DNA Vaccination with HuD Inhibits Growth of a Neuroblastoma in Mice

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ABSTRACT

Some patients with small cell lung cancer (SCLC) or neuroblastoma develop an immune response against HuD, a human homologue of the Drosophila protein, elav, which is expressed in the nucleus and to a lesser degree the cytoplasm of neurons and tumor cells. This immune response is characterized by antibodies (anti-Hu) that at high titers are associated with a disease called paraneoplastic encephalomyelitis/sensory neuronopathy, in which infiltrates of T cells are found in the tumor and nervous system. Although all SCLCs express HuD, anti-Hu antibodies are identified in only 17% of patients with SCLC, usually at low titers, and are associated with indolent tumor growth. To determine whether the anti-Hu immune response causes indolent tumor growth, we developed an animal model using HuD DNA immunization. We found that a plasmid coding for a secreted form of HuD induced a strong and specific anti-Hu response. Immunized animals were challenged by s.c. implantation of a neuroblastoma cell line that constitutively expresses HuD. When compared with controls, mice immunized with the secreted HuD showed significant tumor growth inhibition (51% reduction volume; \(P = 0.0012\)), and 14% of them had complete tumor rejection. Tumors from these animals showed three times more CD3+ lymphocytic infiltrates than those from control mice and had a higher CD8+:CD4+ ratio. None of the animals developed neurological deficits or neuropathological evidence of nervous system pathology. In this mouse model of neuroblastoma, DNA immunization with HuD resulted in tumor growth inhibition but did not induce neurological disease. This model closely mimics the clinical course of more indolent tumor growth seen in patients with the anti-Hu immune response.

INTRODUCTION

Studies in animal models have shown that immunization against tumor antigens can retard growth and occasionally cure established tumors (1-3). In humans, SCLC\(^1\) provides a model of naturally occurring immune responses against cancer (4). One of these immune reactions is targeted against the Hu proteins, which are the human homologues of the Drosophila protein elav (5). Hu proteins are encoded by several genes, including HuD (6), HuC (7), Hel-N1 (8), Hel-N2 (8), and HuR (9). Hu antigens are neuron-specific proteins that are also expressed by SCLC but not by normal lung tissue (6, 10). Although all SCLCs express HuD, only 17% of patients harbor anti-Hu antibodies, usually at low titers (11). Interestingly, in patients with anti-Hu antibodies, the tumor is more likely to be restricted to the lung and mediastinum, to respond better to treatment, and to be associated with survival times longer than those of patients without anti-Hu antibodies (11-13), suggesting that the anti-Hu immune response may inhibit SCLC growth. Among patients with anti-Hu antibodies, a small subset (<1% of SCLCs) who produce high titers of anti-Hu antibodies develop neurological dysfunction (14). Neuropathological findings include neuronal loss, gliosis, and inflammatory infiltrates. This disease, known as paraneoplastic encephalomyelitis/sensory neuronopathy (anti-Hu syndrome), is suspected to result from an immune reaction targeted against the Hu antigens expressed by the tumor but misdirected against neurons (15).

To examine the effects of the anti-Hu immune response on the growth of an HuD-expressing tumor and on the nervous system, we developed a mouse model using immunization with HuD DNA. Because mouse and human HuD proteins have 98% amino acid identity and are identical in the regions containing the antigenic epitopes, we immunized the mice with human HuD DNA (16). The mice were challenged with a neuroblastoma that constitutively expresses HuD because a model of SCLC in immunocompetent mice is not available. Almost 80% of all human neuroblastomas express HuD, and similar to some patients with SCLC, patients with neuroblastoma can develop anti-Hu immune reactions associated with inflammation of the nervous system (17).

We found that the development of an anti-Hu immune response inhibited tumor growth but did not cause neurological symptoms, closely mimicking what is seen in SCLC patients with low titers of anti-Hu antibodies.

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\(^2\)The abbreviations used are: SCLC, small cell lung cancer; IL, interleukin.
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MATERIALS AND METHODS

Plasmids. Three HuD constructs were derived from pHB1.5 using PCR (6). HuF expresses the full-length HuD cDNA, and HuM expresses a truncated HuD protein that corresponds to pHB1.5, bp 116 to 736. This protein contains the first two RNA-binding domains but is lacking the stringer domain and the third RNA-binding domain. The HuDsec construct expresses the full-length HuD cDNA fused in frame at the 5’ end with the IL-2 secretory signal peptide sequence from pBC12/CMV/IL-2, a gift of B. R. Cullen (Duke University, Durham, NC) (18). Constructs were cloned into pcDNA3 (Invitrogen, San Diego, CA) downstream of the cytomegalovirus immediate-early promoter and 5’ to a polyadenylation signal. The resulting plasmids were named pHuF, pHuM, and pHuDsec. DNA for vaccination was purified using Gigaprep (Qiagen, Chatsworth, CA) and resuspended in sterile PBS (Sigma, St. Louis, MO) at a concentration of 1 μg/μl.

In Vitro Expression of HuD. Saos-2 cells (HTB 85; American Type Culture Collection, Rockville, MD) were grown in DMEM containing 15% fetal bovine serum, 0.1 mM nontessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, and 100 units/ml penicillin/streptomycin (Life Technologies, Inc., Grand Island, NY). Cells were transfected with 10 μg of plasmid, using the calcium phosphate precipitation method. Stable transfectants were selected by growth in 200 μg/ml G418 (Sigma). The plasmids. Three HuD constructs were derived from pHB1.5 using PCR (6). HuF expresses the full-length HuD cDNA, and HuM expresses a truncated HuD protein that corresponds to pHB1.5, bp 116 to 736. This protein contains the first two RNA-binding domains but is lacking the stringer domain and the third RNA-binding domain. The HuDsec construct expresses the full-length HuD cDNA fused in frame at the 5’ end with the IL-2 secretory signal peptide sequence from pBC12/CMV/IL-2, a gift of B. R. Cullen (Duke University, Durham, NC) (18). Constructs were cloned into pcDNA3 (Invitrogen, San Diego, CA) downstream of the cytomegalovirus immediate-early promoter and 5’ to a polyadenylation signal. The resulting plasmids were named pHuF, pHuM, and pHuDsec. DNA for vaccination was purified using Gigaprep (Qiagen, Chatsworth, CA) and resuspended in sterile PBS (Sigma, St. Louis, MO) at a concentration of 1 μg/μl.

In Vitro Expression of HuD. Saos-2 cells (HTB 85; American Type Culture Collection, Rockville, MD) were grown in DMEM containing 15% fetal bovine serum, 0.1 mM nontessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, and 100 units/ml penicillin/streptomycin (Life Technologies, Inc., Grand Island, NY). Cells were transfected with 10 μg of plasmid, using the calcium phosphate precipitation method. Stable transfectants were selected by growth in 200 μg/ml G418 (Sigma). For protein immunizations, purified recombinant HuD protein was prepared as described previously (20). Mice were initially immunized with 100 μg of purified recombinant HuD fusion protein and an equal volume of complete Freund’s adjuvant, followed by two injections of 100 μg of HuD protein with incomplete Freund’s adjuvant at 2-week intervals. Tumors were implanted 3 weeks after the last protein immunization.

Tumor Implantation. The Neuro2a cell line, a subclone of the C1300 murine neuroblastoma that was developed in A/J mice (CCL-131; American Type Culture Collection) was maintained in MEM with 0.1 mM nontessential amino acids, Earle’s balanced salt solution, and 10% fetal bovine serum (Life Technologies). For implantation, cells were trypsinized and washed with PBS, and the viability was determined by trypan blue exclusion. Viable cells (2 × 10^6/100 μl PBS) were injected s.c. in the right flank 3 weeks after the last DNA injection.

Tumor volumes were assessed with a caliper every 4 days, using the formula, \( \pi \times \text{length} \times \text{width}^2/6 \) (21), and animals were sacrificed 22 days after tumor challenge. Statistical analysis was performed with the ANOVA repeated measures test using StatView 4.01 software (Abacus Concepts, Berkeley, CA).

Analysis of Anti-Hu Antibodies. Sera were collected by orbital phlebotomy 2 weeks after the third and fourth DNA injections. Purified recombinant HuD protein (20) or lysates of Neuro2a cells (200 μg) were subjected to electrophoresis on a 10% polyacrylamide-SDS gel and transferred to nitrocellulose filters. Filters were blocked in 5% nonfat milk for 12 h at 4°C, cut into strips, and incubated for 2 h at room temperature with serial dilutions of mouse sera in 10% sheep serum. After being washed three times with 0.1% Tween 20 (Sigma), strips were incubated with peroxidase-labeled goat anti-mouse IgG (Amersham Life Science, Cleveland, Ohio) diluted 1:20,000 in 0.1% Tween 20. After additional washing, strips were immersed in enhanced chemiluminescence reagent (Amersham Life Science) and exposed to Hyperfilm (Amersham Life Science), according to the manufacturer’s instructions. Titers of mouse sera were defined by the highest dilution that gave a positive visual signal. Sera from patients with SCLC and paraneoplastic encephalomyelitis/sensory neuropathy were used as a source of anti-Hu antibodies.

Immunohistochemistry. After induction of anesthesia, animals underwent intracardiac perfusion with PBS. Samples of brains and tumors were fixed for 4 h in 4% formalin and embedded in paraffin. Sections (10 μm) were dewaxed in xylene, processed via graded ethanol to distilled water, and stained with H&E. Other samples were embedded in optimal cutting temperature compound (Miles, Inc., Elkhart, IN), snap-frozen in isopentane chilled with liquid nitrogen, and stored at −80°C. Frozen sections (10 μm) were fixed for 10 min in 10% buffered formalin and sequentially incubated at room temperature with 0.3% hydrogen peroxide for 20 min and with 10% normal human serum or 10% normal rabbit serum for 20 min (Jackson Immunoresearch, West Grove, PA). Primary antibodies included biotinylated human IgG from a patient with high titers of anti-Hu antibodies (1:50 in 10% normal human serum), biotinylated hamster anti-mouse-CD3 (1:10 in 10% rabbit serum; clone 5002A; PharMingen, San Diego, CA), rat anti-mouse-CD8α (1:30 in 10% rabbit serum; clone KT15; Serotec, Oxford, United Kingdom), and rat anti-mouse-CD4 (1:30 in 10% rabbit serum; clone RM4–5; PharMingen). Sections were incubated for 90 min with primary antibodies; when the primary antibody was not biotinylated, sections were subsequently incubated for 1 h with biotinylated rabbit anti-rat IgG (1:2000 in 10% rabbit serum; Vector Laboratory, Burlingame, CA). Bound biotinylated IgG was visualized by incubation for 30 min with avidin-biotin-peroxidase complexes (Vectastain ABC complex; Vector Laboratory). The substrate reaction was developed with 0.05% diaminobenzidine hydrochloride (Sigma), 0.5% Triton X-100, and 0.01% hydrogen peroxide in PBS. Between steps, sections were washed with PBS.

Quantitative analysis of CD3+ cells was performed using two different sections for each tumor. In each section, the areas containing the larger inflammatory infiltrates were selected, and the number of CD3+ cells in five fields, each measuring 0.280 mm², were counted. All sections were analyzed independently by two investigators, who were blinded to the immunization history of the animal, and the counts obtained were averaged.

Hot Plate and Tailflick. Animals were examined daily between the first and last vaccination and then 2 weeks after the
immunization protocol was completed by standard hot plate and the tailflick tests (22, 23). All tests were performed in duplicate.

RESULTS

In Vitro Expression of HuD. To confirm that the Hu plasmids expressed immunoreactive Hu proteins, lysates of stably transfected Saos-2 cells were examined by immunoblotting. Saos-2 cells are derived from a human osteogenic sarcoma and do not express Hu proteins (24). After transfection, HuD-reactive proteins corresponding to pHuF (Mr 42,000), pHuDsec (Mr 25,000), and pHuDsec (Mr 45,000) were found to be expressed by these cell lines (Fig. 1). When the media bathing the cells were studied, only the media from cells expressing the pHuDsec construct contained HuD-reactive protein, confirming that this construct results in the secretion of HuD protein from the cell (Fig. 1).

DNA Immunizations Elicited Anti-Hu Immune Responses. Titers of anti-Hu antibodies were measured in mice sera 2 weeks after the second, third, and fourth DNA injections. Plasmids pHuF and pHuM induced antibodies in only 1 of 14 mice. In contrast, the pHuDsec construct induced antibodies in 31 of 34 mice, with antibody titers ranging from 1/200 to 1/64,000 (Fig. 2). In 20% of these animals, antibody titers peaked after the third injection; in 60%, the titers peaked after the third injection and decreased after the fourth injection. In the remaining 20%, antibody titers continued to rise after the fourth injection. The plasmid pcDNA3 (control) did not induce anti-Hu antibodies. Injection of purified recombinant HuD fusion protein resulted in the highest titers of anti-Hu antibodies (up to 1/256,000). No differences in antibody titers were found between the A/J and SWR/J strains of mice.

Sera of Immunized Animals Recognize Mouse Hu Antigens. To examine whether the antibodies induced in mice by the human HuD DNA constructs recognize the mouse Hu antigens, sera were examined using immunoblots of Neuro2a cell extracts. Sera from pHuDsec-immunized animals, but not from control pcDNA3-injected mice, reacted with a protein of Mr ~37,000 in a pattern identical to that of serum from a patient with anti-Hu antibodies (Fig. 3).

DNA Immunization Protects against Neuro2a Tumor Challenge. Three weeks after the last DNA immunization, animals were implanted s.c. with Neuro2a cells. Tumor growth was assessed every 4 days. By day 22 after tumor implantation, 12 of 14 animals immunized with pHuDsec developed measurable tumors. These tumors had volumes that were 51% smaller ($P = 0.0012$) than animals immunized with the control plasmid (n = 14; Fig. 4). Two of the pHuDsec animals did not develop tumors, whereas all 14 pHuF-immunized and all 14 pHuM-immunized animals did develop tumors. Animals immunized with pHuM and pHuF (n = 10, each group) showed a small (16 and 14%) but not significant ($P = 0.40$ and 0.41, respectively) reduction of tumor volumes. No effect on tumor growth was seen in animals injected with recombinant HuD fusion protein.
The inhibition of tumor growth reached maximal significance on days 10 and 14 postimplantation (66% decreased volume for pHuDsec, \( P < 0.0005 \); 50% for pHuM, \( P < 0.05 \); 31% for pHuF, \( P = \text{not significant} \)). In animals immunized with pHuDsec, no correlation was found between tumor volume and antibody titers (data not shown).

**Implanted Tumors Expressed Hu Antigens and Showed Lymphocytic Infiltrates.** A subset of A/J mice preimmunized with control or pHuDsec (\( n = 6 \), each group) were sacrificed 22 days after the tumor challenge, and the expression of Hu antigens by the implanted tumors was assessed by immunocytochemistry. In both groups, all tumor cells in nonnecrotic areas expressed Hu antigens.

Another group of A/J mice injected with pcDNA3 or pHuDsec (\( n = 6 \) each group) were sacrificed 7 days after tumor challenge, and the tumors were examined for lymphocytic infiltrates. One mouse immunized with pHuDsec showed inflammatory reactive tissue with lymphocytic infiltrates at the site where the Neuro2a cells were injected, but no tumor cells were detected. The lymphocytic infiltrates of this tumor were not included in the quantitative analysis of inflammatory cells. Measurable tumors were found in all other animals. CD3+ cell infiltrates were identified in all tumors, either as focal or widespread infiltrates, but the number of CD3+ cells was over three times higher in pHuDsec-immunized animals than in control animals (mean of five fields \( \pm \) SE, 81 \( \pm \) 17 versus 25 \( \pm \) 9 lymphocytes; Fig. 5). The CD4+CD8+ lymphocyte ratio was higher in control mice than in immunized animals (mean \( \pm \) SE, 3.6 \( \pm \) 0.8 versus 1.5 \( \pm \) 0.2). Similar analyses could not be performed later in the experiment (day 20 after tumor challenge) because of the presence of large areas of necrosis in the tumors.

**HuD DNA Immunizations Did Not Induce Paraneoplastic Symptoms.** DNA-immunized animals were examined weekly. None of the animals developed neurological deficits over a 3-month observation period. The tailflick and hot plate assays, used to examine sensory function, were performed 2 weeks after the vaccination protocol was completed and did not show a difference between control and immunized animals (data not shown).

Brains of A/J and SWR/J mice immunized with pHuM, pHuF, or pHuDsec (\( n = 4 \), each group) were examined using H&E staining and immunocytochemistry with anti-CD3 antibodies. None of the animals showed brain inflammation, gliosis, or neuronal loss (data not shown).

**DISCUSSION**

In this study, we demonstrated that vaccination with a plasmid coding for the human HuD gene results in a significant reduction of growth of an HuD-expressing tumor. Vaccinated animals developed anti-Hu antibodies, and their tumors showed...
increased numbers of T-cell inflammatory infiltrates. These results parallel the clinical findings in patients with SCLC who develop anti-Hu immune responses. The tumors of these patients often remain small and localized to the thorax (11, 12), which is unusual for SCLC, and in rare cases, tumor regression has been reported (25, 26). Additionally, the presence of low titers of anti-Hu antibodies at the time of SCLC diagnosis is a strong and independent predictor of complete response to treatment and prolonged survival (12). The tumors of patients with high titers of anti-Hu antibodies and SCLC also appear to have a similar clinical course but often die prematurely from their neurological disorder (14).

A number of prior studies have shown dramatic effects of DNA vaccination on tumor growth. Most of these studies used models in which the tumor cells were transfected with the same gene used for immunization (1, 2). Only a few studies have immunized with a gene that is constitutively expressed by the tumor cells, as we have done (3, 27). Although we immunized mice with human HuD, mouse and human HuD proteins have 98% amino acid identity, with absolute identity in the first and second RNA recognition motifs where the antigenic epitopes are contained (16). In general, the results of experiments immunizing against antigens that are endogenously expressed by tumor cells have been far less dramatic than those achieved with transfected antigens. These differences could in part be attributable to variation in the level of antigen expression or the cellular localization of endogenous proteins compared with proteins expressed from plasmids. Another factor that may explain the differences between immune responses raised against endogenous tumor proteins or transfected gene products is the concurrent expression of MHC class I proteins, which is higher in cells selected for transfection than in the Neuro2a cells, which express low levels of MHC class I proteins (21, 28).

In the present study, protective immunity was developed only in animals vaccinated with a construct that enables secretion of the HuD protein. Normally, HuD is expressed mainly in the nucleus of neurons (10), suggesting that the abnormal cellular localization of the secreted HuD protein was relevant to development of the immune response.

The mechanisms responsible for inhibition of tumor growth in our animals remain speculative. Because vaccination with plasmid DNA will evoke both humoral and cellular immune responses (29), it is possible that growth inhibition was due, at least in part, to CTL-mediated killing. A role of cytotoxic T cell-mediated lysis is supported by the studies demonstrating larger numbers of inflammatory CD3+ and CD8+ infiltrates in the tumors from vaccinated animals than in control tumors. The absence of a direct role of the anti-Hu antibodies in tumor growth inhibition is supported by the lack of correlation between antibody titer and amount of growth inhibition. Furthermore, animals immunized solely with recombinant HuD protein develop higher antibody titers than DNA-vaccinated animals but were not protected against a tumor challenge.

In humans, the intense anti-Hu immune response associated with high titers of anti-Hu antibodies and infiltrates of CD8+ cells in the nervous system is suspected to be the cause of paraneoplastic encephalomyelitis/sensory neuronopathy syndrome (14). This immune response is almost always triggered by SCLCs or neuroblastosomas with high levels of expression of MHC class I and II proteins (17). Efforts to model the paraneoplastic disorder are complicated by the isolation of the nervous system by the blood-brain barrier and the fact that neurons (the only normal cells that constitutively express Hu antigens) are immunoprivileged by the lack of expression of MHC molecules. Attempts to develop the disease in mice, using HuD protein immunization, have resulted in high titers of anti-Hu antibodies but no neurological disease (20), suggesting that other or additional factors are involved in the pathogenesis of paraneoplastic neurological dysfunction. This is confirmed by the present study, where the development of high titers of anti-Hu antibodies did not result in neurological dysfunction. Because a sensory neuropathy is a predominant symptom in the anti-Hu associated paraneoplastic syndrome, we monitored the animals for overt neurological dysfunction and sensory deficits. To avoid missing mild sensory deficits, we used the tailflick and hot plate assays, which measure the reaction latencies to nociceptive stimuli (22, 23).

In conclusion, this study represents one step forward in modeling the effective anticancer immune response found in patients who develop anti-Hu immune responses. The lack of neurological symptoms and pathological abnormalities in the nervous system of the mice provides preliminary evidence that it might be feasible and safe to manipulate the anti-Hu response as part of an immunotherapy for HuD-expressing tumors such as SCLC and neuroblastosoma.

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REFERENCES


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