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Vaccination against tumor cells expressing breast cancer epithelial tumor antigen

(vaccine/recombinant vaccinia virus)

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ABSTRACT **Ninety-one percent of breast tumors aberrantly express an epithelial tumor antigen (ETA) identified by monoclonal antibody H23. Vaccinia virus recombinants expressing tumor antigens have considerable promise in the active immunotherapy of cancer, and we have evaluated the potential of vaccinia recombinants expressing the secreted (S) and cell-associated (transmembrane, T) forms of H23 ETA to elicit immunity to tumor cells expressing ETA. Tumorigenic *ras*-transformed Fischer rat fibroblast lines FR-S and FR-T, expressing the S or T form of H23 ETA, respectively, were constructed for use in challenge experiments. Expression of H23 ETA in these lines was confirmed by Western blotting and immunofluorescence. When challenged by subcutaneous seeding of tumor cells, 97% (FR-S) and 91% (FR-T) of syngeneic Fischer rats rapidly developed tumors that failed to regress. Vaccination with recombinant vaccinia virus expressing ETA-T prior to challenge prevented tumor development in 82% of animals seeded with FR-T cells but in only 61% of animals seeded with FR-S. The vaccinia recombinant expressing the S form was a less effective immunogen, and vaccination protected only 29–30% of animals from developing tumors upon challenge with either FR-S or -T cells. The increased immunogenicity of the recombinant expressing ETA-T was reflected in elevated levels of ETA-reactive antibody in vaccinated animals, confirming that secreted antigens expressed from vaccinia virus are less effective immunogens than their membrane-associated counterparts.**

Tumor-associated antigens have considerable promise as targets for active or passive immunotherapy. Attempts have been made to elicit tumor immunity through inoculation of purified antigens or tumor lysates (1–7) or anti-idiotypic antibodies directed against tumor-associated antigens (8–10). Such approaches have met with only limited success, and we have used instead live recombinant vaccinia viruses expressing tumor antigens to immunize against tumor cells. Inoculation of rodents with live recombinant vaccinia expressing tumor antigens of polyoma virus (11), bovine papillomavirus (12), and human papillomavirus (G. Meneguzzi, C. Cerni, M.-P.K., and R.L., unpublished work) was found to elicit rejection of cognate transformed cells. Although several lines of evidence suggest that an infective agent may contribute to breast cancer, the existence of such an agent has not been confirmed (13). We have therefore focused upon an endogenous protein antigen that is aberrantly expressed in breast cancer. Monoclonal antibody (mAb) H23 was raised against particulate antigens released by T-47D breast tumor cells (14). mAb H23 detects an antigen [H23 epithelial tumor antigen (ETA)] present in 91% of breast tumors examined (14). H23 ETA can be detected in the body fluids of breast cancer patients, where its level correlates with disease status and poor prognosis (15). cDNA (16, 17) and genomic (18) cloning has revealed that a large central segment of the gene encoding H23 ETA consists of a multiple tandem repeat of a 60-nucleotide domain encoding a 20-amino acid sequence motif (17, 18). The number of repeat units differs substantially between individuals and between alleles (16), and copy-number variation has been observed between parent and child (M.H., unpublished data). The presence of similar variable-sized mucin-like glycoproteins in breast cancer and other adenocarcinomas has been reported by other groups (19–27). Sequence data indicate that H23 ETA is similar, if not identical, to the polymorphic episialins or epithelial mucins described by other groups (refs. 28–30; see refs. 31–33 for reviews). Comparison of the cDNA and genomic sequences encoding H23 ETA revealed the existence of two alternative mRNA species presumed to be generated by alternative splicing of a single precursor transcript (17). Translation of the two species is expected to result in two polypeptides that share a common secretion-signal sequence, amino terminus, and repeat array but that differ in their carboxyl-terminal regions. One of the two proteins, the transmembrane (T) form, contains a carboxyl-terminal hydrophobic region (17) that is thought to be responsible for membrane association. The other species, the secreted (S) form, lacks this zone and appears in the culture medium (ref. 17 and unpublished data). Although RNA species corresponding to both forms are detected in breast cancer cells, the possibility that S-form mRNA is a partially spliced precursor of T mRNA has not been rigorously excluded (M.H. and R.L., unpublished data).

We previously constructed vaccinia virus recombinants separately expressing the S and T forms of H23 ETA (VV-ETA-S and VV-ETA-T) and confirmed that the S form was predominantly secreted into the culture medium from VV-ETA-S-infected cells while the polypeptide encoded by VV-ETA-T remained cell-associated (M.H., D.W., M.-P.K., P.C., and R.L., unpublished data). We have now investigated the potential of the recombinants to elicit immunity to syngeneic tumor cells expressing H23 ETA and we report that rats inoculated with VV-ETA-T survive challenge with tumor cells expressing H23 ETA.

MATERIALS AND METHODS

Tumor Cells Expressing H23 ETA. Full-length cDNAs encoding the S and T forms of H23 ETA were constructed by

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Abbreviations: ETA, epithelial tumor antigen; mAb, monoclonal antibody; S, soluble; T, transmembrane.

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reassembly of partial DNA clones (refs. 16 and 17; unpublished data) and inserted between *Bam*HI and *Sal* I sites (S form) or *Bam*HI and *Eco*RV sites (T form) of pHMG (34), a plasmid comprising the promoter region, the untranslated first exon, and the first intron of the mouse housekeeping gene encoding 3-hydroxy-3-methylglutaryl-CoA reductase. The polylinker is followed by a 123-base-pair fragment containing a polyadenylylation signal from simian virus 40 (34). Fischer rat FRras1 transformed fibroblasts (35) were cotransfected with pAG60 [a plasmid determining G418 resistance (36)] and either pHMG-ETA-S or pHMG-ETA-T expression plasmid or the expression plasmid vector pHMG, by a modification (37) of the calcium phosphate precipitation method (38). G418-resistant foci (Geniticin, 500 μg/ml) were subcultured and tested for reaction with mAb H23. Single-cell lines were established by limit dilution from positive clones and flow cytofluorimetry was used to confirm that all cells expressed the antigen (data not shown). FR-0, FR-S, and FR-T cells (expressing no antigen, ETA-S, or ETA-T, respectively) were propagated in Dulbecco's modified Eagle's medium (DMEM, GIBCO) supplemented with 10% fetal bovine serum, penicillin G (100 units/ml), and streptomycin (100 μg/ml).

Vaccinia Recombinants. VV-ETA-S and VV-ETA-T (M.H., D.W., M.-P.K., P.C., and R.L., unpublished work) are vaccinia recombinants separately expressing the S and T forms of H23 ETA under the control of the vaccinia 7.5K promoter. VV-0 is a nonrecombinant (thymidine kinase-deficient) control vaccinia virus. Viruses were propagated on monolayer cultures of BHK21 cells at 37°C in DMEM supplemented with 1% fetal bovine serum and were purified and titered on the same cells according to published protocols (11, 39).

Expression Analysis. Transfected cells were grown as monolayers on chamber slides (Lab-Tek), fixed in cold acetone, and incubated (10 min, 25°C) with mAb H23 at 10 μg/ml in phosphate-buffered saline (PBS: 0.137 M NaCl/3 mM KCl/9 mM Na_2HPO_4/1.5 mM KH_2PO_4, pH 7.4) with 5 mM $MgCl_2$, 5 mM $CaCl_2$, and 1% fetal bovine serum. After washing, immune complexes were visualized by incubation with fluorescein-conjugated rabbit anti-mouse IgG (Miles; 1:50 in PBS with 1% fetal bovine serum) and photographed under UV illumination. For Western blot analysis, 40-μg protein samples of cell extracts (sonication) were analyzed by SDS/PAGE in 3–15% acrylamide linear gradient gels. Following electrophoresis, the separated proteins were electrotransferred to nitrocellulose, and the blot was blocked in PBS containing 5% skimmed milk, incubated with mAb H23 (10 μg/ml) in PBS plus 5% skimmed milk, washed, and incubated with ^{125}I-labeled protein A (Amersham; 10 μCi/ml in PBS plus 5% skimmed milk; 1 μCi = 37 kBq) prior to exposure to x-ray film.

Antibody Titers. Duplicate groups of three male and three female animals were immunized with recombinant vaccinia viruses as described below for challenge experiments. Animals were given booster injections at 10 days and blood was collected by cardiac puncture of anesthetized animals. Serum was collected, clarified, and pooled in groups of three. Microwell dishes (Nunc) were preadsorbed with tumor cells (0.75×10^5 per well) by drying overnight in alkaline medium (50 mM $NaHCO_3$, pH 9.6). Wells were washed, dried, and saturated with PBS plus 1% bovine serum albumin. Serum dilutions (1:50, 1:250, 1:1250) in PBS plus 0.01% Tween 20 were added (60 min, 25°C). After washing, immobilized antibody was detected by incubation with peroxidase-labeled anti-rat immunoglobulin and a commercial development agent as described (39). Color development at 490 nm was determined using an automated microtiter plate reader (Molecular Devices).

Vaccination and Challenge. Four- to five-week-old female or male IOPS Fischer rats (Iffa Credo, Saint Germain sur l'Arbresle, France) were immunized with 10 μl of purified virus containing 2×10^7 plaque-forming units of VV-ETA-S, VV-ETA-T, or VV-0. Immunization was performed by tail scarification (intradermal). Vaccination was repeated at 10 days, and challenge at 14 days was by subcutaneous seeding of tumor cells propagated *in vitro* and resuspended in PBS. Challenge doses (in 100 μl) were as follows: FR-0, 2×10^4; FR-S, 4×10^4; FR-T, 1.5×10^5. All vaccination and challenge procedures were performed under vapor anesthesia (5% halothane in 50% NO/50% O_2). Tumor development was monitored by palpation.

RESULTS

Tumor Cells Expressing H23 ETA. To create an animal model for rapidly growing syngeneic tumors expressing human H23 ETA, we separately introduced full-length cDNAs encoding the S and T forms of H23 ETA into expression vector pHMG. Constructions pHMG-ETA-S and pHMG-ETA-T, and the vector alone, were introduced into Fischer rat FRras1 cells by cotransfection with plasmid pAG60 ($G418^R$) to obtain the stable transfected lines FR-S, FR-T, and FR-0, respectively. Lines positive for H23 ETA expression were analyzed by flow cytofluorimetry to ensure that all cells expressed the antigen, and H23 ETA expression was verified by Northern blot analysis (data not shown). Western blot analysis of extracts of single-cell-derived positive lines was performed to determine whether the expression vector directed the expression of authentic H23 ETA. mAb H23 identified specific protein bands in extracts of cells containing either pHMG-ETA-S or pHMG-ETA-T (Fig. 1*A*). The protein profiles were similar, though not identical, to the mAb H23-crossreactive species synthesized by MCF-7 cells (Fig. 1*B*). Additional bands may be the result of homologous recombination between different repeat units in the ETA coding sequence, as observed in the human genome (16), and/or proteolytic processing of the primary translation product (M.H. and R.L., unpublished data).

To determine whether the T form of H23 ETA is tethered at the cell surface, cell clones harboring pHMG-ETA-T were examined by indirect immunofluorescence for reaction with mAb H23. Strong fluorescence in FR-T cells was observed at the cell membrane and in the cytoplasm (Fig. 2 *E* and *F*), whereas FR-S transfected cells displayed predominantly cytoplasmic staining (Fig. 2 *C* and *D*). Flow cytofluorimetry

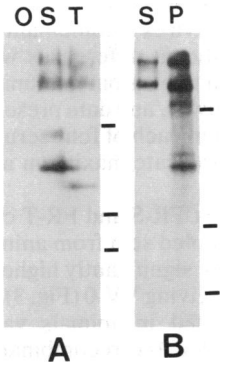

FIG. 1. Western blot analysis of ETA-crossreacting polypeptides in rat tumor cells expressing the S and T forms of H23 ETA. (*A*) Extracts from FR-0 cells (lane O), lane FR-S cells (lane S), and FR-T cells (lane T) analyzed by Western blotting with mAb H23. (*B*) Cell extracts from MCF-7 breast cancer cells separated into supernatant (lane S) and cell-associated (pellet, lane P) fractions prior to Western analysis with mAb H23. Markers indicate the positions of molecular weight standards (M_r 200,000, 100,000, and 68,000).

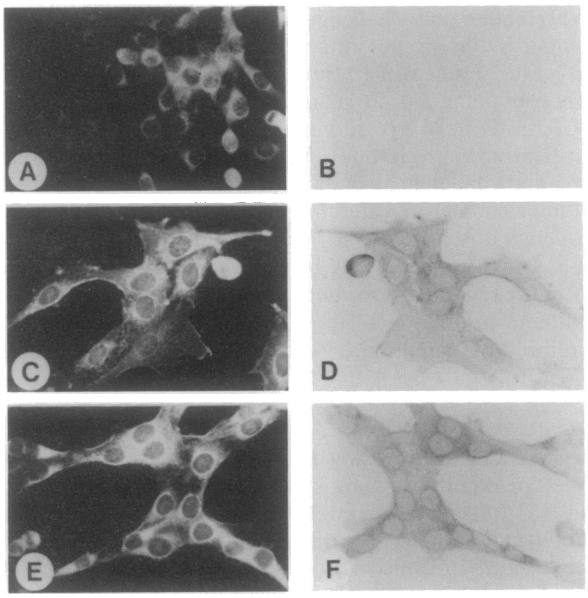

FIG. 2. Immunofluorescence analysis of rat tumor cells expressing H23 ETA. Immobilized control FR-0 (*A* and *B*), FR-S (*C* and *D*), and FR-T (*E* and *F*) cells were counterstained with Evans blue (red fluorescence) and treated with mAb H23 and a second, fluorescein-labeled antibody (yellow fluorescence). Total fluorescence under UV illumination was photographed on color film (*A*, *C*, and *E*, positive photography) and subsequently printed onto red-insensitive film to reveal specific H23 ETA yellow fluorescence (*B*, *D*, and *F*, negative photography, laterally inverted). (×50.)

of the cell clones confirmed that the T form is present on the cell membrane, although some S form was also detected at the cell surface (data not shown). Minor morphological differences were often observed between cells expressing ETA and cells expressing no antigen (compare Fig. 2 *C* and *E* with *A*), though the significance of this alteration is not understood.

Immune Reactivity in Vaccinated Rats. We previously constructed VV recombinants separately expressing H23 ETA S and T forms (M.H., D.W., M.-P.K., P.C., and R.L., unpublished data). To determine whether inoculation of VV-ETA-S and -T leads to an anti-ETA immune response, rats were vaccinated (intradermally) with the two viruses and serum was collected. Sera were examined by ELISA for reaction with immobilized tumor cells. Because immunization with vaccinia virus can elicit a humoral response against host cell components (40), control animals were immunized with VV-0, a vaccinia virus recombinant lacking ETA coding sequences. No significant differences were observed in antibody titers in serum pools from immunized male and female animals (data not shown), and data presented in Fig. 3 are the mean titers obtained in each of four serum pools from a total of 12 animals; bars indicate maximum and minimum values for individual pools.

When tested against FR-S and FR-T challenge cells, readings obtained with pooled sera from animals vaccinated with VV-ETA-S or -T were significantly higher than obtained with sera from animals receiving VV-0 (Fig. 3). ETA-specific titers were more pronounced in animals vaccinated with VV-ETA-T, suggesting that the recombinant expressing the T form is a more effective immunogen. Animals immunized with VV-ETA-T reacted with MCF7 breast cancer cells, whereas serum from animals vaccinated with VV-ETA-S failed to react with these cells. Weak reaction was observed with HeLa cervical cancer cells, which express only low levels of ETA (unpublished data).

Tumor Immunity in Vaccinated Animals. We next examined vaccinated animals for their resistance to challenge with

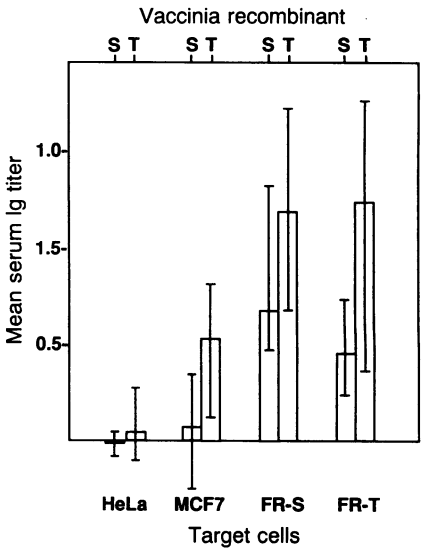

FIG. 3. ELISA analysis of sera from rats vaccinated with VV-ETA-S or -T. Serum pools were obtained from animals separately immunized with VV- ETA-S or -T; serum dilutions were reacted with immobilized tumor cells and developed by using a peroxidase-labeled second antibody (see *Materials and Methods*). Antibody titers are expressed as the optical density developed in the standard assay using a serum dilution of 1:50 and were calculated as the geometric mean of the calculated titers determined from 1:50, 1:250, and 1:1250 dilutions. Vertical bars give maximum and minimum values in four individual serum pools from four animals. Mean titers for background immunoglobulin reacting with non-ETA determinants (equivalent pools from animals vaccinated with VV-0 in the same experiment) were subtracted from the titers presented (background values were 1.93, 1.32, 2.09, and 1.96, from left to right as presented).

syngeneic tumor cells expressing the S and T forms of the H23 ETA. Animals were vaccinated twice with recombinant vaccinia viruses and challenged by subcutaneous seeding of FR-S or FR-T tumor cells or with FR-0 control cells that do not express H23 ETA. Table 1 presents the proportion of animals rejecting their tumors in each experiment and the mean tumor load midway through challenge.

As expected, vaccination was without effect upon tumor development following challenge with FR-0. Similarly, the control vaccinia virus VV-0 did not significantly influence tumor development in animals challenged with FR-0, -S, or -T. Despite a low background of spontaneous tumor rejection in one series of animals, vaccination with VV-ETA-T significantly restricted tumor growth: over three experiments (Table 1, series A–C) 22 of 28 animals (79%) receiving VV-ETA-T and challenged with the FR-T cell line either failed to present tumors or presented small tumors (5–12 mm in diameter) that subsequently regressed. These animals remain tumor-free to date (9 months). The same recombinant appeared somewhat less effective against FR-S tumor cells (17 of 28 animals, 61%). VV-ETA-S, the recombinant expressing the S form of H23 ETA, was a less effective immunogen than VV-ETA-T, and in three experiments (Table 1, series A–C) only 16 of 55 animals (29%) challenged with either FR-S or FR-T rejected their tumors. Surprisingly, in some experiments (Table 1 and data not shown) inoculation of animals with VV-ETA-S appeared to stimulate metastatic spread of tumors in some animals challenged with FR-T cells, and 4 of 10 vaccinated and challenged animals († in Table 1, series B) exhibited rapid metastasis of normally discrete subcutaneous tumor growth.

Results obtained upon separate immunization of male and female animals (Table 1, series A–C) indicated that there might be differences between the responses of males and females. We therefore vaccinated and challenged separate

Table 1. Vaccination against tumor cells expressing H23 ETA

Series	Vaccine (intradermal)	Cells injected (subcutaneous)	Midway mean tumor load,* mm (days post challenge)	Fraction of animals showing rejection	Percent protected animals
A (female)	No virus	FR-0	31 (20)	0/4	0
		FR-S	25 (25)	1/4	25
		FR-T	25 (30)	3/6	50
	VV-ETA-S	FR-0	40 (20)	0/8	0
		FR-S	8 (25)	5/8	63
		FR-T	0.9 (30)	7/8	88
	VV-ETA-T	FR-0	32 (20)	0/8	0
		FR-S	0.4 (25)	7/8	88
		FR-T	0 (30)	8/8	100
B (female)	No virus	FR-S	11 (20)	0/10	0
		FR-T	25 (20)	0/10	0
	VV-ETA-S	FR-S	16 (20)	1/10	10
		FR-T	30 (20)	1/10†	10
	VV-ETA-T	FR-S	1.7 (20)	5/10	50
		FR-T	2.8 (20)	5/10	50
C (male)	VV-0	FR-S	20 (20)	0/10	0
		FR-T	28 (20)	0/10	0
	VV-ETA-S	FR-S	11 (20)	2/10	20
		FR-T	34 (20)	0/9	0
	VV-ETA-T	FR-S	0.1 (25)	5/10	50
		FR-T	2 (20)	9/10	90
D (female)	No virus	FR-T	25 (25)	0/6	0
	VV-ETA-T	FR-T	0 (25)	6/6	100
D (male)	No virus	FR-T	20 (25)	0/6	0
	VV-ETA-T	FR-T	0 (25)	5/6	83

Animals within a single series were vaccinated and challenged in parallel.
*Mean palpable tumor diameter.
†Four of 10 animals showed tumor metastasis.

groups of male and female animals in a separate experiment. As shown in Table 1, series D, there was no significant difference between the tumor rejection rates in males and females.

DISCUSSION

Tumors of epithelial cell origin aberrantly express protein species whose antigenicity is distinct. We recently reported the cloning of genomic and cDNA sequences encoding a polymorphic ETA recognized by mAb H23 (16–18). H23 ETA is detected in 91% of malignant breast tumors, while in normal mammary tissue, as well as in other tissues, H23 ETA expression is weak to undetectable (14). Aberrant expression of H23 ETA in breast cancer encouraged us to investigate the potential of the alternative S and T forms of the antigen as immunogenic targets for active immunotherapy. We previously reported that vaccinia recombinants (41) expressing tumor antigens of viral origin can elicit immunity to challenge with cognate tumor cells (11, 12) and, in some cases, induce rejection of preexisting tumors (11). We therefore constructed VV recombinants separately expressing the S and T forms of H23 ETA (M.H., D.W., M.-P.K., P.C., and R.L., unpublished work). To construct a model for breast tumor cells we further established tumorigenic Fischer rat cell lines separately expressing ETA-S and -T for use in challenge experiments.

Immunization of animals with either VV-ETA-S or -T resulted in ETA-specific antibody; however, the immune response was more pronounced in animals immunized with VV-ETA-T. To determine whether the immune response elicited by vaccination with VV-ETA-S or -T was sufficient to prevent tumor development, challenge experiments were performed. The majority (97% and 93%, respectively), of unvaccinated animals challenged by subcutaneous seeding with the FR-S or FR-T cell lines rapidly developed tumors that failed to regress. When vaccinated with the recombinant viruses expressing ETA-S or -T, however, a substantial proportion of animals rejected their tumors. Eighty-two percent of animals vaccinated with the recombinant expressing ETA-T failed to develop tumors following challenge with the cell line expressing ETA-T (average from Table 1). The recombinant expressing ETA-S protected only 30% of these animals, in support of the view that vaccinia recombinants expressing soluble forms of membrane proteins are less effective immunogens (42, 43). Vaccination against tumor lines expressing ETA-S was also somewhat less effective (29% and 61% overall tumor rejection frequencies in animals vaccinated with VV-ETA-S and -T, respectively), indicating that an association with the cell membrane may be important for both target recognition and immunization. In some experiments, inoculation of animals with VV-ETA-S enhanced tumor metastasis. Although the mechanism of tumor enhancement is unknown, we previously observed similar enhancement of tumor growth in animals vaccinated with polyomavirus LT protein and challenged with tumor cells expressing LT (44).

Anti-tumor immunization was equally effective in males and females, arguing against sexually dimorphic tolerance due to sex-specific expression of an endogenous murine ETA homolog. It is of note that the use of vaccinia recombinants expressing the *neu* oncogene successfully protected mice against challenge with syngeneic cells expressing the rat *neu* gene, whereas rats failed to respond to the same immunogen (45). It is therefore still a matter of conjecture whether vaccinia recombinants expressing human tumor antigens

such as p97 (46), the *neu*/c-*erbB2* gene product (45), or ETA (this work) can elicit a therapeutic anti-tumor response in human. However, the efficient anti-tumor response reported here in rats will encourage further experiments to explore this question.

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