Nucleic Acids Research, 1993, Vol. 21, No. 5 1081–1085

Retroviral vectors displaying functional antibody fragments

Stephen J.Russell[1], Robert E.Hawkins[2] and Greg Winter[1,2]
[1]MRC Centre for Protein Engineering and [2]MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK

Received December 23, 1992; Revised and Accepted February 5, 1993

ABSTRACT

We have made retrovirus particles displaying a functional antibody fragment. We fused the gene encoding an antibody fragment directed against a hapten with that encoding the viral envelope protein (Pr80^{env}) of the ecotropic Moloney murine leukemia virus. The fusion gene was co-expressed in ecotropic retroviral packaging cells with a retroviral plasmid carrying the neomycin phosphotransferase gene (*neo*), and retroviral particles with specific hapten binding activities were recovered. Furthermore the hapten-binding particles were able to transfer the *neo* gene and the antibody-envelope fusion gene to mouse fibroblasts. In principle, the display of antibody fragments on the surface of recombinant retroviral particles could be used to target virus to cells for gene delivery, or to retain the virus in target tissues.

INTRODUCTION

The isolation of genes encoding proteins with known binding properties has recently been facilitated by selection technologies. The genes encoding the protein are packaged such that the encoded protein is displayed on the outside of the package. The package is then selected by its binding affinity (for example an encoded antigen by binding to solid phase antibody), and replicated. The tight linkage between genes and encoded protein allow packages to be selected in rounds of binding and growth, leading to selection factors of more than one in a million. Replicable packages have included mammalian cells—used to isolate the genes encoding lymphocyte surface markers from cDNA libraries[1]—and filamentous bacteriophage—used to isolate the genes encoding antibody fragments[2] and other proteins[3]. The use of filamentous bacteriophage has even led to strategies for building antibodies in bacteria and improving their binding affinities, and so by-passing immunisation[4,5].

Here we have explored the use of retroviral vectors for display of proteins expressed in mammalian cells. Such retroviruses offer an alternative to the use of filamentous bacteriophage as replicable display packages. For example, glycosylated proteins which are unsuitable for display on filamentous bacteriophage may be amenable to selection in a retroviral display system. Retroviral vectors displaying nonviral proteins or peptides may also offer a vehicle for somatic gene therapy[6]. Retroviruses can transfer genes efficiently to cells but they deliver their therapeutic genes to both target and nontarget cells, necessitating local delivery of the recombinant retroviruses to specific target tissues[7,8], or retrovirus-mediated gene transfer to target cells *ex vivo*, followed by reimplantation of these cells[9]. An alternative strategy would be to alter the host range of the virus[10] which is determined in part by the binding properties of the proteins displayed at its surface[11].

In principle, to target the virus to cell surface molecules which are not recognised by natural viral coat proteins, we could incorporate functional nonviral polypeptides into the virion. Nonviral proteins have been incorporated into viral particles, for example, CD4 and chimaeric CD4-envelope proteins into avian retroviruses[12], but the displayed proteins were not shown to be folded. Here we show that a functional antibody fragment can be displayed as a fusion with the retroviral coat protein on the surface of the viral particles and binds specifically to the hapten recognised by the antibody fragment.

MATERIALS AND METHODS

Plasmid construction

The BamHI/ClaI env fragment (nt 6537–7674, nt numbering from Shinnick et al, 1981 (ref 13) from pCRIP (gift from O. Danos[14]) was cloned into the BamHI/ClaI backbone fragment of pZipNeoSV(X) (gift from R. Mulligan[15]) to generate an intermediate plasmid penvBam/Cla.

A SfiI/NotI cloning site was introduced beyond the leader peptide sequence between codons corresponding to the 6th and 7th aminoacids (from the N-terminus) in the mature MoMLV env polypeptide. The oligonucleotides envNotrev (5′-CTG C-AG GAG CTC GAG ATC AAA CGG GCG GCC GCA CCT CAT CAA GTC TAT AAT ATC-3′, complementary to MoMLV env nts 5894–5914 with a 33nt 5′ sequence encoding a NotI site and 21nt complementary to the 5′ tail of envSfifor) and envseq7 (5′-GCC AGA ACG GGG TTT GGC C-3′, complementary to MoMLV env nts 6600–6581) were used to amplify a 739bp fragment from plasmid pCRIP (and encoding downstream of codon 6). A second set of oligonucleotides, envSfifor (5′-TTT GAT CTC GAG CTC CTG CAG GGC CGG CTG GGC CGC ACT GGA GCC GGG CGA AGC AGT-3′, complementary to MoMLV env nts 5893–5873 with a 36nt 5′ overhang encoding a SfiI site and 21nt complementary to the 5′

tail of envNotrev) and revMLVpol (5'-AAT TAC ATT GTG CAT ACA GAC CC-3', complementary to MoMLV pol nts 5277–5249) was used to prime amplification of a 702bp fragment from pCRIP (and encoding upstream of env codon 7). Amplifications were carried out using Vent polymerase and reactions were subjected to 15 PCR cycles at 94°C for 1min, 60°C for 1min and 72°C for 1min. The 702 and 739bp gel-purified PCR products were linked through their complementary 21nt tails to generate an env gene fragment incorporating a SfiI/NotI cloning site: the two fragments were mixed and subjected to three cycles (94°C-1min, 40°C-1min, 72°C-2min) followed by 17 further PCR cycles (94°C-1min, 60°C-1min, 72°C-2min) after addition of ologonucleotides envseq7 and Bglenvrev (5'-TAA TCA CTA CAG ATC TAG ACT GAC ATG GCG CGT-3', complementary to MoMLV pol nucleotides 5766 to 5785 and with the 5' tail incorporating a BglII restriction site). The product, a 905bp fragment, was digested with BglII and BamHI and cloned into the BamHI site of penvBam/Cla (see above) giving the plasmid pSfi/Notenv. Correct assembly of this plasmid was confirmed by restriction analysis and dideoxy sequencing. A SfiI/NotI fragment from pB1.8scFv[16], encoding a functional B1.8 scFv antibody was then cloned into the SfiI/NotI cloning site of pSfi/Notenv to generate the plasmid pNIPenv (Fig 1). Plasmid pDCNeo (Fig 1, a gift from Dr P Allen, Institute of Cancer Research, Fulham Road, London) is a retroviral plasmid which carries the bacterial neomycin phosphotransferase gene. It generates packagable RNA transcripts which are encapsidated into recombinant MoMLV particles and transfer G418 resistance to infected target cells.

Cells and recombinant retroviruses

NIH3T3 fibroblasts, the ecotropic retroviral packaging cell line psi2[17] and the amphotropic retrovirus producer cell line GP+envAm12-BabePuro (a gift from RG Vile, ICRF, Lincolns Inns Fields, London—derived by transfection of GP+envAm12[18] cells with the plasmid pBabePuro[19]) were maintained in DMEM/10%FBS supplemented with 60μg/ml benzylpenicillin and 100μg/ml streptomycin at 37°C in an atmosphere of 5%CO_2. The cells were replated twice weekly using EDTA without trypsin to disrupt the monolayer.

Plasmids pNIPenv and pDCNeo were transfected (or co-transfected) into psi2 cells by calcium phosphate precipitation[20]. Briefly, 2×10^5 cells were plated in 90mm tissue culture plates (Nunc), cultured overnight, washed and fed with 10mls new medium. 10μl plasmid DNA and 50μl 2M $CaCl_2$ (0.2μm-filtered) were diluted in sterile water to a volume of 400μl. The $CaCl_2$/DNA mis was added dropwise to an equal volume of 0.2μm-filtered 2× HEPES-buffered saline (280mM NaCl, 10mM KCl, 1.5mM $Na_2HPO_4.2H2O$, 12mM dextrose, 50mM HEPES, pH adjusted to 7.05 with 0.5N NaOH) and left to stand for 20 minutes at RT. The transfection solution (800μl) was added to the cells which were cultured for 16hrs, washed and re-fed. G418 selection (1mg/ml) was commenced 24 hrs later and continued for approximately 2 weeks.

Transfected colonies expressing surface B1.8 single chain antibody were identified by panning with NIP.BSA-coated beads. Briefly, tosyl activated paramagnetic beads (Dynal, Oslo, Norway. Prod. no. 14004) were coated with NIP.BSA (about 10 NIP-caproate-O-succinimide molecules coupled to each bovine serum albumin molecule[16]), washed extensively in PBS and blocked with DMEM/10%FBS. 90mm tissue culture plates containing up to 50 G418-resistant psi2 colonies were rocked gently for 1 hr at 4°C followed by 1 hr at room temperature with 2×10^7 (50μl) beads in 5mls DMEM/10%FBS. After 5 washes in PBS, positive colonies (heavily coated with paramagnetic beads) were easily identified and were transferred individually for further growth and harvest of cell supernatants.

Recombinant retrovirus titres were determined by transfer of G418 or puromycin resistance. NIH3T3 cells were infected by overnight exposure to 0.45μM-filtered viral supernatants in the presence of 5μg/ml polybrene and colonies resistant to 1mg/ml G418 or 1.25 μg/ml puromycin were counted after 10–14 days. The amphotropic producer cell line GP+envAm12-BabePuro was infected with ecotropic virus by exposing 10^5 cells overnight (twice) to 10 mls of the appropriate producer cell supernatant in the presence of 5μg/ml polybrene.

ELISA for B1.8scFv-MoMLVenv fusion protein

To detect the B1.8-env fusion protein in supernatant of pNIPenv transfected clones, 96-well microtitre plates (Falcon) were coated overnight at RT with 20μg/ml NIP.BSA or BSA alone, blocked for 2 hrs at 37°C in DMEM/10%FCS and washed ×6 in PBS. Culture supernatants, cleared of cell debris by centrifugation at 5000RPM for 15min, were added in triplicate to coated wells and incubated for 2hrs at RT. Wells were washed (PBS×6). The second layer was a goat polyclonal anti-Rauscher MLV env antiserum (Microbiological Associates, Inc. Bethesda), diluted 1/500 in DMEM/10%FCS, and incubated at RT for 1 hr. After 6 washes in PBS, the third layer HRP-conjugated rabbit anti-goat antibody (Sigma) was added, the plates incubated for a further hour at RT, washed×6 in PBS and the reaction developed with ABTS (2'2'-azinobis(3-ethylbenzthiazolone)sulphonic acid). Absorbance readings were measured at 405nm after 20 minutes.

Infectious retrovirus immunosorbent assay (IRISA)

Individual wells in 6-well tissue culture plates (Corning, New York) were coated overnight at 4°C with 100μg/ml NIP.BSA or Ox.BSA (about 14 molecules 2-phenyl-5-oxazolone coupled to each bovine serum albumin molecule, and was a gift from C.Rada), washed 3×PBS, blocked for 2 hrs at 37°C with

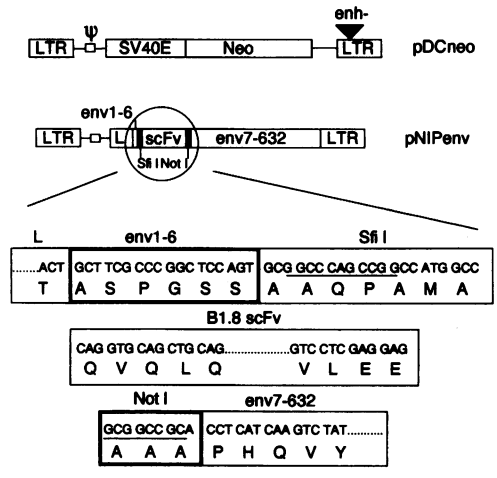

Figure 1. Plasmids pNIPenv and pDCNeo, including sequence and translation of pNIPenv expression vector showing details of fusion between MoMLV*env* and B1.8 scFv. See Materials and Methods for details of construction. LTR = long terminal repeat; L = 33 aminoacid env leader peptide; SV40E = SV40 early promoter, enh$^-$ = enhancer deletion.

DMEM/10%FBS and washed 3×PBS. Virus-containing supernatant (0.45μM-filtered) was added (2 hrs at 37°C), wells were washed 6×PBS and 10^5 NIH3T3 cells were added to each well in 5 mls DMEM/10%FBS containing 5μg/ml polybrene. After 24 hrs incubatiion, G418 or puromycin was added, either before or after replating. 10−14 days later, viable colonies were stained with 50% methanol/5% Giemsa and counted. For inhibition IRISA, virus-containing supernatants were pre-incubated (30 mins at room temperature) with varying concentrations of NIP·BSA.

EM analysis of virus agglutination

0.45μM-filtered virus-containing supernatants were incubated overnight at 4°C with varying concentrations of NIP·BSA. Virions were pelleted by centrifugation at 40,000 rpm for 1 hr, resuspended in 100μl 2% phosphotungstic acid and dropped onto Formvar-coated grids which had previously been coated with a thin layer of carbon. Transmission electron micrographs were taken with a Joel JEM100CX microscope at magnifications ranging from 10,000 to 50,000.

RESULTS

Design of pNIPenv vector

Plasmid pNIPenv (Fig. 1) encodes a chimaeric fusion protein consisting of the ecotropic MoMLV envelope polypeptide $Pr80^{env}$ with a single chain Fv (scFv)[21,22] fragment directed against the hapten 4-hydroxy-5-iodo-3-nitrophenacetyl caproate (NIP)[16] inserted 6 aminoacids from the N-terminus of $Pr80^{env}$. The scFv fragment is flanked by SfiI and NotI sites as in the vector pHEN1[23], to facilitate the cloning of scFv fragments selected from phage display libraries[4]. The 33 aminoacid MoMLV *env* leader sequence is retained, without disruption of the leader cleavage site. The N- and C-termini of the B1.8scFv are connected to adjacent *env* sequences through short linker sequences (Fig. 1). Expression is driven from promoter/enhancer sequences in the 5′ MoMLV long terminal repeat (LTR) and a polyadenylation sequence is provided by the 3′ MoMLV LTR.

Display of antibody fragments on surface of mammalian cells

Plasmid pNIPenv was co-transfected with the retroviral plasmid pDCNeo (which generates a packagable RNA transcript encoding neomycin phosphotransferase and confers G418-resistance, Fig. 1) into the ecotropic retroviral packaging cell line psi2. Control cells were transfected with pDCNeo alone. G418-resistant psi2 transfectants displaying the B1.8 scFv-MoMLVenv fusion protein at their surface were identified by panning with NIP.BSA-coated paramagnetic beads. The cells isolated by panning were heavily coated with the beads, indicating that a functional antibody fragment was displayed on the surface of the transfected cells.

Display of antibody fragments on surface of retrovirus

The retroviruses expressed from the selected clones were titred by transfer of G418 resistance to NIH3T3, and a range of titres noted, for example clones psi2-NIPenv1 (titre 0 G418 t.u./ml) and psi2-NIPenv5 (titre 10^3 G418 t.u./ml). Cell supernatants were then tested by ELISA for presence of the B1.8scFv-env fusion protein (Fig. 2). Using anti-env antiserum as the second layer, specific NIP.BSA-binding activity was detected in supernatants from pooled pNIPenv psi2 clones (titre 10^3 G418 t.u./ml) and from clone psi2-NIPenv5, but not from clone psi2-NIPenv1 or pooled psi2-DCNeo clones (titre 10^3 G418 t.u./ml). This suggested that the functional antibody fragment could be incorporated into virion particles and displayed at their surface.

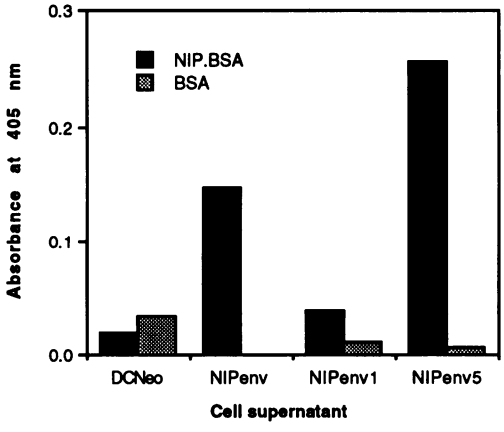

Figure 2. Supernatant ELISA for B1.8scFv-MoMLV*env* fusion protein binding to NIP.BSA.

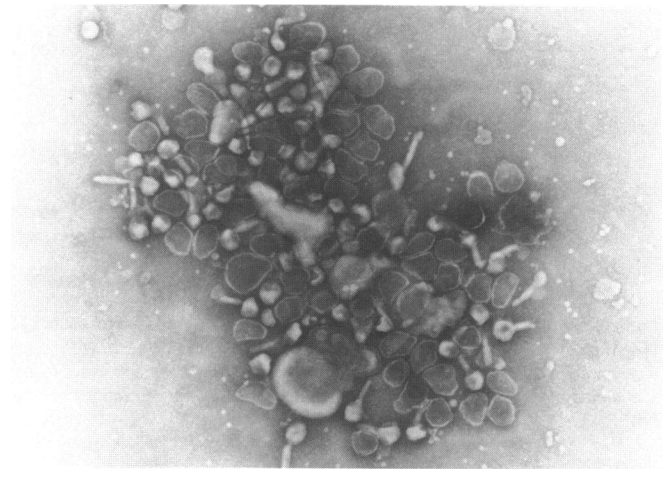

Figure 3. Electron micrograph (× 20,000 magnification) of NIP.BSA-agglutinated virus from psi2-NIPenv5 supernatant.

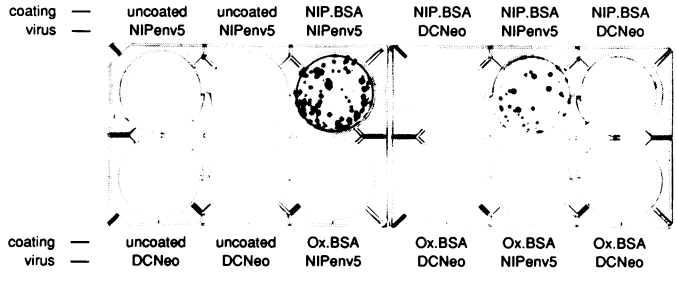

Figure 4. IRISA plate (5% Giemsa). Wells were coated as indicated and bound virus was detected by transfer of G418 resistance to NIH3T3 cells (see Materials and Methods for details). Psi2-NIPenv5-derived virus binds specifically to NIP.BSA-coated wells.

Table 1. Pooled IRISA data for psi2-NIPenv5- and psi2-DCNeo-derived virus binding to NIP.BSA or OX.BSA. Numbers represent Giemsa-stained G418 resistant colony counts (halved for assays which were replated after 24 hrs).

NIPenv5 Input	NIP.BSA	Ox.BSA
1950	14	2
1950	20	1
900	31	0
1560	100	12*
1560	80	11*
4650	125	1
5950	150	2
Totals **18520**	**520**	**29**
DCNeo Input	**NIP.BSA**	**Ox.BSA**
1100	9	7*
625	0	0
625	0	0
1375	2	4
Totals **3725**	**11**	**11**

*polybrene added to initial virus binding reaction

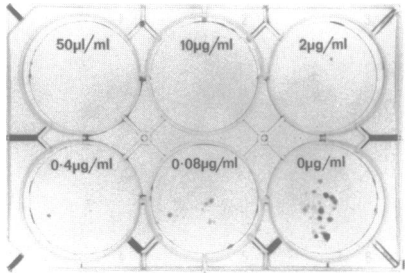

Figure 5. Inhibition IRISA plate (5% Giemsa). All wells were coated with NIP.BSA. Psi2-NIPenv5-derived virus (1ml) was preincubated with varying concentrations of soluble NIP.BSA, as indicated on the photograph, prior to assay. Virus binding is progressively inhibited with increasing concentrations of soluble NIP.BSA.

As a further demonstration, 0.45µM-filtered culture supernatant psi2-NIPenv5-virus was incubated with varying concentrations (0, 0.1, 1.0 and 10.0µg/ml) of NIP.BSA and the resuspended viral pellet examined by electron microscopy for virus agglutination. At 10µg/ml NIP.BSA, numerous large aggregates of MoMLV particles with typical morphology[24] were observed (Fig. 3). Individual virions were closely apposed with a relatively uniform interparticle distance (6–20 nm), indicating crosslinking by NIP.BSA. Similar aggregation was not observed in the absence of NIP.BSA, nor with control psi2-DCNeo supernatant.

Retrovirus particles displaying antibody fragments can package marker genes

As proof that the functional antibody fragment was displayed on infectious retroviral particles, the novel IRISA assay was developed (Materials and Methods). Filtered recombinant psi2-NIPenv5 virus was bound to NIP.BSA-coated plates, washed and NIH3T3 cells added to each well. The bound virus gave rise to G418 resistant colonies (Fig. 4 and Table 1), and the binding of the virus could be competively inhibited by soluble NIP.BSA

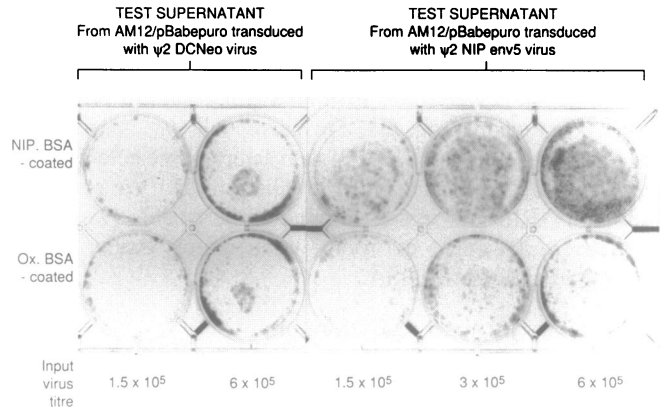

Figure 6. IRISA plate (5% Giemsa). Wells were coated as indicated and bound virus was detected by transfer of puromycin resistance to NIH3T3 cells. Virus expressed by an amphotropic producer cell line infected with psi2-NIPenv5-derived virus binds specifically to NIP.BSA-coated wells.

(Fig. 5). The virus did not bind to phOx-BSA-coated or uncoated tissue culture wells, nor did psi2-DCNeo virus bind to NIP.BSA. This indicates that the virus particles bind specifically to hapten, are infectious and can package a marker gene for transfer into mouse fibroblasts.

Retroviral particles displaying antibody fragments can package the antibody V-genes

The amphotropic producer cell line GP+envAm12-BabePuro was infected with recombinant psi2-NIPenv5 virus and transfer of the hybrid B1.8scFv-MoMLV *env* fusion gene was confirmed by PCR analysis of high molecular weight DNA extracted from these cells (data not shown). As a demonstration of functional expression of the transferred *env* fusion gene, the virus particles expressed by these cells bound more efficiently to NIP.BSA-coated plates than to phOx.BSA-coated plates as indicated by subsequent transfer of puromycin resistance to NIH3T3 cells (Fig. 6). In contrast, GP+envAm12-BabePuro cells infected with Psi2-DCNeo virus gave no signal when analysed by PCR for the *env* fusion gene and expressed virus which did not bind specifically to NIP.BSA. This indicates that psi2-NIPenv5-derived virus particles encapsidate and transfer a functional gene encoding the functional B1.8scFv antibody fragment displayed on their surface.

DISCUSSION

We have shown that a functional antibody fragment can be displayed on the surface of a retroviral particle fused to its envelope protein, and that this confers novel binding specificity on the particle. $Pr85^{env}$, the initial translation product of the *env* gene forms oligomers, undergoes glycosylation and is proteolytically cleaved during its transport through the endoplasmic reticulum and Golgi apparatus to the cell surface where it appears as a small transmembrane C-terminal domain $p15(E)^{TM}$, linked noncovalently or by a disulphide bridge to a larger extracellular domain $gp70^{SU}$(ref 25). Here we fused the scFv fragment close to the N-terminus of $gp70^{SU}$ and we envisage that it is folded and displayed as a separate domain. This choice of fusion site may be important, as an attempt to replace the N-terminal domain of $gp70^{SU}$ with a functional IL2

polypeptide did not succeed[26]. Presumably it will prove possible to incorporate antibody fragments with different binding specificities, and indeed the restriction sites of pNIPenv were designed to facilitate the cloning of other scFv cassettes from filamentous phage vectors[4]. It may also be possible to display other functional nonviral polypeptides (growth factors, cytokines or T cell receptors for example) or short peptide sequences with a variety of receptor binding activities.

We showed that the virus particles that display antibody fragments could also encapsidate the genes of a marker (*neo*), and were infectious as shown by the transfer of G418 antibiotic resistance to murine cells from viral particles immobilised on NIP-BSA coated plates. The infectivity of these particles was expected since they incorporate both the antibody-envelope fusion protein and unmodified envelope protein which is also expressed abundantly in the retroviral packaging cell line. In principle such particles could be used for targeted delivery of a genetic marker to cells (see below). It is not known whether retrovirus can be assembled in which all the subunits of the viral envelope protein are fused to antibody, and if so whether the virus would infect cells.

We also demonstrated that virus particles displaying antibody fragments can encapsidate the genes (pNIPenv) encoding the antibody fragments on their surface and hence have potential as replicable display packages, as with phage antibodies[2]. (These virions could also have been selected directly by including a selectable marker gene on the pNIPenv plasmid). We suggest that it might be possible to evolve new viral tropisms (see below) using repertoires of antibody fragments or peptide sequences displayed on the virus, with cycles of infection and selection.

Retroviral particles displaying antibodies against cell surface antigens should bind preferentially to target cells expressing those antigens, and this may facilitate their infection. For some antigens, the binding of retrovirus-associated antibody fragments to cell surfaces is followed by membrane fusion between virus and target cell: streptavidin-linked biotinylated monoclonal antibodies have been used to link ecotropic retroviruses to the surface of nonpermissive human cells with subsequent transfer of the viral genome into the target cells[27]. This 'molecular bridging' approach was successful (but inefficient) when viral particles were coupled to human MHC class I and class II antigens[27], but not to the human transferrin receptor[28], and suggests that only a limited number of cell surface antigens can function as surrogate receptors for MoMLV particles. In an attempt to identify suitable surrogate receptors, we are currently generating ecotropic retroviral particles displaying antibody fragments against a number of target antigens present on human cells. NIP-derivatised human cells were tested as a model for targeted gene delivery, but became permissive for both modified (displaying an anti-NIP antibody) and unmodified ecotropic viral particles.

Retroviruses displaying antibody fragments might also be used to retain the retrovirus in the vicinity of a tumour, and thereby reduce the systemic spread of recombinant retroviruses. For example, it has been proposed to deliver genes encoding prodrug-activating enzymes to tumours by injection of retroviral vector-producer cells[29,30], and then to administer the appropriate prodrug to kill gene-transduced tumour cells and their untransduced neighbours. As a safety measure, the retroviral particles could be engineered to display antibody fragments directed against an antigen on the tumour cells to enhance their retention within the tumour deposit.

ACKNOWLEDGEMENTS

REH is supported by an MRC Training Fellowship and SJR by an MRC Clinician Scientist Award, with additional support from the Louis Jeantet Foundation and Kay Kendal Research Foundation. We thank C.Rada, R.Mulligan, O.Danos and R.G.Vile for gifts of reagents, and Jim Gray for preparation of electron micrographs.

REFERENCES

1. Stengelin S, Stamenkovic I and Seed B. (1988) EMBO J ., **7**, 1053–1059.
2. McCafferty J, Griffiths AD, Winter G and Chiswell DJ. (1990) Nature, **348**, 552–554.
3. Bass S, Greene R and Wells JA. (1990) Proteins: Struct Funct Genet., **8**, 309–314.
4. Marks JD, Hoogenboom HR, Bonnert TP, McCafferty J, Griffiths AD and Winter G. (1991) J Mol Biol, **222**, 581–597.
5. Hoogenboom HR and Winter G. (1992) J Mol Biol., **227**, 381–388.
6. Friedmann T. (1989) Science, **244**, 1275–1281.
7. Nabel EG, Plautz G and Nabel GJ. (1990) Science, **249**, 1285–1288.
8. Ferry N, Duplessis O, Houssin D, Danos O and Heard JM. (1991) Proc Natl Acad Sci USA, **88**, 8377–8381.
9. Rosenberg SA, Aebersold P, Cornetta K, Kasid A, Morgan RA, Moen R et al. (1990) N Engl J Med., **323**, 570–578.
10. Russell SJ. (1990) Immunol Today, **11**, 196–200.
11. Marsh M and Helenius A. (1989) Adv Virus Res., **36**, 107–151.
12. Young JAT, Bates P, Willert K and Varmus HE. (1990) Science, **250**, 1421–1423.
13. Shinnick TM, Lerner RA, and Sutcliffe JG. (1981) Nature, **293**, 543–548.
14. Danos O and Mulligan RC. (1988) Proc Natl Acad Sci USA, **85**, 6460–6464.
15. Cepko CL, Roberts BE and Mulligan RC. (1984) Cell, **37**, 1053–1062.
16. Hawkins RE, Russell SJ and Winter G. (1992) J Mol Biol., **226**, 889–896.
17. Mann R, Mulligan RC and Baltimore D. (1983) Cell, **33**, 153–159.
18. Markowitz D, Goff S and Bank A. (1988) Virology, **167**, 400–406.
19. Morgenstern JP and Land H. (1990) Nucleic Acids Res., 18, 3587–3597.
20. Graham FL and van der Eb AJ. (1973) Virology, **52**, 456–467.
21. Huston JS, Levinson D, Mudgett HM, Tai MS, Novotny J, Margolies MN et al. (1988) Proc Natl Acad Sci USA, **85**, 5879–5883.
22. Bird RE, Hardman KD, Jacobsen JW, Johnson S, Kaufman BM, Lee SM et al. (1988) Science, **242**, 423–426.
23. Hoogenboom HR, Griffiths AD, Johnson KS, Chiswell DJ, Hudson P and Winter G. (1991) Nucleic Acids Res., **19**, 4133–4137.
24. Dalton AJ, Haguenau F and Moloney JB. (1964) J Natl Cancer Inst., **33**, 255–275.
25. Weiss R, N Teich, H Varmus and J Coffin (eds). (1985) RNA tumour viruses, volume 2. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
26. Russell SJ. (1990) Recombinant viruses expressing lymphokine genes: Their construction and use to modulate growth of transplantable rodent tumours. PhD Thesis. London University.
27. Roux P, Jeanteur P and Piechaczyk M. (1989) Proc Natl Acad Sci USA, **86**, 9079–9083.
28. Goud B, Legrain P and Buttin G. (1988) Virology, **163**, 251–254.
29. Culver KW, Ram Z, Wallbridge S, Ishii H, Oldfield EH and Blaise RM. (1992) Science, **256**, 1550–1552.
30. Stone R. (1992) Science, **256**, 1513.