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Characterization of a Recombinant Vaccinia Virus Expressing Human Melanoma-Associated Antigen p97

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p97 is a cell surface glycoprotein expressed at high levels in most human melanomas but present only in trace amounts in normal adult tissues. We are interested in exploring the possibility of using recombinant vaccinia virus to express a specific tumor-associated antigen as a vaccine against human cancer. To this end, we constructed a recombinant virus, v-p97NY, which contains the entire coding sequence for p97 under the control of the vaccinia virus 7.5K promoter. Upon infection of tissue culture cells, v-p97NY expressed high levels of a membrane-bound glycoprotein immunoreactive with a p97-specific monoclonal antibody. Immunization of mice with this recombinant elicited high-titered antibodies against p97. Spleen cells isolated from these mice proliferated in vitro when stimulated either with purified p97 protein or with syngeneic cells expressing p97 antigen. Delayed-type hypersensitivity was also observed in immunized mice after challenge with p97-expressing cells. These findings indicate the potential usefulness of v-p97NY and similar recombinants in tumor immunotherapy.

There is a long-standing interest among immunologists in developing vaccines for active immunotherapy against tumors. Several approaches have been described, including the use of various preparations of tumor-associated antigens (4, 17, 22, 26) and the use of anti-idiotypic antibodies (9, 16). We chose to examine the recombinant vaccinia virus approach for several reasons. First, this approach has been successfully used to develop potential vaccines against infectious diseases (for a review, see reference 19). Such vaccines are known to elicit not only humoral but also cell-mediated immunity (1), which is of critical importance in tumor immunotherapy (11). Second, immunization with live recombinant virus offers the advantage of copresentation of tumor antigen with host histocompatibility antigens. Viral infection is a highly immunogenic mode of antigen presentation to the immune system, which may result in autoreactivity under certain conditions (23, 24). Such reactivity to a tumor-associated antigen is of importance in the design of a tumor vaccine since many of these antigens are differentiation ("self") antigens which commonly increase immunological suppression rather than T-helper-cell functions. Since the onset of our work, two reports have appeared that further demonstrate the potential of such an approach in tumor immunotherapy (6, 15).

Human melanoma has been used for years as a clinical model for the study of tumor immunotherapy (12, 17; I. Hellström and K. E. Hellström, in J. D. Rodwell, ed., *Targeted Diagnosis and Therapy Series*, in press). There is also well-documented evidence for humoral and cell-mediated immunity against melanoma-associated antigens (11–13). We chose to focus on one such antigen, p97, which is a well-characterized cell surface glycoprotein expressed at high levels in most melanomas (3). cDNA clones of the p97 gene have been isolated, and their nucleotide sequences have been determined (21). This antigen has also been expressed at high levels in various rodent cell lines (G. D. Plowman, Ph.D. thesis, University of Washington, Seattle, 1986), and large quantities of purified p97 can be obtained. We report here the expression of p97 by a recombinant vaccinia virus and the demonstration of its immunogenicity in mice.

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MATERIALS AND METHODS

Cells and viruses. African green monkey kidney cells (BSC-40, a subline of BSC-1 [2]) were propagated in Dulbecco modified Eagle medium (GIBCO Laboratories) supplemented with 10% fetal calf serum (FCS; HyClone), 100 U of penicillin per ml, and 100 μg of streptomycin per ml. CHOSVp97.3 (abbreviated as CHO-p97) was derived from a dihydrofolate-reductase-negative line of Chinese hamster ovary cells (CHO line K-1 [25]) and produces p97 under the control of expression vector SV2p97a (Plowman, Ph.D. thesis). CHO-p97 was propagated in Dulbecco modified Eagle medium plus 8% dialyzed FCS, 150 μg of L-proline per ml, and antibiotics. K1735 (M2 subclone) is a murine melanoma line of C3H/HeN origin (7). M2SVp97.2A (abbreviated as 2A) is a derivative of K1735 cells transformed with vector SV2p97a and expresses human p97 antigen constitutively (Plowman, Ph.D. thesis). SK-Mel 28 is a human melanoma line (27), from which the p97 gene was cloned. The K1735, 2A, and SK-Mel 28 lines were propagated in the same medium as BSC-40 cells.

Vaccinia virus (v-NY) used for the construction of recombinant v-p97NY is a derivative of Wyeth smallpox vaccine (New York City Board of Health strain), plaque purified three times and propagated in BSC-40 cells.

Immunoprecipitation analysis. Cells at confluency or at designated times after virus infection were starved in methionine-free medium for 30 min and then labeled for 2 h with [^{35}S]methionine (specific activity, >800 mCi/ml; New England Nuclear Corp.) at 100 μCi/ml. In designated cultures, tunicamycin (Boehringer Mannheim Biochemicals) was added to a final concentration of 1 μg/ml 1 h before the addition of radioisotopes and was present throughout the labeling period. At the end of this period, the cells were washed with phosphate-buffered saline (PBS) and then lysed in a buffer containing 1% Nonidet P-40, 0.5% sodium deoxy-

cholate, 0.1 M NaCl, 0.01 M Tris (pH 7.4), and 1 mM EDTA. The cell lysates were cleared by centrifugation (30 min at 12,000 × *g*), and portions were used to react with anti-p97 monoclonal antibody (MAb 96.5) or control serum as described (3). Immunoreactive proteins were precipitated with protein A and resolved by electrophoresis on a 10% sodium dodecyl sulfate-polyacrylamide gel (14). Immunoprecipitated proteins were detected by autoradiography of the dried gel.

Quantitation of p97 antigen. Cells were trypsinized briefly, washed with PBS, and divided into tubes, each containing 10^3, 10^4, or 10^5 cells. CHO cells expressing no p97 antigen were used as carrier cells to adjust the cell number to 10^5 per tube. Fab fragments of anti-p97 monoclonal antibody 96.5 were purified and iodinated. Aliquots containing 120 ng (10^6 cpm) of iodinated Fab fragment in FCS were added to a final volume of 50 μl, and the cell suspension was incubated on ice for 1 h. The cells were then washed four times by suspension in PBS plus 10% FCS followed by centrifugation. Radioactivity in washed cells was measured in a Micromedic 4/600+ gamma counter. The number of p97 molecules expressed per cell was calculated as described previously (3) from the radioactivity bound.

Quantitation of anti-p97 antibody. Mice (6-week-old C3H/HeN females, five per group; Charles River Breeding Laboratories, Inc.) were immunized twice at a 4-week interval by tail scarification with 10 μl of crude lysate containing 10^7 PFU of vaccinia virus (v-NY) or its recombinant derivative v-p97NY. Serum samples were collected 2 weeks after the primary immunization and 1 week after the secondary immunization. Anti-p97 antibody was quantitated by enzyme-linked immunosorbent assay as follows. Microtiter plates (Immulon; Dynatech Laboratories, Inc.) were coated overnight with 50 μl of purified p97 (10 μg/ml in PBS). After the antigen was removed, the plates were blocked with 350 μl of 0.5% bovine serum albumin–5% sucrose–0.05 M Tris (pH 8.0) and allowed to dry overnight. Assays were initiated by washing the plate once with PBS, followed by a 1-h incubation with dilutions of test sera or with an anti-p97 monoclonal antibody (MAb 133.2 [3]) as the standard. Antibodies immobilized on the wells were reacted with protein A conjugated to horseradish peroxidase, and the resulting complex was detected by *o*-phenylenediamine, used as the chromogen. Reactivity was measured by A_{490}. A standard curve was constructed to correlate A_{490} readings to the amount of MAb 133.2 added. The amount of p97-specific antibody present in experimental samples was calculated in monoclonal antibody equivalents from the A_{490} readings in the linear range obtained with the appropriate sample dilutions.

Lymphoproliferative assay. Mice were immunized with v-p97NY as described in the previous section. One month after the second immunization, spleens were removed. Spleen cells were mechanically dispersed between frosted glass slides and washed twice in serum-free RPMI 1640 medium (GIBCO). As controls, spleen cells from nonimmunized mice were used. The cells were plated, with or without stimulating antigens, in 96-well round-bottom plates at 10^5 cells per well in 0.2 ml of RPMI 1640 medium containing 0.5% normal mouse serum, 7 mM glutamine, antibiotics, and 2.5×10^5 M β-mercaptoethanol. Cultures were labeled for 6 h on day 4 with 10 μCi of [^{3}H]thymidine (New England Nuclear) per well. Cells were then harvested with a PHD cell harvester, and radioactivity was counted with Optifluor (Packard) in a Beckman LS 3801 counter. Proliferation indices were calculated by dividing the average radioactivity (counts per minute) incorporated in quadruplicate wells stimulated with each antigen by the average counts per minute incorporated in nonstimulated cells.

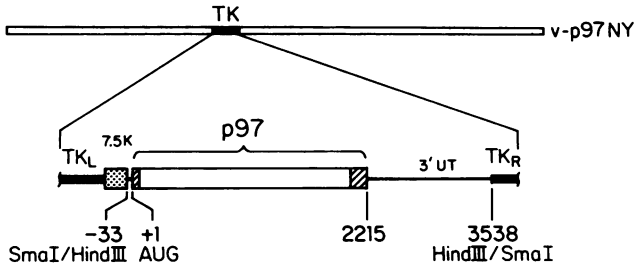

FIG. 1. Structure of v-p97NY genome. The genome of v-p97NY (top open bar) contains a chimeric gene located in the vaccinia virus thymidine kinase (TK) gene (closed bar). This chimeric gene consists of the vaccinia virus 7.5K promoter (stippled bar) ligated to a fragment of human cDNA which contains the coding sequence for melanoma-associated antigen p97 (open and hatched bars), as well as 33 and 1,323 base pairs of 5'- and 3'-untranslated (UT) sequences, respectively. This cDNA fragment was inserted in the *Sma*I site on the plasmid pGS20 (18). The hatched areas at the 5' and 3' termini of the p97 coding region indicate the positions of the putative signal and transmembrane sequences, respectively.

Footpad swelling assay. Mice (five per group) were immunized once with v-p97NY or v-NY as described above. Six days later, the animals were injected with 20 μl of PBS in the right hind paw and with 20 μl of PBS containing 2A (p97$^+$) or K1735 (p97$^-$) cells in the left hind paw (5×10^5 cells per mouse). The thicknesses of the footpads were measured 24 h later in a double-blind manner with a Fowler micrometer. The thickness of the PBS-injected footpad was subtracted from that of the experimental footpad for each mouse. The mean and the standard deviation of the incremental swelling in each group were calculated.

RESULTS

Construction of recombinant virus. A recombinant vaccinia virus, v-p97NY, was constructed by the general scheme described by Mackett et al. (18). This recombinant contains a chimeric gene inserted in the coding sequence of vaccinia virus thymidine kinase. This chimeric gene consists of the vaccinia virus 7.5K promoter followed by a fragment of a human cDNA clone containing the entire coding sequence for p97 antigen (Fig. 1). This fragment also contains 33 and 1,323 base pairs, respectively, of 5'- and 3'-untranslated sequences. The initiation codon for p97 in the recombinant is located 76 base pairs downstream from the 5' terminus of the transcript for the chimeric gene and represents the first such codon in this transcript. Translation started at this codon results in the expression of p97 protein with no additional sequences attached.

Expression of p97 by v-p97NY. To confirm the expression of p97 by this recombinant virus, we analyzed metabolically labeled proteins from v-p97NY-infected cells by immunoprecipitation. Monoclonal antibody against p97 predominantly precipitated a single protein (Fig. 2, lane 3) with the same electrophoretic mobility as that of p97 antigens produced either by transformed Chinese hamster ovary cells (Fig. 2, lane 5) or by human melanoma cell lines (data not shown). Pretreatment of v-p97NY-infected cells with tunicamycin resulted in the synthesis of a polypeptide with a molecular weight of 82,000 (Fig. 2, lane 4) that comigrated with the

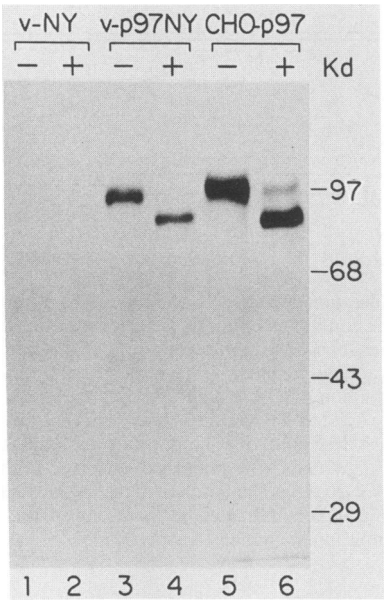

FIG. 2. Radioimmunoprecipitation analysis of p97 protein produced by v-p97NY. Recombinant v-p97NY (lanes 3 and 4) or its parental strain v-NY (lanes 1 and 2) was used to infect BSC-40 cells at a multiplicity of infection of 10. At 12 h postinfection, the cells were labeled with [^{35}S]methionine for 2 h. Cleared cell lysates were reacted with mouse monoclonal antibody against p97, and immunoprecipitated proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Lanes +, Tunicamycin (1 μg/ml) was added to infected cell cultures 1 h before the addition of the radioisotope label. Cultures of a rodent cell line (CHO-p97) expressing human p97 were labeled, and lysates of the cultures were prepared as described above. Proteins from these lysates were immunoprecipitated with monoclonal antibody against p97 and used as controls (lanes 5 and 6). The molecular masses of electrophoretic markers are indicated in kilodaltons (Kd).

unglycosylated form of p97 produced by transformed Chinese hamster ovary cells under similar conditions (Fig. 2, lane 6). The size of this polypeptide was as predicted by the coding capacity of the p97 gene (21). No immunoreactive protein was detected by p97-specific monoclonal antibody in cells infected with parental vaccinia virus (Fig. 2, lanes 1 and 2). These results indicate that v-p97NY was able to direct the expression of a glycoprotein immunologically related to melanoma-associated antigen p97.

To verify the cell surface localization of this protein and to estimate the level of expression, we assayed the ability of v-p97NY-infected cells to bind ^{125}I-labeled monoclonal antibodies specific for p97. The results (Table 1) indicate that approximately 10^6 molecules of p97 analog per cell were expressed on the surface of v-p97NY-infected cells. This

TABLE 1. Surface expression of p97 antigen by cells infected with recombinant vaccinia virus v-p97NY

Cell type	Infecting virus	Radioactivity bound (cpm)	No. of input cells	Molecules of p97/cell (10^3)
BSC-40	v-p97NY	4,200	10^3	1,140.0
BSC-40	v-NY	<200	10^5	<0.5
CHO	None	<200	10^5	<0.5
CHO-p97	None	18,900	10^3	5,140.0
2A	None	8,850	10^3	2,400.0
SK-Mel 28	None	7,372	10^4	190.0

TABLE 2. Production of p97-specific antibodies in mice immunized with recombinant virus v-p97NY

Immunizing virus	p97 antibody titer (monoclonal antibody equivalents [μg/ml])	
	Primary[a]	Secondary[b]
v-p97NY	19 ± 11	70 ± 21
v-NY	0.07 ± 0.01	0.02 ± 0.01

[a] Primary bleed was collected 2 weeks after the first inoculation.
[b] Secondary bleed was collected 1 week after the second inoculation at week 4.

level is higher than that observed for SK-Mel 28 human melanoma cells expressing a high level of p97 (3) but lower than that for two rodent cell lines (CHO-p97 and 2A) expressing the same antigen under control of the simian virus 40 promoter. This result indicates the potential usefulness of the recombinant virus in generating target cells for the study of cell-mediated immunity against p97.

Immunogenicity of v-p97NY. Serum samples from mice immunized with v-p97NY were assayed for the presence of p97-specific antibodies. The results (Table 2) show that such antibodies were generated 2 weeks after a single inoculation with the recombinant virus. The level of this antibody increased after a second inoculation administered at week 4. This level is comparable to that obtained when either purified p97 or rodent cells expressing such antigens were used as immunogens (Plowman, Ph.D. thesis). No p97-specific antibody was generated in animals inoculated with parental vaccinia virus (Table 2).

To demonstrate cell-mediated immunity elicited by v-p97NY, we isolated spleen lymphocytes from control and immunized mice and assayed the ability of these cells to proliferate in vitro upon stimulation by various antigens. The

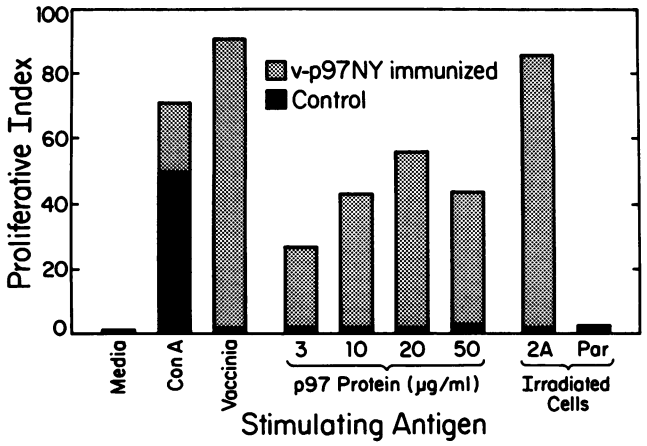

FIG. 3. In vitro proliferation of spleen cells from v-p97NY-immunized mice. Spleen cells from nonimmunized mice (solid bar) or mice immunized with v-p97NY (stippled bars) were harvested and cultured as described in the text. The concentrations of stimulating antigens were as follows: concanavalin A (ConA), 10 μg/ml; UV-inactivated vaccinia virus, 10^6 PFU/ml (before inactivation); gamma-irradiated 2A and parental K1735 (Par) cells, 10^4 per well. Values for [^{3}H]thymidine incorporated were the means for four duplicate wells. The proliferative index was calculated as follows: (counts per minute incorporated into stimulated cells/counts per minute incorporated into nonstimulated cells) × 100.

TABLE 3. DTH to p97 in mice immunized with v-p97NY

Immunizing virus	% Footpad swelling after injection of:	
	2A ($p97^+$)	K1735 ($p97^-$)
v-p97NY	22.2 ± 3.7	1.7 ± 1.5
v-NY	1.3 ± 2.0	2.9 ± 2.6

results (Fig. 3) indicate that the lymphocytes from immunized animals proliferated upon stimulation by the mitogen concanavalin A, purified p97 antigen, or UV-inactivated vaccinia virus. They also responded to stimulation by gamma-irradiated syngeneic cells expressing p97 but not to parental cells. In contrast, spleen cells from control animals proliferated only in the presence of concanavalin A. Since stimulated blast cells from v-p97NY-immunized animals expressed Pan-T and Lyt-1 phenotypes and released interleukin-2 (data not shown), they appeared to be p97-specific T-helper cells.

To determine whether an immune response which may be capable of rejecting tumor cells in vivo (10) is also primed by v-p97NY, we assayed for delayed-type hypersensitivity (DTH) by measuring footpad swelling in mice immunized with v-p97NY or the parental virus v-NY. One week after immunization, footpads were injected with syngeneic tumor cells expressing p97. DTH was detected in the v-p97NY-immunized animals but not in the animals immunized with parental vaccinia virus (Table 3). The response was specific for p97, because injection of syngeneic mouse cells not expressing p97 did not result in any swelling.

DISCUSSION

The use of recombinant vaccinia virus as a novel approach to the development of vaccines has been explored largely in the area of infectious diseases. Recent studies described the use of this approach for immunotherapy and prophylaxis in virus-induced tumor models in rodents (6, 15). We are interested in the potential of this approach for human cancer therapy. This report represents the first description of a recombinant vaccinia virus expressing a differentiation antigen associated with a human tumor.

Viral oncolysates, including those prepared from vaccinia virus-infected melanoma cells, have been used as tumor vaccines for active immunotherapy (5, 26). However, immunological studies of such vaccines are complicated by the complex nature of the immunogen, because antigens may be present in suboptimal amounts, and because molecules with "suppressogenic" properties may exist (20). It is therefore important that recombinant v-p97NY expressed in high quantity a single species of immunoreactive glycoprotein indistinguishable from authentic p97 antigen. Immunization with this recombinant allows studies of immune responses targeted toward this antigen.

Results reported here indicate that both humoral and T-cell-mediated immune responses against a tumor-associated antigen were induced in mice immunized with v-p97NY. Of particular interest is the finding that v-p97NY induced DTH. Attempts to demonstrate footpad swelling in mice immunized with high amounts of purified p97 protein were unsuccessful (data not shown), despite the ability of the purified protein to induce high-titered antibodies to p97 (Plowman, Ph.D. thesis). Since DTH is believed to be important in the recruitment of tumoricidal macrophages in the vicinity of the tumor (8), our results indicate that recombinant virus v-p97NY may be more effective than p97 protein in tumor immunotherapy. This is supported by recent results from tumor challenge experiments in mice (C. D. Estin, U. S. Stevenson, G. D. Plowman, S.-L. Hu, P. Sridhar, I. Hellström, J. P. Brown, and K. E. Hellström, Proc. Natl. Acad. Sci. USA, in press).

Since the objective of this approach is human cancer therapy, it is important to determine whether recombinant v-p97NY can elicit immune responses against human p97 antigen in a species more closely related to humans than mice are. Recent studies indicated that both p97-specific antibodies and DTH were generated in macaques immunized with the recombinant virus (Estin et al., in press). Finally, it is of interest to determine whether a vaccinia virus vector can act as an adjuvant in stimulating an immune response against an antigen expressed on normal tissues, which is important in the realization of tumor vaccines. We believe the approach we have taken and the recombinant virus constructed will be of use in the study of tumor immunotherapy.

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