Expression of a functional single chain antibody on the surface of extracellular enveloped vaccinia virus as a step towards selective tumour cell targeting

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Recombinant vaccinia virus with tumour cell specificity may provide a versatile tool either for direct lysis of cancer cells or for the targeted transfer of genes encoding immunomodulatory molecules. We report the expression of a single chain antibody on the surface of extracellular enveloped vaccinia virus. The wild-type haemagglutinin, an envelope glycoprotein which is not required for viral infection and replication, was replaced by haemagglutinin fusion molecules carrying a single chain antibody directed against the tumour-associated antigen ErbB2. ErbB2 is an epidermal growth factor receptor-related tyrosine kinase overexpressed in a high percentage of human adenocarcinomas. Two fusion proteins carrying the single chain antibody at different NH2-terminal positions were expressed and exposed at the envelope of the corresponding recombinant viruses. The construct containing the antibody at the site of the immunoglobulin-like loop of the haemagglutinin was able to bind solubilized ErbB2. This is the first report of replacement of a vaccinia virus envelope protein by a specific recognition structure and represents a first step towards modifying the host cell tropism of the virus.

Introduction

Vaccinia virus is the most extensively characterized member of the orthopoxvirus family. It exists in two morphologically distinct infectious forms (Appleyard et al., 1971; Ichihashi et al., 1971). Intracellular mature virus (IMV) remains within the cytoplasm of infected cells; extracellular enveloped virus (EEV) is released from the cells and has an extra lipid envelope containing about 10 associated proteins which are absent in IMV. This second form is responsible for the efficient dissemination of the virus in vivo and in vitro (Appleyard et al., 1971; Payne, 1980; Payne & Kristensson, 1985).

One reason for the continued interest in vaccinia virus is that it is possible to insert large segments of foreign DNA into its genome by homologous recombination (Mackett et al., 1982; Panicali & Paoletti, 1982). Several different sites, including the haemagglutinin (HA) gene, have been described for insertion of foreign genes and overexpression of proteins of particular interest (Perkus et al., 1986; Shida et al., 1987). In addition to its use as an in vitro expression system, attenuated vaccinia virus has proven to be a promising candidate for in vivo applications, e.g. as a live vaccine carrier expressing heterologous antigens. More recently, recombinant vaccinia virus expressing cytokine genes has been proposed for cancer therapy (Peplinski et al., 1995; Whitman et al., 1994). For such an application it would be a considerable advantage if the virus could be specifically targeted at certain cells. Towards this goal, we are generating vaccinia virus recombinants expressing engineered cell attachment proteins. This should allow the selective infection and destruction of cells expressing particular surface markers, as is the case for certain tumour cells.

In this study, we have fused a single chain antibody (scFv) to the extraviral portion of an envelope protein. The viral HA was selected as a target for insertion of scFv for two reasons. Firstly, the existence of infective haemagglutination-negative virus mutants indicates that this protein tolerates modifications without interfering with EEV formation (Ichihashi & Dales, 1971). Secondly, the extraviral NH2-terminal portion of this molecule contains an immunoglobulin-like domain (Jin et al., 1989). It was therefore tempting to replace this domain with an scFv directed to a specific cell surface marker.

The scFv used in this study was derived from the monoclonal antibody FRP5 which is specific for ErbB2, a member of the ErbB/EGF receptor-related family of receptor
tyrosine kinases (Wels et al., 1992b). Overexpression of ErbB2 is observed in human tumours arising at various sites including the breast and ovaries, where it correlates with an unfavourable patient prognosis (reviewed by Hynes & Stern, 1994). Its differential expression on the surface of tumour cells and the fact that it is internalized upon binding of antibody (Harwerth et al., 1992) could render this protein an interesting attachment site for a modified vaccinia virus. Such a virus might have the potential to target malignant tumour cells and/or for targeted gene delivery for tumour vaccination approaches.

Methods

Cells and viruses. The rabbit kidney cell line RK13 (ATCC no. CCL 37) and the African green monkey kidney cell line CV1 (ATCC no. CCL 70) were cultured in Dulbecco’s modified Eagle medium (DMEM). The human breast adenocarcinoma cell line SK-BR-3 (ATCC no. HTB 30) was grown in RPMI 1640. The media were supplemented with 10% foetal calf serum (FCS) and 100 U/ml and 100 µg/ml penicillin and streptomycin, respectively. Vaccinia virus strain IHD-J was used in this study.

Antibodies. The rabbit antiserum anti-scFv(FRP5) and the monoclonal anti-ErbB2 antibody were produced as described (Wels et al., 1992b; Harwerth et al., 1992). The monoclonal anti-haemagglutinin antibody 1H831 (Shida, 1986) was kindly provided by H. Shida (Kyoto University School of Medicine, Japan). The rabbit anti-vaccinia virus core protein 4a antiserum was produced as described (Wittke et al., 1984).

Construction of chimeric haemagglutinin genes. The vaccinia virus HA gene (Shida, 1986) was amplified by PCR using the oligonucleotide primers 1 (5’ GCCGCTCGAGCTGACGATGTGCTTCATG 3’) and 2 (5’ GTCGCAGCGGTCTCAACATTITTTAAG 3’). The resulting fragment was subcloned into pBlueScript (pBS; Stratagene) via the XhoI and SalI sites contained in the primers, and the resulting construct was termed pBS–VHA. The plasmid pWW152-5 containing the anti-ErbB2 scFv(FRP5) sequence has been described by Wels et al. (1995).

The scFv sequence was inserted into the vaccinia virus HA gene according to the strategy depicted in Fig. 1. Firstly, a fragment containing the 5’-terminal sequence of the gene and the upstream flanking sequence included in pBS–VHA was amplified by PCR using the T3 primer of pBS and primer 3 (5’ AGATCCTAAGGCTTCTAGTCTGAGG 3’). Primer 3 introduces the restriction sites HindIII and BglII downstream of nucleotide 517 of the HA gene (Shida, 1986). The fragment obtained was fused to two different 3’ HA fragments, according to the different insertion sites of the scFv sequence in constructs A and B. Both 3’ fragments were produced by PCR using primer T7 of pBS as downstream primer. As upstream primers, primers 4 (5’ AAAAGCTTACGAGATTAGTGCTGATGACAC 3’, corresponding to bp 521–535) and primer 5 (5’ AAAAGCTTACGAGATTAGTGCTGATGACAC 3’, bp 565–579), were used for constructs A and B, respectively. The upstream and downstream segments were ligated via HindIII and subcloned into pBS. The scFv sequence excised with HindIII and BamHI from pWW152-5 was inserted into the artificially introduced HindIII and BglII sites of these constructs. The resulting plasmids containing the scFv sequence at two different positions of the HA gene were termed pVVHA-scFv:A and pVVHA-scFv:B.

Production and isolation of recombinant virus. To obtain vaccinia recombinants containing the scFv–HA fusion gene, CV1 cells were infected with vaccinia virus IHD-J at an m.o.i. of 0-1 and transfected with the plasmids pVVHA–scFv:A or B linearized with XhoI. IMV was recovered and used to reinfect CV1 cells. After overnight incubation, the cells were washed and incubated with the anti-scFv(FRP5) antisera for 1 h. After three washes with PBS, bound antibody was labelled with a fluorescein isothiocyanate-conjugated goat anti-rabbit IgG antibody. The labelled cells were then subjected to fluorescence activated cell sorting (FACS), and fractions containing cells with fluorescence intensities corresponding to the positive control were collected. These cells were lysed, and the recovered IMV was analysed by PCR using the scFv specific primers 7 (5’ CAACGTGACAGCTGCTGGACC 3’) and 8 (5’ CCAATTTTGTGCCGGACC 3’) or the HA specific primers 1 and 2. This IMV was used to infect CV1 cells for a further round of FACS enrichment. After four FACS cycles, the recovered IMV was subjected to plaque purification. Plaques originating from infection with pure recombinant virus were identified by PCR using the HA specific primers 1 and 2.

Sequencing of recombinant viruses. In order to confirm the genome structure of the scFv insertion in the recombinant viruses, the HA gene was isolated on viral DNA by PCR and subcloned into pBS as outlined above for the wild-type gene. The DNA boundaries at the scFv insertion sites were then sequenced by double-strand dideoxy sequencing.

Virus preparation. RK13 cells were infected with wild-type or recombinant vaccinia virus at an m.o.i. of 10 in PBS (3 ml per 175 cm² T-flask) under gentle agitation at room temperature. After 1 h, the inoculum was replaced by 10 ml culture medium, and the cells were incubated for 24–48 h.

For EEV preparation, the culture supernatant was harvested and clarified by 5 min centrifugation at 750 g. The EEV was precipitated for

Fig. 1. Construction of plasmid vectors for insertion of the anti-ErbB2 scFv encoding sequence into the vaccinia virus HA gene. The oligonucleotide primers used for PCR amplification are designated (bold) as described in Methods. The inserted scheme shows the protein structure of wild-type and recombinant HA, indicating amino acid positions referring to the wild-type sequence: wt, IHD-J wild-type HA; A, HA-scFv fusion proteins; TM, transmembrane domain; hatched box, HA signal peptide.
30 min at 15000 g in a swing-out rotor and washed with PBS. The virus was resuspended in PBS (50 µl per 175 cm² T-flask) and photometrically quantified by calculating 64 µg per A₆₅₀ unit (Payne, 1979).

For IMV, the infected cells were washed and resuspended in PBS (2 ml per 175 cm² T-flask). The cells were lysed either by Dounce homogenization or by multiple passages through a 26 gauge needle, followed by sonication for 30 s in a sonication waterbath. The cell debris was removed by centrifugation at 750 g for 5 min, and 200 µl of supernatant was centrifuged through a 500 µl cushion of 36% sucrose in an Eppendorf centrifuge for 30 min. The IMV was resuspended in 200 µl PBS for further purification.

To obtain pure virus, 200 µl of the EEV or IMV suspensions was overlayed onto a discontinuous CsCl gradient (1.2 ml of 1.3 g/ml, 1.0 ml of 1.25 g/ml, 1.8 ml of 1.2 g/ml) and centrifuged in a Kontro TST 55.5 rotor at 30000 g for 30 min at 4 °C. The viral bands were collected, diluted with at least two volumes of PBS, precipitated by centrifugation and resuspended in 200 µl PBS for further analysis.

EEV devoid of membranes was prepared as follows: CsCl-purified EEV was incubated in 1% Brij-58 (Sigma) for 10 min at room temperature under gentle rotation (Payne, 1978). The membrane-stripped EEV was recovered by centrifugation through an equal volume of 36% sucrose.

### Western blotting

Vaccinia virus protein (1–5 µg per lane) was fractionated on 10% SDS–polyacrylamide gels and transferred to a nitrocellulose membrane by electroblotting. After 1 h of blocking in Tris-buffered saline containing 0.05% Tween 20 (TBS–TWEEN) and 5% non-fat dried milk, the membranes were incubated with rabbit anti-scFv(FRP5) antiserum, monoclonal anti-HA antibody, or anti-vaccinia virus core protein 4a antiserum, all diluted 1:1000 in TBS-Tween, for 1 h at room temperature. After washing with TBS–TWEEN, the membranes were incubated with anti-rabbit or anti-mouse IgG antibody conjugated with horse-radish peroxidase (diluted 1:3000) for 1 h at room temperature. Bound secondary antibody was detected using the enhanced chemiluminescence kit from Amersham.

### Proteinase K digestion

EEV (20 µg) in a total of 40 µl PBS was treated with proteinase K concentrations ranging from 0–2 µg/ml for 30 min at 37 °C. At the end of the incubation, the reactions were diluted with 1 ml PBS and stopped with PMSF at a final concentration of 0.5 mM. The EEV was recovered by centrifugation in an Eppendorf centrifuge for 30 min at 4 °C, resuspended in 50 µl of SDS gel loading buffer, and boiled immediately. The viral lysates (10 µl per lane) were loaded on 10% polyacrylamide gels and analysed by Western blotting.

### ErbB2 binding

Cell membranes containing the ErbB2 receptor were prepared as follows: SK-BR-3 cells in PBS were lysed by freezing and thawing and subsequent sonication for 30 s. The lysate was clarified by centrifugation at 5000 g for 5 min.

Ninety-six-well plates (Nunc Immunoplate MaxiSorp) were coated with recombinant or wild-type EEV (5 µg per well) in PBS at 4 °C overnight. After three washes with PBS, the viruses were fixed with 2% formaldehyde for 30 min at 4 °C. After one wash, non-specific binding sites were blocked with 5% non-fat dried milk in PBS for 1 h at room temperature. The viruses were then incubated with triplicate samples of SK-BR-3 membranes at various dilutions in PBS, for 1 h at room temperature. Alternatively, various amounts of coated virus were incubated with a constant amount of membrane (15 µg per well). The wells were then washed and incubated with the monoclonal anti-ErbB2 antibody at a dilution of 1:1000 for 1 h. After washing, anti-mouse IgG antibody conjugated with horseradish peroxidase was added (dilution 1:1000). Using 1,2 phenylenediamine as a substrate, bound ErbB2 protein was quantified in an ELISA reader at 490 nm. Each binding experiment was performed at least three times.

#### Results

##### Construction of recombinant viruses expressing a single chain antibody fused to haemagglutinin

The sequence encoding an anti-ErbB2 scFv antibody was inserted into the vaccinia virus HA gene at two different positions (Fig. 1). The two fusion proteins were designed to contain the scFv structure at the NH₂ terminus, nine codons downstream of the signal sequence cleavage site, either as an insertion within the complete HA sequence (construct A) or in place of the immunoglobulin-like NH₂-terminal domain (construct B). As a rapid way to introduce these constructs into the viral genome and to eliminate the authentic HA gene at the same time, we decided to perform a direct gene replacement using standard vaccinia recombinant techniques. Homologous recombination within the sequences flanking the HA gene had the further advantage that the introduction of the modified gene did not alter its authentic genomic context, which might have affected its transcriptional regulation.

A disadvantage of this method is that it does not provide any selective means to isolate recombinant virus. However, since HA appears at the surface of the host cells during the course of vaccinia virus infection, it was possible to enrich for cells infected with recombinant virus by FACS using an anti-scFv(FRP5) idiotype antiserum.

##### Purification of recombinant virus

Surprisingly, even after four rounds of FACS enrichment no distinct peaks corresponding to cells expressing the scFv-HA fusion protein were detected (data not shown). This is probably due to a low affinity of the rabbit antiserum to the native form of the scFv, since the serum had been raised against the denatured scFv produced in bacteria. Nevertheless, the cell fractions showing the highest fluorescence intensities after each FACS cycle were selected. In order to monitor the enrichment of recombinant virus, the viral DNAs obtained from the selected cell fractions were analysed by PCR using primers specific for the scFv insert. For constructs VVHA–scFv:A and B, the correct amplification product of 0.7 kb, which was not detected in the initial virus populations, appeared after four rounds of FACS (Fig. 2a). The upper two bands in the control represent non-specific amplification products which occurred reproductively using these primers on the corresponding plasmid DNA. In order to assess the relative contents of contaminating wild-type virus in the preparations of recombinants A and B, the DNA of this final population was further analysed by PCR using the HA gene specific primers 1 and 2 (see Methods). As the major amplification product, these primers revealed the 1.8 kb fragment corresponding to the wild-type gene, while minor bands of higher molecular mass corresponding to the scFv-HA fusion genes confirmed the presence of recombinant virus for both constructs A and B (Fig.
Fig. 2. PCR analysis of recombinant virus. Viral DNA obtained from cells after four rounds of FACS selection, before (a, b) and after (c) a subsequent plaque purification step, was subjected to PCR amplification. Primers 5 and 6 (see Methods) were used to identify the scFv insert (a), and primers 1 and 2 were used to analyse the wild-type or recombinant HA genes (b, c).

wt, IHD-J wild-type virus; p-A, plasmid pBS-VVHA:A; p-B, plasmid pBS-VVHA:B; A, recombinant A; B, recombinant B. Molecular size standards are indicated in kb.

Expression of the single chain antibody in extracellular viral particles

The fact that FACS using an anti-scFv antibody allowed enrichment of cells infected with recombinant virus already indicated that the scFv–HA fusion protein was expressed and transported to the surface of infected cells like wild-type HA. In order to test whether the recombinant protein was also incorporated into EEV particles, wild-type and recombinant EEVs from preclarified cell culture supernatant were analysed by immunoblotting (Fig. 3a). The anti-scFv antibody recognized specific bands in lysates from both recombinants A and B that were absent in the lane containing wild-type virus (Fig. 3a, right panel). The molecular masses of these bands corresponded to those expected for the respective fusion proteins. A monoclonal anti-vaccinia virus HA antibody stained the same bands, in addition to the authentic protein in wild-type EEV (Fig. 3a, centre panel). This experiment further confirmed the absence of wild-type HA in the preparations of recombinants A and B. Although identical amounts of each virus were loaded per lane, as confirmed with an antibody to core protein 4a (Fig. 3a, left panel), both anti-HA and anti-scFv antibodies revealed more HA–scFv fusion protein in recombinant B than in recombinant A. It is not clear whether this is due to a lower expression level, or to a less efficient incorporation of construct A into the EEV envelope. Since the same effect was observed using two independent antibodies, it is unlikely that it is due to simple alterations in epitope accessibility between constructs A and B. Interestingly, upon infection at identical m.o.i., recombinant A also yielded only about 60% of the amount of EEV particles obtained with wild-type virus and recombinant B (Fig. 4). This finding further suggested that construct A somehow interfered with envelopment and EEV release.

In order to confirm that the HA–scFv fusion proteins are associated with EEV particles, and do not originate from
contaminating cellular components in the clarified culture supernatant, additional Western blots were performed on purified virus (Fig. 3b). Indeed, the anti-scFv antibody detected the HA–scFv fusion proteins in CsCl-purified EEV, but not in IMV, for both recombinants. Furthermore, an extraction of recombinant EEV with Brij-58, a mild detergent used to selectively solubilize EEV membranes (Payne, 1978), completely removed the modified HA molecules.

Altogether, these results clearly demonstrated that the HA–scFv fusion proteins were associated with the EEV membranes of the two recombinants. Since the scFv structure was inserted near the NH$_2$ terminus of its HA carrier, which is a type I glycoprotein (Shida, 1986), one would expect the scFv to be exposed at the surface of the EEV envelope. This represents a crucial condition for specific targeting to the cellular ErbB2 receptor protein. To confirm its surface topology, the scFv on recombinant EEV was exposed to increasing amounts of proteinase K. The proteinase sensitivities of the scFv structure and, as a control for an internal protein, of the core protein 4a (Sarov & Joklik, 1972) were monitored by Western blotting (Fig. 5). Whilst core protein 4a completely resisted, the scFv–HA fusion proteins were highly susceptible to proteinase K, as was wild-type HA. Thus, these results clearly indicate that the scFv structure was exposed at the surface of recombinant EEV.

**Binding of ErbB2 to the single chain antibody on recombinant virus**

The capability of the scFv, expressed on the surface of recombinant EEV, to bind the ErbB2 antigen was assessed in an ELISA-based microwell assay (Fig. 6). Wells coated with EEV particles were incubated with a membrane lysate originating from the human breast cancer cell line SK-BR-3 that expresses high levels of ErbB2. After washing, bound ErbB2 was detected using a specific antibody. Although we had shown that both recombinants A and B express the scFv on their surface, binding of ErbB2 protein to recombinant A remained within the range of the background levels obtained with wild-type virus. In contrast, recombinant B clearly bound ErbB2 antigen in a concentration dependent manner. This strongly indicated that, using construct B, the anti-ErbB2 scFv fused to the viral HA and displayed on the surface of recombinant EEV retained the ability to specifically bind its corresponding antigen.

**Discussion**

Our long-term goal is to generate a vaccinia virus with a specific host tropism restricted to defined cells and tissues. Targeted infection and elimination of malignant cells by means
of such a virus might provide a novel and efficient tool for cancer therapy. In this report we have shown that it is possible to express a functional scFv at the surface of EEV, fused to the extraviral portion of HA.

The vaccinia virus HA is the best studied protein of the EEV envelope. Modifications within this protein that rendered the virus haemagglutination-negative had no effect on virus replication and infectivity (Seki et al., 1990; Payne, 1979; Ichihashi & Dales, 1971). In addition, disruption of the HA sequence by insertion of heterologous genes allowed pro-
duction of recombinant viruses (Shida et al., 1987), which further demonstrates that the HA protein is not necessary for virus propagation. These observations prompted us to insert the scFv into the immunoglobulin-like domain close to the NH₂-terminus of this extraviral protein. In case replacement of the single immunoglobulin-like loop by the scFv double loop (construct B) affected expression of the fusion protein, we also fused the scFv to the full-length HA (construct A). For both constructs, pure recombinants were obtained by plaque purification after several rounds of FACS enrichment.

Infection with recombinant A not only yielded significantly lower amounts of EEV, as compared to wild-type virus and recombinant B, but also lower amounts of HA fusion protein on the virus particle. Thus, despite the fact that HA is not essential for vaccinia virus infection and replication, modification of the NH₂-terminus part of the protein by addition of the scFv seemed to affect HA incorporation into the viral envelope as well as virus production, envelopment or release.

Nevertheless, for both constructs A and B, the scFv–HA fusion proteins were exposed at the EEV surface. Katz et al. (1997) very recently showed that a heterologous antigen can be targeted to the EEV surface via fusion to the transmembrane and cytoplasmic domains of the B5R protein. However, in contrast to our approach, their fusion gene was inserted into the TK locus and expressed using a relatively weak promoter. This resulted in co-expression of the chimeric with the native B5R protein, and the fusion protein reached only about 6% of the expression level of its authentic counterpart. Our study represents the first report of a vaccinia virus envelope protein that can be entirely replaced by a corresponding fusion protein without affecting its envelope topology and expression level, as in the case of recombinant B.

Although the ErbB2 specificity of the grafted scFv has been well established (Moritz et al., 1994), only recombinant B containing the structure in place of the NH₂-terminal immunoglobulin-like loop of HA was able to bind its target. Inserted into the short stretch between the loop and the NH₂-terminus of the protein (construct A), the scFv did not bind ErbB2. Here, the proximity of the HA loop might sterically interfere with antigen binding by the two variable domains of the scFv, or the scFv might be misfolded. For the display of other heterologous structures, the site of insertion into the haemagglutinin gene will therefore have to be tested individually. It is possible, however, that the immunoglobulin-like loop of HA represents a versatile general insertion site.

Expression of a recognition structure at the surface of recombinant EEV will not be sufficient for restricting vaccinia infectivity to a certain cell type, since the recombinants still contain the as yet unidentified wild-type host cell attachment protein, allowing infection of a broad range of cells. It is therefore not surprising that we did not observe any preferential infectivity of the scFv–HA recombinants to cells expressing ErbB2 (data not shown). Elimination or at least reduction of the infectivity mediated via this ligand and in recombinant B will thus be the essential next step towards specific cell targeting. Only scarce and controversial information is currently available on the first steps of vaccinia EEV infection, i.e. cell attachment and penetration (Payne & Norrby, 1978; Ichihashi, 1996). Most recent investigations have focused on the mechanism of infection by IMV (Ichihashi & Oie, 1996; Chang et al., 1995; Ichihashi et al., 1994; Lai et al., 1991). However, since the extracellular enveloped form of the virus represents the biologically relevant infectious agent disseminating to distant cells and tissues of an organism (Blasco & Moss, 1992; Payne, 1980), in vivo cell targeting will require more detailed studies on the component of the viral envelope mediating the first interaction with the host cell. A

Fig. 6. Binding of ErbB2 protein to EEV from wild-type virus (□) or recombinants A (■) and B (▲). ELISA plates were coated with either 5 µg per well of EEV (a) or with serially diluted virus (b). Viruses were fixed, and either various amounts of lysed SK-BR-3 cells (a) or a constant amount of 15 µg per well (b) were added. The amount of specifically bound ErbB2 protein was determined using an anti-ErbB2 antiserum, as described in Methods.
recent report (McIntosh & Smith, 1996) identified a group of envelope glycoproteins encoded by the A34R gene as potential candidates, since deletion of this gene increased the production of EEV, but decreased its infectivity five to sixfold. It will be interesting to test whether the infectivity of an A34R deletion mutant carrying the functional scFv can be restored by host cells expressing ErbB2. Since ErbB2 was shown to be rapidly internalized and degraded upon stimulation with a monoclonal antibody (Yarden, 1990), virus bound via this scFv may passively enter the host cell together with the endocytosed receptor.

Immunotherapy and gene therapies of cancer have attracted increasing attention during the past few years. The conjugation of radioisotopes (for a review see Mach et al., 1991) or toxins (Wels et al., 1992a) to antibodies recognizing specific tumour antigens offer an interesting approach for the targeted destruction of malignant cells (for a review see Boden & Joshi, 1995). In the case of ErbB2 expressing metastatic breast cancer, a monoclonal antibody has recently been shown to be effective on its own in a clinical study (Baselga et al., 1996). Alternatively, stimulation of the anti-cancer immunity of a patient via overexpression of cytokines by the tumour cells in vivo provided promising results (for a review see Verbik & Joshi, 1995). Targeted gene delivery in vivo requires an efficient and specific vector, such as a retrovirus modified in its host tropism (Kasahara et al., 1994). Pseudotyped murine and avian retroviruses expressing chimeric envelope proteins containing single chain antibodies allowed specific infection of cells expressing the respective cell surface proteins (Somnia et al., 1995; Marin et al., 1996; Chu & Dornburg, 1995). Fusion of heregulin (a peptide ligand specific for the ErbB receptor family members ErbB3 and ErbB4) to the envelope protein rendered a murine retrovirus infectious for human breast cancer cells expressing the target protein (Han et al., 1995). However, a major technical constraint for a clinical use of modified retroviruses is the limited virus titres obtained from cell cultures.

Vaccinia virus has been used for almost two centuries as a live vaccine in humans and, in most cases, has proven to be well tolerated. As in the case of the smallpox vaccine, injected IMV would locally infect some host tissue and produce the therapeutic EEV in vivo. EEV would then find and infect its specific target cells, which would result in a further release of EEV. The use of tumour cell specific and replicating virus might therefore lead to an autonomous amplification of the therapeutic agent, until all target cells are eliminated or the infection is controlled by the immune system of the host organism. On the other hand, recombinant vaccinia viruses have previously been proposed as vectors for in situ expression of cytokines and costimulatory molecules in tumour cells (Pepinski et al., 1995; Hodge et al., 1994; Whitman et al., 1994; Lee et al., 1994). Targeted delivery of the corresponding genes would provide a crucial improvement in terms of efficiency and safety.

We would like to thank Jacqueline Goenaga, Alexandra Tirelli and Séverine Beck for technical assistance, and Dr Elmus Beale for critical reading of the manuscript. This work was supported by the Swiss National Science Foundation, Grant 31–43434.95, by Cancer Research Switzerland, Akt 312, by the Sandoz-Stiftung, by the Schweizerische Krebsliga. SKL 185–9–1995, and by the Jubilaeumsstiftung der Schweizerischen Lebensversicherungs- und Rentenanstalt.

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Received 25 April 1997; Accepted 10 July 1997


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