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Immunology

Effective tumor immunotherapy directed against an oncogene-encoded product using a vaccinia virus vector

(immunotherapy/*neu* oncogene/vaccinia virus)

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ABSTRACT **We have constructed a vaccinia virus recombinant that expresses the extracellular domain of the rat *neu* oncogene-encoded protein, a 185-kDa transmembrane glycoprotein termed p185. Strain NFS mice immunized with this recombinant virus developed a strong antibody response against the *neu* oncogene product and were fully protected against subsequent tumor challenge with *neu*-transformed NIH 3T3 cells. No tumor immunoprotection was found when recombinant virus-immunized mice were challenged with Ha-*ras*-transformed NIH 3T3 cells. These data indicate that immunization with a single oncogene-encoded antigen can fully and specifically protect animals against tumor cells bearing this antigen.**

The discovery over the past decade of cellular oncogenes has provided one explanation of the molecular mechanisms responsible for neoplastic conversion of many cell types (1). Nevertheless, these genes and similarly acting cellular elements can, at best, explain only part of the process of tumor formation. Before growing out into a tumor, the transformed cells must confront and evade physiological mechanisms designed to defend the host against cancer.

Prominent in these defenses are presumably immune mechanisms that involve specific recognition and elimination of tumor cells. These mechanisms are poorly understood: the nature and importance of immunological effector mechanisms are unclear, and the identities of tumor cell markers that may be recognized by these effectors remain mostly elusive.

We have created a model of tumor cell recognition by endowing tumor cells with a specific, single antigenic marker distinguishing these cells from those of adjacent normal tissue. This marker derives from the extracellular domain of the rat *neu* oncogene-encoded protein p185 (2). By transfecting NIH 3T3 mouse cells with the rat oncogene, we cause these cells to undergo neoplastic transformation and at the same time to express the oncogene-encoded p185 antigen at the cell surface (2, 3).

The ectodomain of this rat p185 antigen constitutes a highly immunogenic determinant in tumor-bearing NFS mice; these mice invariably mount a strong serum response to this protein (2). Tumors formed in NFS mice from *neu* transfectants initially grow rapidly but ultimately regress. This regression is not seen with tumors formed from other types of oncogene-transfected cells and can be attributed, at least partially, to recognition of the *neu* transfectants by the host immune system.

The immune mechanisms that effect this regression of *neu*-transformed cells and establish anti-tumor immunity remain undefined. Possibly the p185 antigen alone suffices to induce the anti-tumor response. Alternatively, this antigen may only provoke an effective response when acting in concert with other unrelated, transformation-specific antigens displayed by the oncogene-transformed cells. To test this hypothesis, we immunized mice with the p185 antigen alone using a vaccinia virus vector to present antigen to mice that were subsequently challenged with oncogene-transformed cells. Immunization against the *neu*-encoded antigen alone served as effective prophylaxis against tumors formed by *neu* oncogene-transfected cells.

MATERIALS AND METHODS

Virus and Cells. CV-1 cells were obtained from the American Type Culture Collection (ATCC CCL70) and were grown in Eagle's minimal essential media (MEM) supplemented with 10% fetal calf serum. Vaccinia virus strain VZ2 contains the *Escherichia coli lacZ* gene inserted at the *Bam*HI site in the vaccinia virus *Hin*dIII F region (4). This virus expresses β-galactosidase and forms blue plaques in the presence of the chromogenic substrate Bluo-gal (Bethesda Research Laboratories).

Construction of a Chimeric Donor Plasmid for *in Vivo* Recombination. pEVAC is a recombinant plasmid that contains a 2.5-kilobase (kb) *Pst* I fragment corresponding to the middle portion of the vaccinia virus *Hin*dIII F fragment (5); this *Pst* I fragment is inserted into the *Pst* I site of a derivative of pEMBL18 (6) lacking a *Bam*HI restriction site. Adjacent to the *Bam*HI site in the vaccinia virus fragment is an early vaccinia promoter, designated *Bam*-F, which has been used previously to express a variety of antigens (7). This vector was used to insert the rat neu cDNA described by Bargmann *et al.* (8). To disable the oncogenic function of the *neu*-encoded protein, we deleted internal sequences [between the *Bam*HI site at nucleotide (nt) 2175 and the *Bgl* II site at nt 3250] in the neu cDNA sequence (8). This deletion removes the region specifying the tyrosine kinase domain of the *neu*-encoded protein; additionally, it generates a frameshift mutation downstream of the kinase domain, creating a new stop codon shortly after the *Bgl* II site at nt 3250. The resulting construct was designated pEVAC-neu.

Construction, Identification, and Purification of Recombinant Vaccinia Virus. The recombinant vaccinia virus was constructed as previously described (9). In short, CV-1 cells (10^6 cells per 6-cm plate) were infected with vaccinia virus VZ2 at a multiplicity of infection of 2 and were incubated for 40 min at 37°C. Cells were then transfected with 27 μg of calcium orthophosphate-precipitated pEVAC-neu DNA. After a further incubation for 16 hr at 37°C, virus was harvested and viral titers were determined.

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Abbreviations: pfu, plaque-forming unit; mAb, monoclonal antibody.

The DNA used for this transfection could recombine with homologous sequences in the *Hin*dIII F region of the parental VZ2 genome, thereby replacing the *lacZ* gene. As a result, recombinant virus appeared as white plaques in the presence of Bluo-gal, while the parental virus VZ2 appeared blue. White plaques were picked, and five cycles of plaque purification were done. One of the isolated recombinant viruses, designated ABT9-4, was amplified and analyzed for expression of the truncated *neu*-encoded protein.

ELISA. Serum antibody responses to vaccinia were detected using a solid-phase ELISA. Sucrose gradient-purified vaccinia virus (WR strain) at a protein concentration of 10 μg/ml in 0.05 M carbonate buffer, pH 9.6, was used to passively coat microtiter wells. After 2 hr at 37°C, the solution was aspirated, and dilutions of test sera were added to the wells. Following a 1-hr incubation at 37°C, the wells were washed three times with phosphate-buffered saline supplemented with 0.05% Tween 20 and were then incubated with horseradish peroxidase (HRP)-labeled goat anti-mouse IgG (Jackson ImmunoResearch, Avondale, PA) at a dilution of 1:5000. Rat sera were tested using an HRP-labeled $F(Ab)_2$ goat anti-rat IgG, also at a dilution of 1:5000. After incubation with the second antibody, the wells were again washed three times with PBS/Tween, and color was developed using 3,3,5,5'-tetramethylbenzidine (TMB, Sigma). Ten micrograms of TMB was dissolved in 1 ml of dimethyl sulfoxide, and 100 μl of this solution was added to 5 ml of acetate citrate buffer, pH 6.0, with 10 μl of 3% H_2O_2 (vol/vol). Color was allowed to develop for 5 min, after which the reaction was stopped by adding 2.5 M H_2SO_4. Absorbance was read at 450 nm in a Dynatech (Alexandria, VA) Minireader II plate reader.

Serum antibody responses to the rat p185 protein were determined similarly, using a cell lysate of DHFR/G8 cells (DHFR, dihydrofolate reductase) to coat the microtiter wells. DHFR/G8 cells overexpress the nontransforming rat p185 protein (10). ELISA titers against the p185 protein are reported as the last dilution that gives an OD reading at least 0.05 unit above background binding OD. ELISA titers against vaccinia virus are defined as the serum dilution that results in a half-maximum OD.

RESULTS

Construction of a *neu*-Containing Recombinant Vaccinia Virus. The *neu* oncogene was initially detected by transfection of DNAs of chemically induced rat neuroblastomas into NIH 3T3 mouse cells (2, 11). The resulting transfectants were found to be tumorigenic in NFS mice. These mice also were found to mount a strong humoral immune response against the extracellular portion of the p185 protein specified by the transfected rat gene (2). This p185 protein has many properties of a growth factor receptor. Besides the extracellular domain, it has a transmembrane domain and an intracellular domain with sequences that share homology with proteins having tyrosine kinase activity (8, 12). The *neu*-encoded protein found in the oncogene-transfected cells differs from its normal counterpart by a single amino acid substitution in the transmembrane domain of the protein (13).

We used a vaccinia virus vector to present the *neu* oncogene-encoded antigen to the immune system of NFS mice. Vaccinia vectors have been used to generate immunity to a number of transmissible agents, largely enveloped viruses; these include rabies virus, hepatitis B virus, influenza viruses, Friend leukemia virus, and human immunodeficiency virus (14–18). In each case, cloned genes specifying viral glycoproteins have been stably introduced into a nonessential region of the vaccinia virus genome. These introduced genes, the transcription of which is driven by vaccinia virus promoters, are expressed concomitantly with the vaccinia genes in cells infected by the viral vector. The proteins specified by these transduced genes appear to undergo proper maturation and post-translational modification.

Because the ectodomains of these viral glycoproteins resemble biochemically that of p185 protein, we reasoned that the vaccinia vector system could also be used to generate immunity to the *neu*-encoded p185 protein. Specifically, we anticipated that the antigen would be displayed at the surface of vaccinia infected cells, mimicking its configuration on the surface of oncogene-transformed cells. Accordingly, we hoped for the development of immunity to cells displaying this protein.

To test these ideas, we adapted a cDNA clone of the *neu* oncogene for introduction into the vaccinia vector. We first removed the bulk of the sequences specifying the cytoplasmic domain of the protein. By deleting the kinase domain, we presumably disabled the oncogenic effector functions of p185 while leaving intact the immunogenic ectodomain. The truncated neu cDNA clone, encoding the ectodomain, the transmembrane anchor domain, and ≈50 amino acid residues of the intracellular domain, was then joined with the *Bam*-F promoter of vaccinia virus. The resulting construct was designated pEVAC-neu (Fig. 1). This chimeric gene was then introduced into vaccinia virus by homologous recombination (*Materials and Methods*). The resulting chimeric virus was termed ABT 9-4.

Expression in Infected Cells. To test whether the manipulated *neu* gene encoded by the recombinant vaccinia virus is expressed in infected cells, CV-1 cells were infected at a multiplicity of infection of 10 plaque-forming units (pfu) per cell with either the ABT 9-4 recombinant virus or an equal dose of WR wild-type vaccinia virus. Directly after infection, [^{35}S]cysteine was added, and infection was allowed to proceed for 6 hr. Following this, infected cells were lysed and lysates were immunoprecipitated with the anti-p185 monoclonal antibody (mAb) 7.16.4 (3). This antibody reacts with a still undefined determinant located in the ectodomain of the protein. As can be seen in Fig. 2, ABT 9.4-infected cells, but not wild-type vaccinia virus-infected cells, produce a 100-kDa protein that is precipitated by the mAb 7.16.4. The M_r of the precipitated protein agrees well with that calculated for

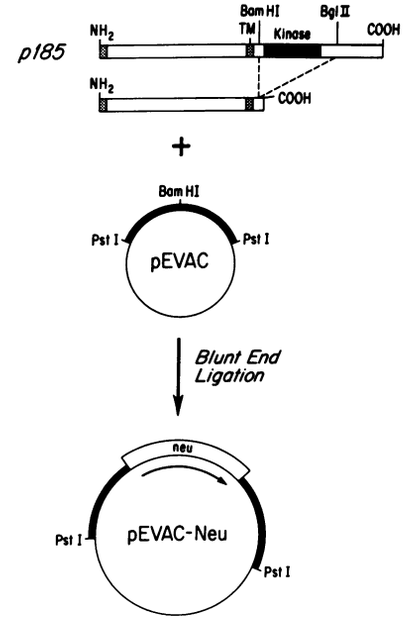

FIG. 1. Schematic representation of the construction of the pEVAC-neu plasmid. (*Top*) Schematic representation of the p185 gene. TM, trans-membrane domain; black box, domain homologous to proteins with tyrosine kinase activity. The cDNA lacking a kinase domain was inserted at the *Bam*HI site of pEVAC.

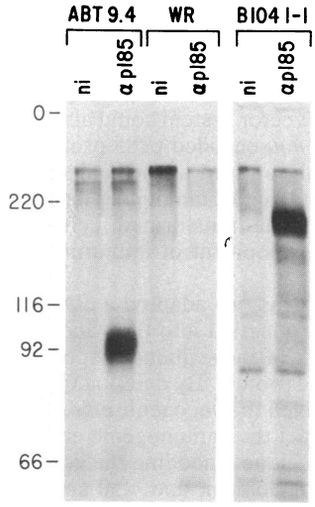

FIG. 2. Expression of internally deleted p185 in vaccinia virus-infected cells. CV-1 cells were infected with either wild-type vaccinia virus (strain WR) or ABT9-4 recombinant virus. Infected cells were labeled with [^{35}S]cysteine. Labeled lysates of infected cells were immunoprecipitated with an anti-p185 mAb and separated by NaDodSO$_4$/acrylamide gel electrophoresis. A lysate from B101-1-1 cells (expressing the transforming p185 protein) was added as a control. Lanes: ni, lysates precipitated with a nonimmune mouse serum; α p185, lysates immunoprecipitated with the 7.16.4 mAb. Positions of the M_r markers are indicated.

the protein specified by the truncated *neu* gene. We conclude that the ABT 9-4 recombinant directs the synthesis of a truncated p185 molecule.

Immune Reactivity in Virus-Infected Mice. The ability of inbred mice to respond to a foreign antigen differs widely between strains. Accordingly, we first tested various mouse strains for their ability to mount an immune response against the rat p185 protein. Eight-week-old mice of various strains were inoculated i.p. with 10^8 pfu of either wild-type vaccinia virus or an equal dose of ABT9-4 recombinant virus. After 4 weeks, a booster injection of 10^8 pfu of virus was given, and sera were collected 2 weeks later. The production of antibodies against the *neu* oncogene product was followed using an ELISA assay as described. Table 1 shows that not all mouse strains can respond to the rat p185 protein. We assume that these differences are due to strain differences in major histocompatibility complex haplotypes. For example, neither mouse strain of the *H-2^d* haplotype responded to the *neu*-encoded product. In subsequent experiments we concentrated on NFS mice as a model. These mice are closely related to the strain from which the NIH 3T3 cell line arose; thus they represent a reasonable host for oncogene-transformed NIH 3T3 tumor cells.

Table 1. Antibody titers of different mouse strains to rat p185 protein after vaccination with vaccinia recombinant ABT9-4

Strain	Haplotype	Immunity	ELISA titer
BALB/c	*H-2^d*	Nonimmune	0
		Immune	0
C3H/HeN	*H-2^k*	Nonimmune	0
		Immune	≥1:80
DBA/2N	*H-2^d*	Nonimmune	0
		Immune	0
NFS	Outbred	Nonimmune	0
		Immune	1:40
NzW/LacJ	*H-2^z*	Nonimmune	0
		Immune	≥1:80
SM/J	*H-2^v*	Nonimmune	0
		Immune	0
Swiss	Outbred	Nonimmune	0
		Immune	≥1:80

Mice were immunized i.p. with 10^8 pfu of ABT9-4 recombinant virus. After 4 weeks mice were boosted with a similar dose of virus. Indicated titers were obtained with sera collected 2 weeks after immunological boost. ELISA assays were done as described.

We next determined the kinetics of the development of immunity against the p185 protein in NFS mice immunized with the vaccinia virus recombinant. NFS mice were immunized with a single s.c. injection of 10^8 pfu of the ABT9-4 recombinant virus. Control mice were immunized in parallel with an equal dose of wild-type vaccinia virus. To monitor the development of immunity, sera were collected at weekly intervals, and the sera were tested for the ability to precipitate p185 from labeled cell lysates.

Fig. 3 shows that infection by the recombinant vaccinia virus resulted in high titers of antisera against p185 within 3 weeks. Further, the serum titers slightly increased in the following week. A similar pattern was found for the development of antibodies directed against vaccinia virus in these mice as measured in an ELISA assay (data not shown). As expected, no reactivity against p185 was developed when mice were exposed to the wild-type vaccinia virus (Fig. 3, lanes 2 and 3). These data made it clear that a single injection of recombinant vaccinia virus leads to the efficient induction of anti-p185 antibody within a period of 4 weeks.

Tumor Rejection in Immune Mice. Subsequent experiments were designed to test whether immunization of NFS mice with the ABT9-4 recombinant virus had an effect on the ability of *neu*-transformed NIH 3T3 cells to establish tumors. These NIH 3T3 derivatives, termed B104-1-1, carry the rat *neu* oncogene and display substantial amounts of oncogene-encoded p185 on their surface (2).

Young adult NFS mice were injected i.p. with 10^8 pfu of wild-type or recombinant vaccinia virus and challenged with various doses of B104-1-1 tumor cells 4 weeks postimmunization. Both viruses provoked a similar anti-vaccinia virus

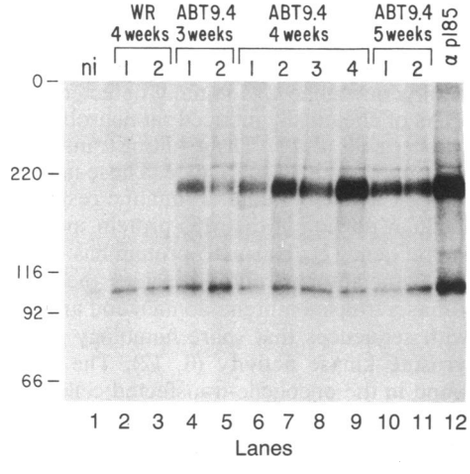

FIG. 3. Development of antibody response to p185 protein in vaccinia virus-infected mice. Mice were immunized with a single inoculation of either wild-type or ABT9-4 recombinant virus. Serum from immunized animals was collected after 3, 4, and 5 weeks; these sera were then used to precipitate p185 from ^{32}P-labeled lysates of DHFR/G8 cells. These cells overexpress the nontransforming version of p185. Lanes: 1, nonimmune mouse serum; 2 and 3, sera from mice immunized with wild-type vaccinia virus collected 4 weeks postimmunization; 4 and 5, sera from mice immunized with ABT9-4 recombinant virus, 3 weeks postimmunization; 6–9, sera from ABT9-4 immunized mice, 4 weeks postimmunization; 10 and 11, sera from ABT9-4 immunized mice, 5 weeks postimmunization; and 12, mAb 7.16.4.

immune response in these mice, as measured in a vaccinia virus ELISA assay (data not shown). The growth of tumors at the site of injection was followed in time and is presented in Fig. 4.

In mice immunized with wild-type virus B104-1-1, cells grow progressively for the first 12–19 days, after which the tumors begin to regress spontaneously and finally disappear completely after about 5 weeks. A similar pattern of tumor growth and rejection was observed when nonimmunized NFS mice were injected with an equal dose of B104-1-1 cells (data not shown). Because tumor regression is not seen when these cells are injected in athymic nude mice, tumor regression is probably caused by the spontaneous development of immunity against these cells.

A quite different result was obtained when B104-1-1 cells were injected into NFS mice immunized with the ABT9-4 recombinant virus (Fig. 4 *A* and *B*). Following injection of a tumor cell dose of 2×10^6 cells per animal, no tumor developed at the site of injection; at a dose of 10^7 cells per animal, a small nodule developed at the site of injection within 5 days that quickly disappeared in the next several days. These results show that immunization with the vaccinia virus recombinant drastically inhibits the outgrowth of p185-expressing tumor cells.

No difference in tumor outgrowth was seen when mice immunized with either wild-type virus or recombinant virus were challenged with Ha-*ras*-transformed NIH 3T3 cells. This showed that the immune protection brought about by the recombinant vaccinia virus is specific for tumor cells displaying the *neu* oncogene-encoded p185 (Fig. 4*C*).

Immune Reactivity in Rats. The introduction of the *neu*-transformed NIH 3T3 cells into NFS mice represents an experimental artifice in that these tumor cells present an immunogenic rat protein to the mouse host. The immunogenicity of this protein appears to induce the eventual rejection of tumors formed from these cells (Fig. 4 *A* and *B*). This situation would seem to contrast with one arising in an animal bearing an autochthonous tumor or a tumor deriving from fully syngeneic cells. In these latter cases, no antigen of allogeneic origin is presented, and potently immunogenic proteins are usually not displayed by the tumor cells.

This reasoning caused us to question whether immunity to p185 antigen and associated tumor rejection could be developed in a fully syngeneic system. Thus we attempted to immunize BDIX rats (Animal Genetics and Production Branch, National Cancer Institute, Bethesda, MD) with the vaccinia recombinant expressing the p185 antigen. The neuroblastomas in which the *neu* oncogene arose were induced in rats of this strain (19). Though the ectodomain encoded by the vector-born *neu* gene is identical to that of the normal p185 expressed in the BDIX rat, the possibility remained that the single amino acid substitution present in the transmembrane domain of the oncogene-encoded protein might confer immunogenicity on this protein. This amino acid substitution is specified by the truncated *neu* gene borne by the vaccinia vector.

To measure the effectiveness of the vaccinia recombinant virus in rats we immunized the following rat strains with ABT9-4 recombinant: BDIX, Fisher, Lewis, Sprague–Dawley, Wistar–Kyoto. Weanling rats were immunized by i.p. injection of 10^8 pfu of wild-type vaccinia virus or ABT9-4 recombinant virus followed by a second i.p. injection of 10^8 pfu of virus 3 weeks later. Two weeks after the booster injection animals were bled, and their sera were tested for reactivity with vaccinia virus antigens. Both strains of virus grew well in these rats, provoking equivalent antivaccinia serum response (titers $\geq$1:10,000 in a vaccinia virus-ELISA were found in all rat strains immunized). These sera were also tested for the ability to precipitate either the normal or the transforming version of the p185 protein from lysates of *neu*-transfected cells. No reactivity against either protein was found in any of the rats tested (data not shown).

Although no humoral immunity against p185 was elicited in these rats, these rats might still display an effective antitumor response. Accordingly, we tested whether immunization of BDIX rats by the ABT9-4 virus would result in inhibition of the growth of B104 neuroblastoma cells. These B104 cells derived directly from a chemically induced tumor of a BDIX rat and express the transforming version of p185 (19). Exposure of the BDIX rats to the ABT 9-4 virus led to no significant inhibitory effect on the growth of injected B104 tumor cells (data not shown). It appears that the ectodomain of the *neu*-p185 that is expressed in vaccinia vector-infected cells is not immunogenic in BDIX rats, in that neither anti-p185 serum response nor anti-tumor immunity was observed. It remains possible, however, that recombinants that express the *neu*-encoded p185 protein at a higher level, possibly used in combination with drugs that reduce tolerance of animals against a self product, may be more effective

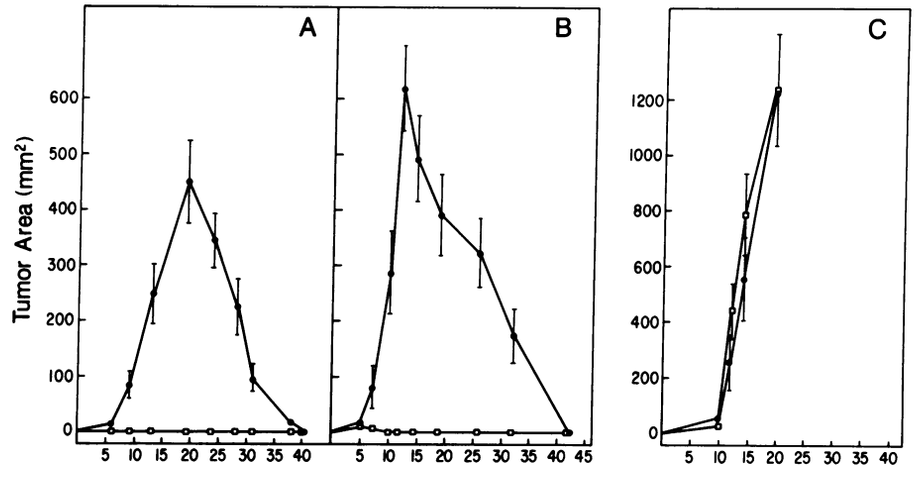

FIG. 4. Tumor challenge of mice vaccinated with vaccinia virus recombinants. Young adult NFS mice were immunized with a single injection of 10^8 pfu of wild-type vaccinia virus or ABT9-4 recombinant virus. Four weeks later, these mice were challenged with either 2×10^6 (*A*) or 1×10^7 *neu*-transformed NIH 3T3 cells (*B*). As control, a group of immunized mice was challenged with Ha-*ras*-transformed NIH 3T3 cells (*C*). Each group consisted of 10 mice. Data are represented as the average tumor area ± SD. ●, Wild-type vaccinia-immunized mice; □, ABT9-4 recombinant-immunized mice.

in inducing immunity in syngeneic animals. Also, alternate routes of vaccination may more effectively stimulate cellular immune responses. Animals vaccinated intradermally with recombinant vaccinia viruses often mount a better, more protective immune response than if vaccinated i.p. (ref. 17; unpublished data). Here, however, the presence of an amino acid substitution in the transmembrane domain of the p185 protein appears to be insufficient to overcome a tolerance that the rat immune system shows towards this protein.

DISCUSSION

We describe the construction of a vaccinia virus recombinant expressing the extracellular domain of the rat *neu* oncogene and its use in tumor immunotherapy. Our data indicate that the recombinant vaccinia virus-induced immunity results in the full protection of mice from subsequent tumor challenge with cells that express the rat *neu* oncogene. The immunological effector mechanisms involved in inducing anti-tumor immunity have not been studied extensively. Our data indicate that the vaccinia virus recombinant can induce significant antibody titers against the rat *neu* oncogene protein in vaccinated mice. The effect of high levels of circulating antibodies against the *neu*-encoded p185 molecule may be 2-fold. First, they may contribute to immunological rejection of the *neu*-transformed tumor cells. Furthermore, it was shown by Drebin *et al.* (20) that addition of anti-p185 antibodies to the culture medium of *neu*-transformed cells resulted in reversion of the transformed phenotype of these cells, presumably by downmodulation of the cell surface expression of the *neu* oncogene product. Anti-p185 antibodies may have a similar effect on *neu*-transformed cells *in vivo*, which may further contribute to the prevention of tumor outgrowth. *In vivo* treatment of nude mice with an anti-p185 mAb resulted in the inhibition of tumorigenicity of *neu*-transformed cells (21). However, inhibition of tumorigenicity in these experiments was partial, indicating that antibody treatment alone is insufficient to cause complete regression of the tumor induced by the *neu*-transformed cells. In the present study complete protection against tumor challenge with *neu*-transformed cells was seen, suggesting that an immune mechanism other than humoral immunity was induced by the vaccinia virus recombinant. Supporting this view is the finding that vaccinia virus vectors can effectively induce T-cell mediated immune responses in immunized animals (17).

Our data demonstrate that immunization with a single, well-defined antigen can confer protection against tumor cells bearing this antigen. This contrasts with other experimental models in which animals are immunized with tumor cells or tumor cell extracts in which a complex mixture of antigens provokes immunity.

The present experiments show that vaccinia virus can serve as an effective vector for inducing immunity against antigen-bearing cells. A similar result was recently obtained by Lathe *et al.* (22) using polyoma virus-encoded antigens. The success of our experiments appears to stem from the allogeneic origin of the antigen that induced an immune response in the vaccinated host. However, present results provide no assurance that such immunity will develop if the antigen in question differs only subtly from similar proteins found in the normal tissues of tumor-bearing hosts. Distinct tumor-associated antigens have been well documented in the syngeneic tumors of mice and in human tumors of autochthonous origin (23). The presence of such tumor-associated antigens, however, probably reflects expression of genes that are normally expressed at a low level in other cells of the body, or at a different time of development. As such, it is unlikely that they are strongly immunogenic, and attempts to provoke anti-tumor immunity by vaccination with these antigens may be more difficult.

One encouraging result has recently been obtained by Hearing *et al.* (24), who showed that immunization of mice with a purified mouse melanoma-specific antigen conferred resistance to subsequent challenge with mouse melanoma cells. How the immunization with the melanoma antigen can provoke an immune response in a fully syngeneic host remains unclear. In our experiments induction of immunity was unsuccessful under analogous conditions. The data of Hearing *et al.* do suggest, however, that tumor-associated antigens can be successfully used as targets for tumor immunotherapy. Vaccinia virus may be an efficient tool for presenting these antigens to the immune system of the tumor-bearing host.

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