RESEARCH ARTICLE

In vivo evaluation of an EIAV vector for the systemic genetic delivery of therapeutic antibodies

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Lentiviral-based vectors hold great promise as gene delivery vehicles for the treatment of a wide variety of diseases. We have previously reported the development of a nonprimate lentiviral vector system based on the equine infectious anaemia virus (EIAV), which is able to efficiently transduce dividing and nondividing cells both *in vitro* and *in vivo*. Here, we report on the application of EIAV vectors for the systemic delivery of an antibody fusion protein designed for the treatment of cancer. The therapeutic potential of a single chain antibody against the tumour-associated antigen, 5T4, fused to immune enhancer moieties has been demonstrated *in vitro* and here we evaluate the genetic delivery of a 5T4 scFv fused to B7.1 (scFvB7) using an EIAV vector. The kinetics and concentration of protein produced following both intravenous (i.v.) and intramuscular (i.m.) administration was

determined in immune competent adult mice. In addition, the immune response to the EIAV vector and the transgene were determined. Here, we show that a single injection of EIAV expressing scFv-B7 can give rise to concentrations of protein in the range of 1–5 µg/ml that persist in the sera for more than 50 days. After a second injection, concentrations of scFv-B7.1 rose as high as 20 µg/ml and levels greater than 2 µg/ml were present in the sera of all mice injected i.v. after 210 days despite the detection of antibodies against both the transgene and viral envelope for the duration of this study. These results demonstrate the potential of EIAV as a gene therapy vector for long-term production of therapeutic recombinant proteins.

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Introduction

The treatment of a wide variety of diseases can be approached using systemic administration of therapeutic proteins, including the clinical use of antibodies against tumour-associated antigens to treat cancer (eg herceptin).^{1,2} However, there are problems associated with the repeated administration of protein to achieve lasting therapeutic levels at the sites where they are required, particularly for small proteins with short half-lives. These include bolus effects, inconvenience to the patient and the cost of manufacture of sufficient amounts of recombinant protein. Gene therapy delivery systems are now being developed to overcome these problems, employing a variety of vectors and designing potentially more effective therapeutic gene products.

Specific problems associated with whole antibody therapy, such as immunogenicity in humans and poor tumour penetration, are being addressed using single chain antibodies (scFvs), which are the smallest antibody fragments to retain full antigen-binding capacity. Since

they can be expressed as single recombinant proteins, they provide ideal building blocks for fusion proteins with therapeutic and/or tumour localizing properties.³ ScFvs specific to tumour-associated antigens (TAAs) have been shown to penetrate tumours more readily than whole antibodies⁴ and recombinant antibody fragments that consist of fusions with biologically active tumoricidal agents such as GM-CSF⁵ and TNFα⁶ have been designed to target and treat tumours.

Gene transfer vectors that have been employed to systemically deliver scFvs *in vivo* include first-generation adenoviral vectors used to produce scFvs specific to TAAs such as carcinoembryonic antigen (CEA)⁷ and ErbB2.⁸ However pre-existing immunity to adenovirus, its association with liver toxicity, the transient nature of expression and the inability to repeat administration presents practical difficulties with using this genetic delivery system in humans.^{9,10} Others have engineered the viral coat of retroviruses to express a scFv specific to CEA to then target the virus specifically to malignant cells and deliver a tumoricidal protein.¹¹ However, use of retroviruses is limited in that they can only transduce rapidly growing cells so that slow growing malignancies may escape surveillance.¹² Lentiviruses have the advantage that they can be produced to relatively high titres and are able to transduce both dividing and nondividing cells.^{13,14} This allows long-term expression of the gene or genes of interest. Their large capacity (8–9 kb) means that they can accommodate the majority of genes that have been proposed for gene therapy.^{15,16}

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One example of such viruses is the nonprimate lentivirus equine infectious anaemia virus (EIAV). EIAV belongs to the nonprimate subgroup within the *Lentiviridae*. It has the simplest genome of all the lentiviruses, encoding only three accessory genes, unlike HIV, which encodes six. EIAV infects all *Equidae* causing anaemia but no immunodeficiency and there has been no documented case of EIAV infecting humans. A recombinant EIAV vector containing the minimal requirements for genetic delivery has previously been described^{17,18} and has been used for gene transfer in the CNS to correct a rat model of Parkinson's disease.¹⁹ More recently, long-term treatment of diabetes insipidus via administration of EIAV expressing arginine vasopressin (AVP) into the supraoptic nuclei of the hypothalamus has been demonstrated.²⁰ EIAV vectors have therefore been used in varied applications to correct disorders of the CNS. All vectors used in these studies were pseudotyped with the glycoprotein of vesicular stomatitis virus (VSV-G) serotype Indiana.

To date there has been no description on the use of EIAV to deliver therapeutic proteins systemically. In this study, we describe the use of an EIAV vector for the genetic delivery of scFvs. The scFv used for this study is specific to the tumour-associated antigen, 5T4, which is a human oncofetal protein expressed on a large number of malignancies but absent on most normal adult tissue.^{21–23} ScFv fusion proteins specific to 5T4 have previously been described²⁴ including the 5T4 scFv fused to the murine homologue of the immunostimulatory molecule, B7.1 (scFvB7). In this study, we use this molecule to evaluate the ability of EIAV to genetically deliver a secreted protein, systemically, *in vivo*. We administered two injections, 78 days apart and monitored the levels of fusion protein in the sera of immune competent mice. In addition, antibody responses to both the vector and transgene were determined.

After a single i.v. injection of EIAV-scFvB7 pseudotyped with VSV-G, serum concentrations of 1–5 µg/ml persist for up to 50 days. Lower amounts are produced when the same vector is injected i.m. and take longer to reach levels seen after an i.v. injection. After subsequent administration of vector, by either route, fusion protein could still be detected at 7 months. Antibodies, specific to VSV-G and to the transgene could also be detected throughout the study.

This study is the first to describe the systemic administration of an EIAV vector that gives long-term

expression of a secreted protein. This demonstrates the potential of this delivery system for the gene transfer of other therapeutic proteins in which long-term production of high concentrations are required for effective therapy.

Results

An EIAV vector for the genetic delivery of an scFv specific to 5T4

To allow evaluation of the EIAV vector in a self-antigen system, we have cloned a murine scFv fused to murine B7.1 into the EIAV vector for subsequent evaluation in mouse tumour models. The 5T4 scFv protein fused to the myc-His tagged scFvB7 has been described previously.²⁴ The EIAV vectors used are based on the minimal EIAV vector system, produced using the three-plasmid transfection system comprising a *gag/pol* expression plasmid, a plasmid expressing the VSV-G envelope protein and the genome plasmid.¹⁸ The genome plasmid, pSMART2, is based on the pONY8.0 series of plasmids,²⁵ but in addition contains a central polypurine tract (cPPT) upstream of the internal cytomegalovirus (CMV) immediate-early enhancer/promoter and the woodchuck hepatitis post-transcriptional regulatory element (WPRE) downstream of the transgene (Figure 1). Both these elements were included to increase viral titre and protein expression, respectively. Three different genome plasmids were used; one encoding the transgene of interest (scFvB7), one for β -galactosidase (*lac Z*) and one for green fluorescence protein (GFP).

Quantification and binding activity of scFv secreted by cells transduced with EIAV-scFvB7

The concentration and specificity of scFv produced by the EIAV vector was evaluated *in vitro* following transduction of a murine adenocarcinoma tumour cell line CT26.²⁶ Cells were transduced with vector particles at an MOI of 10 and then passaged six times. The cells were then grown in serum-free media and tissue culture supernatants collected over a period of 7 days. The concentration of scFvB7 secreted into the media at each time point was determined using an ELISA in which scFvB7 is captured onto plates coated with 5T4 (Figure 2). To determine the molecular weight of the scFvB7 produced, tissue culture supernatants were harvested

Figure 1 EIAV genome plasmid. The configuration of the pSMART2 genome plasmid encoding the myc-His-tagged scFvB7 fusion protein is shown. B7.1-EC, murine B7.1 extracellular domain; Linker, (gly₄ser)₃ amino-acid flexible linker; myc, myc epitope; His, 6 histidines. SIN LTR, self-inactivating long terminal repeat to prevent production of full-length vector RNA in transduced cells; cPPT/CTS, central polypurine tract to improve transduction of nondividing cells; RRE, Rev response element; WPRE, woodchuck hepatitis post-transcriptional regulatory element.

Figure 2 The scFvB7 protein accumulates in the tissue culture medium of cells stably transduced with EIAVscFvB7. The transduced cell lines CT26neo-EIAV-scFvB7 and CT26neo-EIAV-LZ were each seeded at 1×10^5 /well of a 12 well plate in 1 ml serum-free medium. Tissue culture supernatants were collected at the time points indicated and diluted fourfold. Using known quantities of recombinant scFvB7 as standard, the concentration of scFv secreted into tissue culture media of transduced cells was quantified.

from the same transduced cell line after 4 days of culture and concentrated before analysis by SDS-PAGE. After probing with antibodies either specific to B7.1 or to the C-terminal myc tag, a ~ 85 kDa band was detected (data not shown). This confirmed the expected molecular weight of the secreted product, and that the EIAV vector system used supports continued expression of the full-length scFv fusion protein several weeks after transduction *in vitro*. These bands were absent in lanes loaded with tissue culture supernatant obtained from cells transduced with EIAV expressing the control transgene, *Lac z* (EIAV-LZ).

We have previously shown that the scFvB7 fusion protein is able to bind simultaneously to 5T4 and to CTLA-4, a ligand specific to B7.1, at the cell surface.²⁴ To demonstrate the ability of the secreted scFvB7 produced by an EIAV vector to bind to the native form of its ligand, a 5T4 transfectant of the melanoma cell line K1735hu5T4 was transduced with EIAV-scFvB7. After 4 days of culture, cells were harvested and the amount of scFvB7 bound to 5T4 at the cell surface was determined by flow cytometry (Figure 3). The presence of 5T4 at the cell surface of K1735hu5T4 was confirmed by use of the conformationally sensitive mouse anti-5T4 monoclonal antibody (mAb), H8.²⁷ The binding of the anti-B7.1 mAb to K1735hu5T4, but not K1735wt, indicates that the scFvB7 is being secreted from the cells and is binding back to the 5T4 at the cell surface. The binding of CTLA4-Ig to the 5T4-positive cell line demonstrates simultaneous binding of the scFv to 5T4 and the binding of B7.1 to its ligand CTLA-4. The amount of B7.1 detected at the cell surface is equivalent to the amount of H8 detected by FACS.

Intravenous delivery of scFv using EIAV results in long-term detection of the secreted transgene

One of the problems encountered with use of therapeutic antibodies is the requirement for repeated injections to attain sustained levels of protein that will be effective therapeutically, especially when the protein has a very short half-life. Adams *et al*²⁸ have shown that the equivalent of 22 ng/ml is sufficient to saturate the TAA, Her2/neu on SK-OV-3 tumours established in a murine model. To determine whether EIAV vectors could be used to produce levels of protein in sera that could be considered equivalent to concentrations of antibody known to be therapeutic, EIAV vectors expressing either scFvB7 or LZ were injected i.v. into immune competent Balb/c mice. Sera were isolated from blood samples and concentrations of scFvB7 determined by ELISA. The scFv is highly specific for the 5T4 antigen bound to the ELISA plate; however, to take into account any nonspecific binding to the plate of other antibodies induced by the treatment (eg anti-VSV-g antibodies), net concentrations of scFvB7 protein were determined by subtracting background levels of binding seen with sera from mice injected with the EIAV-LZ control vector. The average values for these were, in general, less than 20% of the average for the EIAV-scFvB7-injected mice (data not shown).

Figure 4 shows the serum concentrations of scFv in individual mice where a single i.v. injection of 1×10^7 transforming units (TU) EIAV-scFvB7 resulted in between 1 and 3 μ g/ml of scFv 28 days after injection. By day 43 in the majority of mice, protein levels had peaked to an average of 1.9 μ g/ml. By day 70, average serum concentrations had fallen to 40% of this value. Thus, a single injection of EIAV-scFvB7 was sufficient to give rise to average serum levels of scFv of between 1 and 2 μ g/ml over a 70-day period. To determine whether a second injection of EIAV-scFvB7 could enhance levels of fusion protein in the serum, the same preparation of EIAV lentivector was administered 78 days after the first injection. After 7 days, serum concentrations of scFvB7 rose to an average of 8.4 μ g/ml. The serum levels of scFv decreased by 40% by day 99 and were maintained at an average of over 4 μ g/ml through to day 211. An exception to this was seen on day 162 when a significant decrease in levels was observed. Thus, two i.v. injections of EIAV-scFvB7 have resulted in high concentrations of circulating scFv that persisted for over 130 days postinjection.

Serum concentrations of scFv following intramuscular genetic delivery of EIAV

As an alternative to i.v. administration, a more localized i.m. route was also studied by injecting 5×10^6 TU into each muscle in the hind limbs of Balb/c mice so that each mouse received 1×10^7 TU. Sera were isolated from blood samples taken at regular intervals and the concentration of scFvB7 in each was determined. Average values obtained from mice injected with the vector expressing the control transgene were subtracted from scFv determined in individual mice injected i.m. with EIAV-scFvB7. As for the i.v.-treated mice, these were, in general, less than 20% of the average for the EIAV-scFvB7-injected mice (data not shown).

Figure 3 Ligand-binding specificity and recognition of the secreted scFvB7 protein produced by EIAV-scFvB7. The 5T4-positive cell line, K1735-5T4 (a), and the 5T4-negative cell line K1735wt (b) were each transduced with EIAV-scFvB7 at an MOI of 10. Cells were then incubated with an anti-5T4 monoclonal antibody (H8), a FITC-conjugated anti-B7.1 mAb or a CTLA4-Ig fusion protein. H8 and CTLA4-Ig were then detected with a rabbit anti-mouse Ig-FITC antibody.

Figure 4 Detection of scFv in the sera of mice following i.v. administration of EIAV expressing the single chain antibody scFvB7. 1×10^7 TU of either EIAV-scFvB7 or the control vector EIAV-LZ was injected on day 0 and day 78 (arrows). Tail bleeds were taken on days shown and scFv concentrations in the sera of individual mice determined. Plot shows net values for scFv following subtraction of background binding seen in the sera of mice injected with EIAV-LZ.

Figure 5 shows the levels of protein produced following i.m. genetic delivery. After the first injection, serum concentrations of scFvB7 increased slowly to an average of 0.8 $\mu\text{g}/\text{ml}$ by day 42 after which levels began to decline. A second i.m. administration was given on day 56 after serum concentrations began to fall in the majority of mice. Similarly to the second i.v. injection, this resulted in a significant increase in scFvB7 in three out of the five mice studied, so that 8 days later serum concentrations had increased, to between 15 and 20 $\mu\text{g}/\text{ml}$. The other two mice also had increased levels of scFvB7, but only up to $\sim 2 \mu\text{g}/\text{ml}$. Levels then began to decrease so that by day 160, two out of five mice had

levels of between 8 and 14 $\mu\text{g}/\text{ml}$, while the remaining three had levels below 2 $\mu\text{g}/\text{ml}$. The distinct difference in the patterns observed between the mice may be due to variations in the precise location of the injections, for example with respect to the proximity of blood vessels within the muscle.

Antibody titres to VSV-G

The EIAV vectors used in this study were pseudotyped using the glycoprotein VSV-G, which is a highly immunogenic protein.²⁹ In order to determine how the immune response to the EIAV viral envelope influences

Figure 5 Detection of scFv in the sera of mice following i.m. administration of EIAV expressing the single chain antibody scFvB7. 5×10^6 TU of EIAV-scFvB7 or of the control vector EIAV-LZ was injected into each hind limb on day 0 and day 56 (arrows). Tail bleeds were taken on days shown and scFv concentrations in the sera of individual mice determined. Plot shows net values for scFv following subtraction of background binding seen in the sera of mice injected with EIAV-LZ.

transduction efficiency after a second administration, we determined the antibody titres to VSV-G using ELISA.

Antibody titres to VSV-G are shown in Figure 6a (i.v.) and 6b (i.m.) which demonstrates that mice injected i.v. with EIAV-scFvB7 had an anti-VSV-G titre of between 1 in 32 000 and 1 in 64 000 on day 7. In the majority of these mice, titres had decreased by half on day 43. Up to this time point, serum concentrations of scFvB7 had peaked in the majority of mice studied. As expected, a second i.v. injection of EIAV-scFvB7 on day 78 significantly enhanced antibody titres. The first time point measured after the second injection (day 85) showed an increase of at least eight-fold. These increased antivector titres persisted until the last time point measured (day 120), indicating that the existence of antivector envelope antibodies did not prevent successful transduction and transgene expression upon a second administration of the vector.

A very similar anti-VSV-G responsive pattern was observed after i.m. administration (Figure 6b). Thus, both i.v. and i.m. routes of readministration result in elevated anti-VSV-G antibody titres that persisted for the duration for which they were measured.

Antibody titres to murine scFvB7

To determine the antibody titre to the scFvB7 transgene product, ELISAs were carried out on pooled serum samples from the i.v.-injected mice and compared to pooled serum samples from EIAV-LacZ-treated mice (Figure 7). Anti-scFvB7 antibody titres were undetectable 7 days after injection but by day 70 end point titres of 1/250 could be detected. After the second injection, levels rose to 1/2000 and had decreased to 1/500 by the end of the study. Similar results were also obtained using the serum from individual mice and in comparison to normal mouse serum rather than EIAV-LacZ treated mice (data not shown). The scFv transgene also

contained a c-terminal myc-His tag. To determine if the antitransgene response was directed to the nonself tag component, mouse serum was preincubated with myc-His peptide prior to addition to the ELISA plate. This showed that approximately 50% of the antitransgene antibody response was directed to the myc-His tag (data not shown).

Cellular immune responses to transgenes expressed by EIAV

It is possible that induction of a cytotoxic T-cell response to the transgene could reduce expression *in vivo*, by destruction of transduced cells. However, we were unable to assess CD8 response to the transgene because the H^{2d} epitopes for scFvB7 are unknown. Thus, we employed an EIAV vector expressing β -galactosidase (β -gal; LZ) as a model foreign antigen, since the immunodominant epitope for this protein has been well characterized in Balb/c mice and would therefore allow us to assess the immune response to a known epitope. Mice were injected i.v. with either EIAV-LZ or a control construct expressing GFP EIAV-GFP. Attenuated vaccinia virus expressing β -galactosidase (MVA-LZ) was included as positive control.³⁰ The CD8⁺ immune response was measured by ELISPOT using the H-2^d restricted β -gal peptide TPHPARIGL (p876–884).³¹ Responses to the mitogen PHA were included as a control and in general were less strong than might be expected. Figure 8a illustrates that comparable antigen-specific immune responses to the immunodominant β -gal peptide after 14 days were found in both mice injected i.v. with EIAV-LZ and those with MVA-LZ (Figure 8b).

Comparison of transduction sites *in vivo*

The observed differences in scFvB7 levels after a single injection of EIAV via the i.v. and i.m. routes of administration may be due to differences in biodistribu-

Figure 6 Antibody titres to the VSV-G envelope of EIAV-scFvB7 are enhanced upon second administration of vector. Total antibody end point titres to VSV-G following tail vein (a) or i.m. (b) injections are shown.

Figure 7 Antibody titres to the scFvB7 transgene after i.v. administration. Serum samples from the time points shown were pooled and the antibody titres determined by ELISA as compared to EIAV-LZ treated mice.

tion of EIAV particles. In order to determine the tissue distribution achieved using each route, EIAV-LZ was injected into Balb/c mice as described in previous experiments. A higher dose of 1×10^8 TU was used to maximize detection of EIAV sequences within extracted DNA. DNA was extracted from the liver, heart, spleen, lung and the muscle of the hind limb 29 days after injection. This DNA was then analysed for the quantity of EIAV sequences and murine GAPDH by real-time PCR, which will measure the amount of vector that has entered a tissue and been converted to DNA. This DNA may be integrated or episomal. We chose a time point of approximately 4 weeks to increase the probability of detecting integrated DNA. Tissue sections were taken for PCR and primers specific to the packaging signal of EIAV were used in a quantitative PCR (QPCR) assay to

Figure 8 EIAV-LZ pseudotyped with VSV-G induces transgene-specific T cells. Balb/c mice were injected i.v. with 1×10^7 TU of EIAV-LZ and spleens removed 14 (a) and 31 (b) days later. IFN- γ secretion in response to the immunodominant β -gal peptide, TPHPARIGL, as measured by ELISPOT, is shown.

estimate the copies of DNA derived from EIAV in each of the tissues studied.³² The copy number of murine GAPDH was used to control for the amount of DNA in each reaction. Figure 9 illustrates that the major site of integration following injection i.v. is the liver followed by the spleen. In contrast, the major sites of transduction after i.m. administration of EIAV is the hind limb muscle. The copy numbers of EIAV present in the remaining tissues studied (lungs and heart) were much lower and on the whole fell below 1 copy/million GAPDH copies. These observations are in agreement with an earlier study where VSV-G-pseudotyped HIV vectors were found to mainly target the spleen, liver and bone marrow of mice injected i.v.^{33,34}

Discussion

Previous evaluation of HIV-1 lentivectors following systemic delivery have demonstrated long-term expression of the transgene for up to 1 year, depending on the transgene and host.^{33–35} An alternative to HIV-1 is the EIAV-based vector, which has been developed, in part, because HIV-1 is a human pathogen and as a result may face extra hurdles in clinical use. In addition to this, the potential advantages of EIAV are its relative simplicity compared to HIV-1, the inability of the parent virus to replicate in human cells and the nonlethal nature of the infection in its natural host, the genus *Equidae*. In this study, we assessed the ability of a systemically administered EIAV vector to give long-term expression of a secreted protein. Genetic delivery of therapeutic proteins by long-term expression vectors could have significant advantages over repeated administration of recombinant protein. To illustrate this potential application, we used the 5T4 scFv-B7.1 fusion protein to evaluate production of systemic antibody from an EIAV expression vector, *in vivo*.

The first aim of this study was to determine the concentration and longevity of serum concentrations of scFvB7 produced in immune competent mice using the EIAV vector. A study with a VSV-G-pseudotyped HIV-1 vector expressing human Factor IX (hFIX) demonstrated circulating levels of the recombinant protein in the order of 10–100 ng/ml after administration of 2×10^8 TU into either nude or immunocompetent mice. Levels of hFIX reduced more rapidly in the immunocompetent mice compared to the nude mice,³⁵ possibly due to the induction of an immune response against the transgene

Figure 9 Comparison of EIAV distribution following i.v. and i.m. administration. A total of 1×10^8 TU of EIAV-LZ was injected and organs were removed 29 days later. Genomic DNA was isolated and EIAV-LacZ copies per million GAPDH copies were determined by real-time PCR.

product. The explanation that the reduction in transgene product is due to clearance of expressing cells by the immune system is underlined by a recent study monitoring both hFIX and GFP expression by HIV-based lentivectors.³⁶ Expression of GFP in immunocompetent mice lead to production of GFP-specific CD8⁺ cells; however, in GFP transgenic mice, such cells were not found and QPCR demonstrated the persistence of the vector in the liver and spleen in the transgenic mice compared to clearance in the nontransgenics. Additionally, expressing hFIX under a CMV promoter lead to high levels of serum hFIX protein, which became undetectable after 2 weeks, concomitant with a high titre of anti-hFIX antibodies. However, using a liver-specific promoter lead to much longer-term expression with only a low titre of anti-hFIX antibodies in some mice. Here, we found that a single i.v. injection of 1×10^7 EIAV-scFvB7 resulted in average serum protein concentrations ranging between 1 and 6 µg/ml over a 40-day period. A second administration significantly increased these levels, which then decreased, but were still in the range of 2–8 µg/ml 130 days later. Thus, it is possible to attain high levels of secreted transgene following a single i.v. injection of EIAV-scFvB7, and that following a second injection scFv levels are further enhanced. Administration of the vector intramuscularly was also investigated as this could potentially be a less invasive route of administration in humans. Overall, a similar pattern of scFvB7 levels in the serum was observed. The levels after the first injection were on average a little lower than for i.v. However, after a second i.m. injection, serum concentrations increased between five- and 10-fold and, similarly to i.v., the concentration then decreased. In three out of five mice, this fall was quite rapid resulting in levels lower than 2 µg/ml. However, in two mice the fall was less dramatic so that serum concentrations persisted between 5 and 14 µg/ml until day 160 after the initial injection.

An explanation of the differences in serum concentrations of scFv observed following a single injection via the two routes could be that different sites are targeted by the different routes of administration used. To investigate this, we compared the distribution of EIAV 29 days after administration by both routes using real-time QPCR. We found that i.v. administration resulted in better transduction of the liver and spleen than was seen with mice injected i.m. EIAV copies following i.m. administration were mainly localized to the site of injection with very little transduction of the liver. Thus, the presence of vector in the liver after i.v. administration may be partly responsible for the higher levels of serum scFv observed in these animals after a single injection, since that may allow better dissemination of the protein in contrast to a more localized production in the muscle.

Overall, we have seen µg/ml concentrations of scFvB7 following a single administration of EIAV lentivector that are increased after a second injection and persist, in total, for at least 7 months. It is interesting that in some cases the second injection lead to a 10-fold increase in serum levels, which then declined and fluctuated for the duration of the study at values between peak and presecond injection levels. One explanation of this large increase could be that there is a threshold of detection because the systemic protein may be sequestered by different tissues or compartments and thus not circulate freely. Subsequently, after the first injection, only a

proportion of the protein produced may be detected; however, when the second injection is given a much larger proportion of the protein is now in circulation and thus detectable in the sera. Since others have found that transgene product levels can be affected by host immune responses, we investigated both cellular and humoral responses to EIAV-scFvB7.

A single injection of EIAVscFvB7 resulted in detectable anti-VSV-G antibodies, which increased significantly when a second injection was given. The antibodies detected by the ELISA used include IgG and IgM subclasses specific to VSV-G Indiana. Both subclasses have been shown to be effective at neutralizing VSV infections in mouse models.^{37,38} We also investigated anti-GAG responses, but the levels of antibody detected were much lower than for VSV-G (data not shown).

It is unlikely that the drop in scFvB7 serum levels that occurred after the second injection was due to the clearance of transduced cells by antibodies specific to VSV-G since transduced cells will not express the VSV-G. However, it is possible (as has been seen by others) that an immune response to the transgene could result in clearance of expressing cells. In this study, we found that antibody titres to scFvB7 were undetectable on day 7 but could be detected by day 70, albeit to levels that were very much less than seen for VSV-G. After the second injection of EIAV-scFvB7, antibody titres increased and remained detectable for the duration of the study. The increase in antibodies to scFv may have resulted in partial clearance of circulating scFvB7. The fact that the scFv and B7 are murine should reduce the level of immune response and may well explain the relatively high levels of secreted protein. The myc-His tag is not native and therefore may be a target for the immune system. In fact, preabsorbing the pooled sera with a myc-his peptide prior to analysis for anti-transgene antibodies reduced the titre by approximately 50%. This suggests that a significant portion of the immune response is against the non-native elements of the recombinant protein and thus for clinical applications the tag component would obviously not be included.

The influence of the anti-tag antibodies on the transgene protein serum levels is unclear since the antibodies may be against single epitopes and thus not aggregate and bind to Fc receptors. If this were the case, they may then be carried through into the ELISA to measure transgene protein and artificially increase the value obtained. However if they did bind Fc receptors, then some protein would be removed from the serum by presentation to the immune system via macrophages, monocytes or complement fixation and thus the levels of protein measured would be lower than if the tag were not present. It is quite possible that both these scenarios could occur and that the balance between the two will vary in individual animals. Thus overall, there is a clear increase in transgene protein levels after the second injection, although the ELISA measurement may not be an exact reflection of the magnitude of this increase.

We found that a single injection of EIAV-LZ at the same dose used to deliver scFvB7 was sufficient to induce a cellular immune response to a nonself protein, β -gal. Since VSV-G-pseudotyped HIV-1-based lentiviral vectors are known to transduce antigen-presenting cells (APCs) *in vivo*³⁹ this was not unexpected. The relatively high transduction of spleen following i.v. injection

suggests that in addition to the liver this organ is a major site of transgene production and as such is an optimal site for priming of antivector and transgene responses. Taken together, these observations point toward the induction of an immune response to VSV-G, and the transgene scFvB7, which do not prevent production of scFv but may instead reduce the persistence of high levels of scFv initially observed after a second injection. Alternatively, a proportion of the cells transduced may have a high turnover and thus be removed from the system after a relatively short period of time. In addition, longer lasting, transduced cells may reach equilibrium in the amount of recombinant protein expressed.

One way in which the immune response to vector components can be reduced is to employ purification steps as part of the production process. For example, a recent study by Baekelandt *et al*⁴⁰ has shown that removal of extraneous viral components from lentiviral preparations by sucrose gradient ultracentrifugation significantly reduces the antibodies induced to the HIV-1 vector and improves transgene expression. Others have shown that purification of lentiviral gene transfer vectors provides highly purified vectors that reduces cell toxicity and significantly improves gene transfer in primary cells.⁴¹ We are currently employing a similar procedure to purify EIAV vectors that are intended for clinical use to remove cellular and subcellular components including VSV-G that is surplus to requirements for packaging EIAV. This together with the use of reduced amounts of VSV-G plasmid during production should not only improve the safety profile of these vectors but also reduce the potential clearing effects of antivector immune responses on subsequent vector administrations.

However, immune responses to the viral envelope or transgene may not completely explain the fluctuations in scFvB7 levels that occurred after the second i.v. injection. In particular, the decrease measured on day 162 in all five mice requires some explanation. A simple explanation may be the presence of an asymptomatic infection, which could cause upregulation of the B7.1 ligands CD28 and CTLA-4, thus absorbing the scFvB7 protein from the serum at that time point. Therefore, this effect could be specific to the scFvB7.

In this study, we have demonstrated the long-term expression of a secreted protein from a minimal EIAV vector following systemic administration. This highlights the potential therapeutic application of these vectors for prolonged systemic production of antibody fragments or other secreted proteins.

Materials and methods

Virus production and titration

EIAV vectors were produced using a vector system as described previously.^{18,20} Vectors were produced in 10 layer cell factories (Nalgene) each seeded with 5×10^8 HEK293T in DMEM-10 (Sigma Aldrich) containing 10% FBS, 1% MEM nonessential amino acids, 2 mM glutamine and 10 U/ml penicillin, 10 mg/ml streptomycin (DMEM-10). The next day, the genome plasmid was cotransfected into the human kidney cell line, with the plasmid encoding the envelope protein VSV-G (pRV67),

together with either pONY3.1 that encodes Gag/Pol. Each cell factory was transfected with 230 µg of genome plasmid, 57.5 µg of env plasmid and 230 µg of gag/pol plasmid using Fugene 6 (Pharmacia). After 18 h, 10 mM sodium butyrate (Sigma Aldrich) was added. After 7 h, this media was replaced with fresh DMEM-10 lacking sodium butyrate. Tissue culture supernatants were collected after an additional 18 h and passed through 0.45 µ filters. Supernatants were first concentrated by spinning at 6000 g for 16–18 h at 4°C. The pellet was then resuspended in PBS before a final centrifugation step at 4°C for 90 min at 20 000 r.p.m. Viral supernatants were typically concentrated 2000-fold by resuspending in 0.5 ml of TSSM formulation buffer (20 mM Tromethamine, 100 mM NaCl, 10 mg/ml sucrose and 10 mg/ml mannitol). Virus was titrated using TaqMan PCR to measure RNA content³² and to determine biological titre by FACS analysis for GFP or B7.1 and by x-gal staining for Lac Z.

Transduction of tumour cell lines in vitro

The CT26 cell line expressing stably transfected with pIRESneo (Invitrogen) to express neomycin resistance has been described.³⁰ The K1735 cell line expressing 5T4 was produced in the same manner, using pIRESneo containing the 5T4 cDNA. Cells (5×10^4) were transduced with EIAV vector in 12-well plates at an MOI of 10 in 200 µl of RPMI containing 8 µg/ml of polybrene. Following incubation at 37°C in 5% CO₂ for 3–5 h, an additional 1 ml of RPMI (no polybrene) was added to each well. Once cells became confluent, they were transferred to T25s using cell dissociation medium.

FACS analysis

Cells transduced with EIAV-scFvB7 were harvested once confluent (3–4 days of culture in T25s) using cell dissociation medium. Expression of 5T4 was determined using a mouse monoclonal antibody (mAb) H8 that is specific to human 5T4.²³ Cells (5×10^5) were incubated with 100 ng of H8 for 1 h at 4°C. A FITC-conjugated rabbit anti-mouse Ig (DAKO) was then used to detect 5T4 at the cell surface. Murine B7.1 was detected by incubating 5×10^5 cells with FITC-conjugated rat anti-mouse B7.1 (Becton Dickinson) following the manufacturer's instructions. Cells were also labelled with FITC-conjugated isotype control to control for nonspecific binding. The binding function of B7.1 fused to scFv was evaluated using the high-affinity fusion protein CTLA4-Ig (Becton Dickinson). After 1 h at 4°C, CTLA4-Ig was detected using FITC-conjugated rabbit anti-mouse Ig (DAKO). At the end of each labelling step, cells were washed twice. Cells were finally fixed using 2% paraformaldehyde in PBS before acquisition on FACS-Calibur (Becton Dickinson).

Capture ELISA for scFv specific to 5T4

ELISA plates (Immunogen) were coated using recombinant 5T4 purified from tissue culture supernatants of a transfectant CHO cell line expressing truncated 5T4 (5T4TM–). A measure of 100 µl containing 1 µg/ml of 5T4 in PBS was dispensed into each well and incubated overnight at 4°C. Plates were blocked using blocking buffer (0.05% Tween in PBS (wash buffer) containing 10% FBS) and diluted samples or known amounts of scFvB7 protein²⁴ were added in triplicate (50 µl/well) to the

same plate. Following incubation at room temperature for 1–2 h, plates were washed six times and 250 ng/ml of anti-mouse rat B7.1 was added to each well. After 1 h at room temperature, plates were washed as before and incubated with 500 ng/ml of anti-rat HRP (Dako). Finally, after subsequent washes, 100 µl/well TMB (tetramethylbenzidine) substrate was added to each well and once sufficient colour had developed the reaction was stopped using 2 M HCl. Plates were read at 450–570 nm.

Systemic delivery of EIAV to adult mice

Female Balb/c mice (ages 8–10 weeks) were obtained from Harlan UK Ltd, Blackthorn, Bicester, Oxon OX25 1TP, England. 1×10^7 TU of EIAV was injected i.v. in a volume of 200 µl and i.m. injections consisted of a single injection of 5×10^6 TU into each hind limb so that a final dose of 1×10^7 TU was administered. Tail bleeds of no more than 200 µl were performed in accordance with Home Office guidelines. Serum was removed following an ultracentrifuge spin at 20 000 g for 10 min and stored at –20°C. Samples used for quantifying levels of scFv were diluted so that OD measurements fell within the linear portion of the standard curve.

ELISA to measure antibody titre to VSV-G

ELISA plates were coated with VSV-G obtained from tissue culture supernatants of cells infected with the VSV-Indiana strain. A 1:100 dilution of VSV-G in coating buffer (0.1 M NaHCO₃, 0.1 M NaCO₃, 0.01% NaN₃, pH 9.6) was dispensed into each well (50 µl/well) and incubated overnight at 4°C. Plates were blocked as described for the 5T4 scFv capture ELISA and serial dilutions of serum samples added to plates in duplicate (40 µl/well). Following incubation at room temperature for 1–2 h, plates were washed as before and 500 ng/ml of anti-mouse HRP (Dako) in PBS Tween added. Plates were developed as described previously and plates read at 450–570 nm. An end point titre is defined as the dilution at which the OD of the test sample is two-fold higher than the signal obtained for normal serum taken from naïve age-matched mice.

ELISA to measure antibody titre to scFvB7

ELISA plates were coated with purified scFvB7-myc-His protein²⁴ using 50 µl of 100 µg/ml in coating buffer per well and treated as described for the VSV-G ELISA. In experiments to investigate the role of the myc-his tag in the antibody response, pooled serum (diluted 1/62.5) was preincubated with an myc-his peptide (EQKLI-SEEDLNSAVDHHHHHH) in 10–20 molar excess compared to the target antigen on the plate. An irrelevant peptide was used as a control.

ELISPOT assay

Female Balb/c mice, 8–10 week old, were injected i.v. with 1×10^7 PFU MVA or 1×10^7 TU of EIAV, and after 14 or 31 days, the spleens from two mice per vaccination group were pooled and homogenized in RP-10 that consisted of RPMI 1640 (Sigma Aldrich) supplemented with 2 mM L-glutamine, 10 U/ml penicillin, 10 mg/ml streptomycin, 10% foetal bovine serum (all from GIBCO BRL). Erythrocytes were lysed using Tris ammonium chloride solution and washed twice in RP-10. Lymphocytes were seeded onto MAIP N45 plates (Millipore) at

1×10^5 cells per well in 100 μ l RP-10. Plates had previously been coated with 7–15 μ g/ml of anti-IFN- γ antibody (MabTech) overnight at 4°C, washed twice with RPMI followed by a blocking step in RP-10 for 30 min at room temperature. Antigens were added to cells in triplicate: β -gal THPPARIGL (p876–884) peptide at 0.5 μ g/well; phytohaemagglutinin (PHA) at 0.1–1 μ g/well. Secreted IFN- γ was detected following the manufacturer's instructions provided with the MabTech ELISPOT kit. Plates were developed using 50 μ l/well of alkaline phosphatase chromogenic substrate (Pharmacia) as instructed by the manufacturers and washed thoroughly with tap water once spots became apparent. Plates were air dried in the dark and spots counted using an automated ELISPOT counter.

PCR analysis of tissues obtained from mice injected with EIAV

Mice were killed 4 weeks after i.v. or i.m. injection of EIAV-LZ and the following tissues were extracted and immediately frozen in liquid nitrogen: liver, heart, kidney, spleen, lung and muscle of the hind limb. Sections (0.8 μ) of each organ were performed using a clean blade for each organ. Sections were homogenized in 80 μ l of PBS using a tissue homogenizer (Sigma Aldrich). Care was taken to use new tips for each tissue homogenized. DNA was isolated from homogenates equivalent to approximately 10 mg of spleen or to 25–30 mg of each of the other tissues using the DNeasy Kit (Qiagen). An RNase A step was included according to the manufacturers instructions to increase the purity of DNA isolated. DNA was quantified by spectrophotometry. Real-time PCR on the ABI 7000 TaqMan was performed using 200–250 ng of DNA per reaction. Primer probe sets specific to the EIAV packaging signal were as follows: forward primer: 5' ATT GGG AGA CCC TTT GAC ATT G 3' (900 nM); reverse primer: 5' ACC AGT AGT TAA TTT CTG AGA CCC TTG TA 3' (300 nM); probe: 5' (FAM) CAC CTT CTC TAA CTT CTT GAG CGC CTT GCT (TAMRA) 3' (100 nM). Primers and probe to murine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used on DNA from the same samples and PCR reactions read on the same plate: forward primer: 5' CGG ATT TGG CCG TAT TGG 3' (300 nM); reverse primer: 5' TGG CAA CAA TCT CCA CTT TGC 3' (300 nM); probe: 5' (FAM) CCT GGT CAC CAG GGC TGC CAT T (TAMRA) 3' (100 nM). The EIAV genome plasmid pONY8Z and the GAPDH plasmid 2.1 (containing the GAPDH sequence from the murine cell line A9) were used to generate standard curves. Each consisted of five serial 10-fold dilutions that ranged from 8×10^4 to eight EIAV copies or 3×10^7 – 3×10^3 GAPDH copies. The conditions for each reaction were 50°C for 2 min and 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min.

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