Patient 2 did not receive diphtheria and tetanus (DT) immunization, and evaluations are incomplete.

#Baseline Ig titers were measured prior to DC_{RNA} and DT immunization. Normal ranges are provided in parentheses.

† Tetanus and diphtheria IgG titers were measured pre- and post (28–58 days)-DT immunization. Protective titers are shown in parentheses. Tetanus titer of 4 EU/ml and diphtheria titer of 0.6 EU/ml are the upper limits of sensitivity for the assay.

about the baseline immune function of pediatric brain tumor patients. Although immunodeficiency in cancer patients has been previously reported (Bersagel, 1971), the cause of deficiency is still under debate and likely to be heterogeneous. However, there is evidence that suggests that brain tumor patients are immunosuppressed through several mechanisms. Glioma cells have increased TGF-β expression, which inhibits cytotoxic T lymphocyte activity as well as inhibiting B cells, natural killer cells, lymphokine-activated killer cells, and macrophages (Ashley and Bigner, 1997). Immunosuppressive cytokines IL-6 and IL-10 have been shown to be produced by glioma cells in vitro (Cinque et al., 1992; Tada and de Tribolet, 1993) and in vivo (Huettner et al., 1995).

It has been shown that MoDC vaccine preparations become more antigenic after the addition of cytokines to further differentiate them into mature DCs (Dhodapkar et al., 2000). Our preparation of MoDCs did not include an additional maturation step beyond antigen uptake. Therefore, to determine if our vaccine was producing an immunosuppressive effect, we performed an IL-10 ELISA on patient serum samples throughout treatment. We found that none of the patients tested had increased IL-10 levels. It has been shown that in vivo DCs mature after antigen uptake or exposure to inflammatory cytokines and subsequently upregulate costimulatory molecules on their cell surface. Recently, it has been shown in melanoma patients that in vitro–derived mature DCs were able to migrate to T cell areas of lymph nodes while immature DCs were not (De Vries et al., 2003). Phenotypically mature DCs are defined as CD11c+, CD80+, CD86+, and CD1a+ (Stockwin et al., 2000). The phenotypes of our RNA-pulsed MoDCs include HLA-DR^{high}, CD86+, CD11c+, CD1a−, and a population of CD11c− cells (data not shown). Therefore, our MoDCs do express some maturation molecules after antigen uptake, without a maturation step.

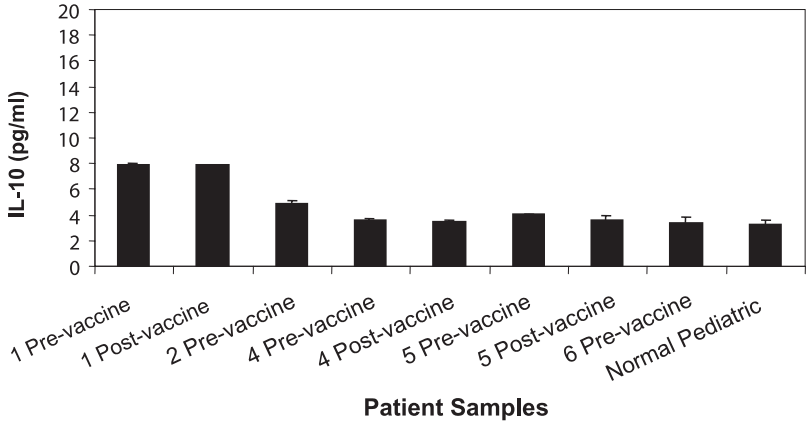

Fig. 4. IL-10 levels pre- and post-DC_{RNA} vaccination. Patient serum samples were assayed for induction of IL-10 levels. No samples were above detectable levels post-DC_{RNA} vaccination. Error bars represent standard deviations of the mean from triplicate samples.

The results of this study have demonstrated that our methodology for producing DC-based antitumor vaccines using a single apheresis is both safe and feasible in a pediatric population with recurrent brain malignancies. Similarly to others, we have shown tumor responses in a minority of patients who have received DC_{RNA} vaccine. In addition, we have performed an examination of the baseline immune status of this cohort of patients, revealing that immunocompetency is impaired compared with normal controls. This finding may have important implications for immunotherapy applications. Our study suggests that future examination of the effects of brain tumors and required therapies on the immune system is warranted. As well, the development of more effective DC vaccine methodologies may be required before significant impact is achieved in treating pediatric brain cancer.

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References

Ashley, D.M., and Bigner, D.D. (1997) Recent advances in the biology of central nervous system tumors. *Curr. Opin. Neurol.* **10**, 445–451.

Ashley, D.M., Faiola, B., Nair, S., Hale, L.P., Bigner, D.D., and Gilboa, E. (1997) Bone marrow-generated dendritic cells pulsed with tumor extracts or tumor RNA induce antitumor immunity against central nervous system tumors. *J. Exp. Med*. **186**, 1177–1182.

Bergsagel, D.E. (1971) An assessment of massive-dose chemotherapy of malignant disease. *Can. Med. Assoc. J.* **104**, 31–36.

Bullard, D.E., Thomas, D.G., Darling, J.L., Wikstrand, C.J., Diengdoh, J.V., Barnard, R.O., Bodmer, J.G., and Bigner, D.D. (1985) A preliminary study utilizing viable HLA mismatched cultured glioma cells as adjuvant therapy for patients with malignant gliomas. *Br. J. Cancer* **51**, 283–289.

Cinque, S., Willems, J., Depraetere, S., Vermeire, L., and Joniau, M. (1992) "In vitro" effect of interleukin-1 beta on human glioma cell lines: Regulation of cell proliferation and IL-6 production. *Immunol. Lett.* **34**, 267–271.

De Vries, I.J., Krooshoop, D.J., Scharenborg, N.M., Lesterhuis, W.J., Diepstra, J.H., Van Muijen, G.N., Strijk, S.P., Ruers, T.J., Boerman, O.C., Oyen, W.J., Adema, G.J., Punt, C.J. and Figdor, C.G. (2003) Effective migration of antigen-pulsed dendritic cells to lymph nodes in melanoma patients is determined by their maturation state. *Cancer Res.* **63**, 12–17.

Dhodapkar, M.V., Krasovsky, J., Steinman, R.M., and Bhardwaj, N. (2000) Mature dendritic cells boost functionally superior CD8(+) T-cell in humans without foreign helper epitopes. *J. Clin. Invest.* **105**, R9–R14.

Geiger, J.D., Hutchinson, R.J., Hohenkirk, L.F., McKenna, E.A., Yanik, G.A., Levine, J.E., Chang, A.E., Braun, T.M., and Mule, J.J. (2001) Vaccination of pediatric solid tumor patients with tumor lysate-pulsed dendritic cells can expand specific T cells and mediate tumor regression. *Cancer Res*., 8513–8519.

Heiser, A., Dahm, P., Yancey, D.R., Maurice, M.A., Boczkowski, D., Nair, S.K., Gilboa, E., and Vieweg, J. (2000) Human dendritic cells transfected with RNA encoding prostate-specific antigen stimulate prostate-specific CTL responses in vitro. *J. Immunol.* **164**, 5508–5514.

Hosking, C.S., and Balloch, A. (1983) A formula to assess lymphocyte responsiveness in vitro. *Birth Defects Orig. Article Ser.* **19**, 21–23.

Huettner, C., Paulus, W., and Roggendorf, W. (1995) Messenger RNA expression of the immunosuppressive cytokine IL-10 in human gliomas. *Am. J. Pathol.* **146**, 317–322.

Mitchell, A.E., Elder, J.E., Mackey, D.A., Waters, K.D., and Ashley, D.M. (2001) Visual improvement despite radiologically stable disease after treatment with carboplatin in children with progressive low-grade optic/thalamic gliomas. *J. Pediatr. Hematol. Oncol*. **23**, 572–577.

Murphy, J.B., and Sturm, E. (1923) Conditions determining the transplantability of tissues in the brain. *J. Exp. Med.* **38**, 183–197.

NCI. National Cancer Institute, Cancer Therapy Evaluation Program (1999) Common Toxicity Criteria—Version 2.0. Rockville, Md.: NCI Division of Cancer Treatment and Diagnosis (available at http://ctep.cancer.gov/reporting/ctc.html).

Porgador, A., and Gilboa, E. (1995) Bone marrow-generated dendritic cells pulsed with a class I-restricted peptide are potent inducers of cytotoxic T lymphocytes. *J. Exp. Med.* **182**, 255–260.

Romani, N., Reider, D., Heuer, M., Ebner, S., Kampgen, E., Eibl, B., Niederwieser, D., and Schuler, G. (1996) Generation of mature dendritic cells from human blood. An improved method with special regard to clinical applicability. *J. Immunol. Methods* **196**, 137–151.

Sampson, J.H., Crotty, L.E., Lee, S., Archer, G.E., Ashley, D.M., Wikstrand, C.J., Hale, L.P., Small, C., Dranoff, G., Friedman, A.H., Friedman, H.S., and Bigner, D.D. (2000) Unarmed, tumor-specific monoclonal antibody effectively treats brain tumors. *Proc. Natl. Acad. Sci. USA* **97**, 7503–7508.

Shelton, M.S., Meek, F., and Hoskings, C.S. (1974) Serum immunoglobulin levels in children. *Aust. J. Med. Technol.* **5**, 113.

Shirai, Y. (1923) Transplantations of rat sarcomas in adult heterogeneous animals. *Jpn. Med. World* **1**, 14–15.

Simonsen, O., Bentzon, M.W., and Heron, I. (1986) ELISA for the routine determination of antitoxic immunity to tetanus. *J. Biol. Stand.* **14**, 231–239.

Stockwin, L.H., McGonagle, D., Martin, I.G., and Blair, G.E. (2000) Dendritic cells: Immunological sentinels with a central role in health and disease. *Immunol. Cell Biol.* **78**, 91–102.

Tada, M., and de Tribolet, N. (1993) Recent advances in immunobiology of brain tumors. *J. Neurooncol*. **17**, 261–271.

Yu, J.S., Wheeler, C.J., Zeltzer, P.M., Ying, H., Finger, D.N., Lee, P.K., Yong, W.H., Incardona, F., Thompson, R.C., Riedinger, M.S., Zhang, W., Prins, R.M., and Black, K.L. (2001) Vaccination of malignant glioma patients with peptide-pulsed dendritic cells elicits systemic cytotoxicity and intracranial T-cell infiltration. *Cancer Res*. **61**, 842–847.