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Vaccinia Virus Recombinants: Expression Vectors and Potential Vaccines

M. MACKETT
Cancer Research Campaign
Department of Molecular Biology,
Paterson Institute for Cancer Research,
Christie Hospital NHS Trust,
Wilmslow Road, Withington,
Manchester M20 9BX, U.K.

1. INTRODUCTION

At the end of the 1970s and beginning of the 1980s when smallpox vaccination was coming to an end, vaccinia virus and poxviruses were set to become the forgotten family of viruses. Surprisingly, with the development of techniques to allow expression of foreign genes in vaccinia, the virus has enjoyed something of a renaissance. Indeed, the generation of vaccinia recombinants has almost become a cottage industry, with literally hundreds of foreign genes being inserted and expressed by the virus. A literature search on Medline using "vaccinia" and "recombinant" as keywords since 1983 reveals well over 1000 references. The vast majority of these describe one or more vaccinia recombinants, some describe 10 or more.

Eukaryotic growth factors, polymerases, ion channels, tumour markers, cell surface proteins, oncogenes, bacterial enzymes and structural proteins, protozoan surface proteins and well over 100 different virus gene products from all families of eukaryotic viruses have been expressed by vaccinia recombinants. This widespread use of vaccinia virus as a vector owes much to the ease of generating recombinants, the authenticity of the foreign gene product and the potential uses of the recombinants once available. For example, most of the genes of HIV-1 (human immunodeficiency virus), HIV-2 and SIV (simian immunodeficiency virus) have been introduced into vaccinia. The utility of the vector system is illustrated by the expression of the HIV-1 envelope glycoprotein (gp160). The glycoprotein will fuse CD4 positive cells (1), assembles into higher order structures (2, 3), is cleaved into gp120 and gp41 (4, 5) and is transported to the cell surface, all features of the authentic glycoprotein. gp160 expressed by vaccinia is antigenically indistinguishable from the authentic glycoprotein (6) and recombinants or fixed recombinant infected cells have been used to immunize animals and humans (see Tables VI and VII) (7–13). The nature of the cytotoxic T-cell response and the specificity of antibody-dependent cellular cytotoxicity in HIV-1 infection has also been analysed using vaccinia recombinants (6, 14–17). Furthermore, a variety of recombinants expressing variants of gp160 have been generated in order to study CD4 binding, glycosylation, oligomerization, cleavage and antigenicity of the altered molecules (18, 19). The cleavage of gp160 into gp41 and gp120 has also been shown to be achieved by the cellular protease furin. This was demonstrated using vaccinia recombinants expressing furin or gp160. Co-infection of cells with the

TABLE I Genes Expressed by Vaccinia Virus Encoded T7 RNA Polymerase in a Transient Assay

Gene	Refs
Rat 2A sodium channel α-subunit	103
Drosophila shaker K^+ channel	103–105
Mouse muscle nicotinic acetylcholine receptor (AChR)	103
Rat brain 5HT1C receptor	103
Human serotonin 1A receptor	106
Human cystic fibrosis membrane conductance transport regulator	82
Dengue virus proteins and deletion derivatives	84
Influenza M1	172
Pro-opiomelanocortin	173
Insulin growth factor/insulin receptor hybrids	174
Varicella zoster virus gpI and gpIV	175
Hepatitis C virus polyprotein and deletion derivatives	176
Bunyamwera virus L protein	177
γ-Aminobutyric acid transporter	178
Sindbis virus proteins	179
Vesicular stomatitis virus N, P, L, M and G	83
Human immuno deficiency virus type-1 gp41	180
Murine coronavirus haemagglutinin–esterase	181
Parainfluenza virus F and HN	182
CD4 (T cell surface marker)	79

two recombinants resulted in complete cleavage of gp160 into gp120 and gp41 (20). The cleavage could also be inhibited by chloromethylketone, a known inhibitor of furin.

The possibility of using vaccinia recombinants as live vaccines has aroused a great deal of interest and some controversy. This idea has arisen because of the role that vaccinia played in the smallpox-eradication campaign. Vaccinia was an effective vaccine partly due to its ease of manufacture and its low cost coupled with its simplicity to distribute and administer. Recombinant vaccinia viruses can be generated that express antigens derived from pathogenic organisms and in many instances they are able to protect against the pathogen. For example, vaccinia recombinants expressing the rabies virus glycoprotein protect foxes (21), racoons (22) and skunks (23) against challenge with rabies virus even if the vaccine is presented in baited food (24). Field trials of this recombinant using baited food in Belgium have been very successful (24) and other countries have set up similar vaccination programmes.

This chapter examines the improvements in technology over the past 10 years, the wide variety of experimental uses recombinants have been put to and progress in using the live recombinants as vaccines.

TABLE II Virus Genes Expressed by Vaccinia Virus Recombinants

Virus	Gene expressed	Post-translational Modification*	Cell location or biological activity	Refs
Herpes simplex virus type 1	gB	G	Surface	184–187
	gC	G	Surface	188–190
	gD	G	Surface	143, 150, 184, 185, 189, 191–195
	gE	G	Surface	196
	gG	G	Surface and nuclear membranes	197
	gH	G	Surface only if co-expressed with gL	198, 199
	US 7 (gI)	G	Surface and nuclear membranes	196, 200
	gL	G	Surface only if co-expressed with gH	199
	ICP0		Nucleus – transactivates expression of transfected marker genes	201, 202
	ICP4		Nucleus – transactivates	201, 202
	ICP 27			203
	Thymidine kinase		Active enzyme	45, 49, 204–206
Herpes simplex type 2	gB	G	Surface, multimers formed	207
	gD	G	Surface	207
	vp16 tegument protein			207

Bovine herpes virus	gI, gIII	G	Surface	208, 209
	gIV	G	Surface	210, 211
Equine herpesvirus 1	gB	G	Surface and forms oligomers	212
	gp13 (gC)	G	Surface	213
	gp14	G	Surface	214
Pseudorabiesvirus	gp50 (gD)	G	Surface	215, 216
	gp63, gI, gX	G	Surface	216
	gII, gIII	G, proteolytically processed	Surface	217
Murine cytomegalovirus	IE pp89	P	Nucleus	218, 219
	gB	G	Surface	220
Epstein–Barr virus	gp340	G	Surface	85, 221
	gp78/55	G	Surface	222
	BZLF1		Nucleus, transactivates	223
	EBNA2A, B		Nucleus	224, 225
	EBNA3 (3a)		Nucleus	
	EBNA4 (3b)		Nucleus	
	EBNA5 (leader protein, LP)		Nucleus	
	EBNA6 (3c)		Nucleus	
	LMP1		Surface	
	LMP2		Surface	

TABLE II *(Cont)*

Virus	Gene expressed	Post-translational Modification*	Cell location or biological activity	Refs
Varicella Zoster virus (VZV)	IE62			226–228
	gI	G	Surface	175, 227, 228
	gIV	G	Surface	175, 228
	gV	G	Surface	228
	gene 61 (vMW 120)		Zinc binding protein	229
Cytomegalovirus (CMV)	IE 72Kd		Surface	230
	gB	G	Surface	231
	gH	G	S, Surface only if co-expressed with UL115	232, 233
	UL115	G	S, Surface only if co-expressed with gH	233
HHV6	gH	G	S, Surface only if co-expressed with gL	234
	gL	G	S, Surface only if co-expressed with gH	234

*In many cases, glycosylation and correct transport of the foreign gene implies that cleavage of a signal peptide has taken place.
G, glycosylation; P, phosphorylation; S, secretion.

2. CONSTRUCTION OF RECOMBINANTS

For detailed reviews of the biological properties of vaccinia virus see ref. (26). Vaccinia virus is a large double-stranded DNA virus with a genome of nearly 200,000 base pairs. The entire sequence of the Copenhagen strain and much of the WR strains is available (27) and shows that the virus has approximately 200 densely packed genes coding for a wide variety of gene products. These genes range from a multigenic RNA polymerase (28), a thymidine kinase (29), a DNA polymerase (30) and uracil DNA glycosylase (31) to genes with homologies to the interleukin-1β (IL-1β) receptor (32, 33) and even genes with homology and activities similar to those of complement control proteins (34, 35, 329). A number of these genes are involved in several different mechanisms designed to avoid recognition and elimination by the host immune system (reviewed in ref. (118).

Virus particles have a brick-like or oval morphology composed of an inner dumbell-shaped core surrounded by an outer protein coat. The core contains many virus-coded enzymes allowing the virus to synthesize functional capped, methylated and polyadenylated mRNA from a vaccinia template. This ability, along with other virus-coded enzymes such as a DNA polymerase allow the virus to replicate exclusively in the cytoplasm of the infected cell. Unlike other DNA viruses that replicate in the nucleus vaccinia DNA is non-infectious and the virus uses unique regulatory sequences with an apparent absence of RNA splicing.

2.1. Plasmid Insertion Vectors

To simplify the construction of recombinant viruses expressing foreign genes, a series of plasmid vectors have been developed in a number of laboratories. Some of those available are shown in Fig. 1. The essential features of the basic insertion vector plasmids are a vaccinia promoter (mRNA initiation site devoid of translational initiation site) adjacent to convenient restriction enzyme sites flanked by vaccinia DNA (often the vaccinia virus thymidine kinase (TK) gene). The gene to be expressed is cloned in one of the restriction enzyme sites and the whole construct transferred to vaccinia virus by homologous recombination in infected cells. In the recombinant virus transcription will start at the authentic vaccinia mRNA start site and translation will initiate at the site provided by the foreign gene. cDNAs need to be used as there is no evidence that vaccinia will splice genes.

The original insertion vectors suffer from several limitations mainly due to difficulties in identification of recombinants against a high background
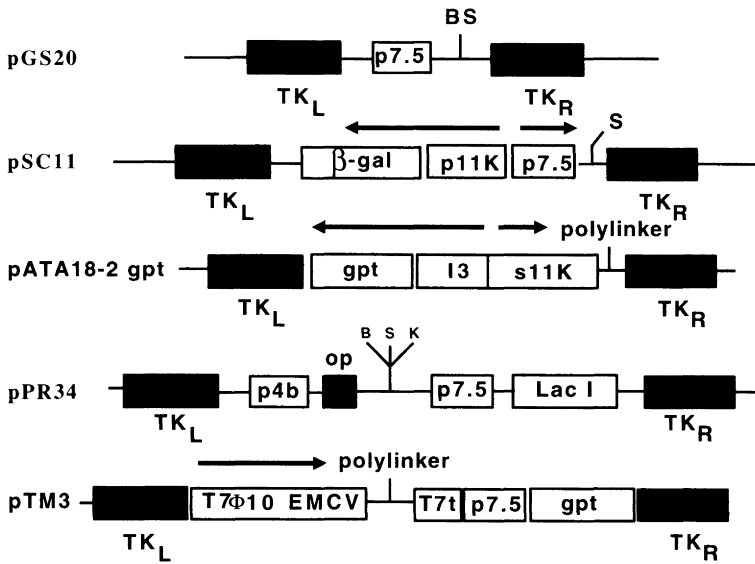
Fig. 1. Vectors for insertion of foreign genes into vaccina virus. All the vectors shown here insert into the thymidine kinase (TK) gene of the virus. pGS20 (50), pSC11 (36). Shaded boxes indicate the left-hand (L) or right-hand (R) region of the TK gene. Arrows indicate the direction of transcription of the various units. p11K is a promoter for a late structural gene (51) and s11K is an engineered version of the promoter (52), p7.5K is a promoter that is active before and after DNA replication (53), I3 is an intermediate gene promoter in the Hind III I fragment of vaccinia, 19K is an early promoter located within the inverted terminal repeats of vaccina virus, T7φ10 is the bacteriophage T7 capsid gene promoter and T7t is the terminator from the same gene. Expression from these promoters is directed by T7 RNA polymerase provided by a second recombinant virus (54). EMCV is the encephalomyocarditis virus ribosome entry site (55). β-gal is the E. coli β-galactosidase gene allowing recombinant virus to be detected on the basis that it co-expresses the enzyme which in the presence of the X-gal will give a blue plaque. gpt is the E. coli guanine phophoribosyltransferase gene which confers resistance to MPA and allows recombinants expressing the gene to be plaqued in the presence of the MPA. S (SmaI), B (BamHI) and K (KpnI) are sites for the restriction endonucleases indicated. pATA 18-2 gpt originated in Dr H. Stunneberg's laboratory (EMBL, Heidelberg).

(often 1000-fold excess) of parental wild-type non-recombinant virus. If the TK locus is used then a TK negative phenotype can be used to select recombinants because of their ability to grow in the presence of 5-bromodeoxyuridine. However, as spontaneous TK mutants arise with a frequency of 1 in 10^3 to 1 in 10^4, a DNA-based screen to distinguish recombinants is necessitated. This can take the form of a PCR for the foreign gene on DNA isolated from a single plaque or Southerns of DNA isolated from small monolayers of cells infected with the progeny of a single plaque.

Insertion into non-TK sites was originally dependent on screening for recombinant DNA. However, co-insertion vectors are now available which allow insertion of a marker gene or a gene allowing dominant selection along with the gene of interest in any non-essential region of the virus genome. Figure 1 shows some of these co-insertion vectors using either *Escherichia coli* β-galactosidase (*36, 37*) or gpt (*38, 39*).

Additional, less frequently used, methods for generating recombinants include co-insertion of the gene of interest with the neomycin resistance gene and selection with G-418 (*40, 41*), co-insertion of the gene of interest with the hygromycin resistance gene and selection with hygromycin (*42*), selection based on host resistance gene (*41*) where the parent virus will not grow on a particular cell line and the plasmid used to generate the recombinant restores the parent virus growth properties and plaque morphology (*43*) where the parent virus gives a small plaque phenotype which is restored by the transfected plasmid.

The site of insertion is directed by the vaccinia DNA in the insertion vector and obviously must be from a non-essential region of the genome if infectious virus is to be produced. Several regions have been used to generate recombinants including a region towards the left hand end of the virus genome that is deleted in some mutants (*44*), and internal position within the *Hind* III F fragment of the WR strain of virus (*45*), the virus haemagglutinin gene (*46*), the 19K EGF homologue gene (*47*), a variety of other positions (see ref. (*48*)) as well as the TK gene (*49, 50*).

The choice of insertion vector is crucial as this will determine both the time and level of expression as well as the site of insertion into vaccinia virus. The most important criteria on which to base the decision is the use which the recombinant will be put to. It may not be necessary to make a recombinant since the transient system produces reasonable levels of protein and may be all that is required to study the protein of interest. If high levels of expression are required then either a late structural promoter, e.g. the 11 kDa promoter (*51*) or the T7 system (*54, 55*) should be used. Under some conditions foreign genes expressed by late promoters will not stimulate cell-mediated immunity or act as targets for cytotoxic T-cells (*56*).

A complication for expression of foreign genes can be the presence within the foreign gene coding sequence of signals that terminate early gene transcription in vaccinia. Thus, if the gene is expressed under control of an early promoter premature termination occurs at a TTTTTNT sequence and the protein will not be produced. HIV-1 gp160 has two such sequences and it was shown (*57*) that if the termination signals were mutated while conserving the coding sequence, increased levels of gp160 were produced from the early–late 7.5 kDa promoter and the virus produced higher levels of anti-gp160 antibody when immunizing mice.
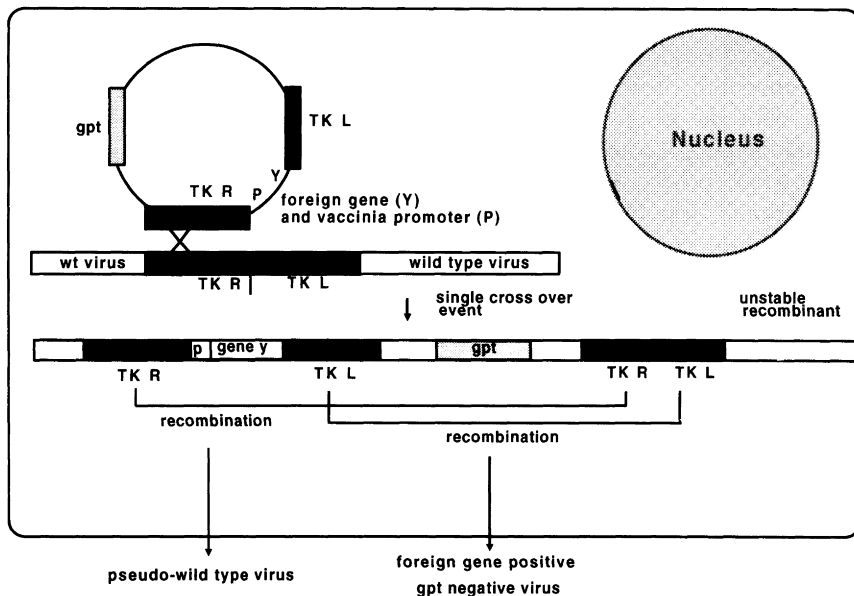
Fig. 2. Selection of recombinant viruses by transient dominant selection. The plasmid contains the vaccinia virus thymidine kinase (TK) gene interrupted by a hypothetical foreign gene Y driven by a vaccinia virus promoter (P) with the *E. coli gpt* gene located distal to the vaccinia virus sequences. When recombination between the plasmid and virus genome occurs by a single crossover the whole plasmid is inserted into the virus genome. The virus is unstable because of the presence of direct repeats and resolves to either WT virus or a recombinant with gene Y inserted within the TK locus. All three types of virus genome are viable and can be packaged, but only virus containing the *gpt* gene can form plaques under selective conditions.

2.2. Transient Selection

Two methods have been described which allow selection of recombinants based on the transient expression of a marker gene. The first method (58) was described using β-galactosidase and involves flanking the marker gene with direct repeats. A plasmid insertion vector containing the 7.5 kDa gene driving expression of β-galactosidase, followed by the same promoter driving the measles fusion protein gene was constructed. Recombinant fowl poxviruses were isolated by plaquing in the presence of X-gal. As recombination between the repeats in poxviruses is highly efficient, further passage resulted in the loss of the β-galactosidase. The recombinant virus still contains the measles fusion gene, but if recombinants expressing a second gene were required then β-galactosidase could be used as a marker for co-selection of the recombinant expressing both genes.

The second method, referred to as transient dominant selection (59), involves the construction of a plasmid transfer vector where the *E. coli gpt*

gene is linked to a promoter and is placed distal to vaccinia virus DNA which contains the gene of interest driven by a second promoter. After transfection of this plasmid into infected cells MPA-resistant viruses are selected. Resistance to MPA can only be acquired if a single cross-over event occurs (see Fig. 2). Because this virus contains direct repeats at the site of insertion it is unstable and on passage loses with similar efficiencies (depending on the relative lengths of DNA from vaccinia flanking the genes) either the *gpt* gene or the whole plasmid. DNA from plaques can be screened by PCR for which of the two events has occurred. The major advantage of this is that the final virus does not contain the *E. coli gpt* gene and, therefore, it may be used sequentially to make successive genome alterations at different loci. Even if multiple mutations are not needed it is desirable under some circumstances not to have the marker present, e.g. if the virus were to be used as a vaccine. A further useful situation occurs if the virulence of particular viruses is to be compared. As sister viruses, recombinant and wild type, are generated from the same parent and therefore the wild type virus is an ideal control for the recombinant virus.

2.3. Reverse *gpt* Selection (*60*)

This method is useful for eliminating *E. coli gpt* from a vaccinia recombinant. This may be desirable if dominant selection with gpt is to be used at another chromosomal locus or if a pseudo-wild-type virus is required as a control for a recombinant that has a deleted or mutated gene. The method relies on the inability of vaccinia recombinants expressing *E. coli gpt* to plaque in the presence of 6-thioguanine (6TG). Hypoxanthine phosphoribosyltransferase (HPRT) negative lines need to be used for the plaque assay as HPRT will incorporate 6TG into DNA, a toxic event. If a plasmid which by homologous recombination would delete *gpt* is transfected into a cell line infected with the *gpt*-expressing virus, progeny virus can be screened for the loss of *gpt* simply by plaquing in the presence of 6TG.

2.4. DNA Reactivation as a Method for Generating Recombinants

Although poxvirus DNA is non-infectious it is possible to reactivate denatured poxviruses by homologous or heterologous helper poxviruses. This phenomenon reported decades ago (*61*) was dependent on intact virus substructures and was not achieved with naked DNA until 1980 (*62*). Recently, it was shown that fowlpox-virus-infected cells can package vaccinia DNA and rescue infectious vaccinia virus from the DNA (*63*). This allows direct insertion of foreign DNA into vaccinia virus if restriction enzymes are used that cleave vaccinia DNA at a single site. The system
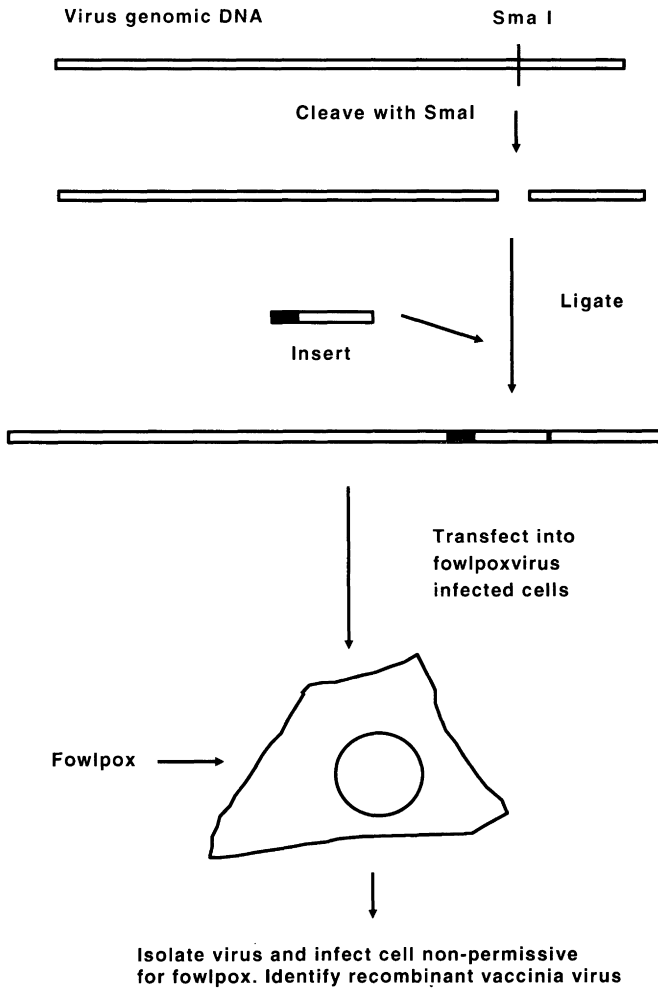
Virus genomic DNA   **Sma I**

Cleave with SmaI

Ligate

Insert

**Transfect into
fowlpoxvirus
infected cells**

Fowlpox ⟶

**Isolate virus and infect cell non-permissive
for fowlpox. Identify recombinant vaccinia virus**

Fig. 3. Generation of recombinants from virus DNA. Isolated vaccinia DNA is cleared with a restriction enzyme that has a single site within the virus genome (e.g. Sma1). A foreign gene is placed under control of a vaccinia promoter (insert) and ligated together with the cleaved genomic DNA. The ligation reaction is transfected into fowlpox infected cells where recombinant DNA can be packaged. Recombinant virus is then grown on a cell line in which fowlpox cannot replicate, i.e. any non-avian line. Recombinant virus can be distinguished from wild-type virus by analysis for foreign gene DNA or expression.

(Fig. 3) is similar to the use of λ phage arms followed by ligation with the insert/library and packaging *in vitro*. In the vaccinia case the arms are those of vaccinia and the packaging occurs *in vivo* due to the helper fowlpox virus. The advantage of this system is that it eliminates the need for cloning into insertion vectors and offers potential to clone large fragments or toxic

genes that could not be cloned in bacteria, as well as the possibility of generating eukaryotic expression libraries.

3. CONTROL OF THE EXPRESSION OF FOREIGN GENES

3.1. Natural Promoters

The kinetics and level of expression of a foreign gene depend primarily on the promoter chosen to drive expression. In general, late promoters for structural genes are stronger than the early promoters. When the 11 kDa late structural gene was used to drive the β-galactosidase gene the yields of recombinant product were in the order of $1-2\,mg^{-1}/l$ of infected cell culture (64). The 7.5 kDa promoter gives a 10- to 20-fold lower level of β-galactosidase than the 11 kDa promoter (64). (For further features of vaccinia virus transcription and promoters see refs. (65) and (66). Several other vaccinia promoters have been used to drive foreign gene expression. These include the 19 kDa early promoter, the 4 b late structural promoter, the cowpox inclusion body protein promoter and several synthetic promoters, e.g. H6 (41). The vast majority of genes, however, have been expressed by the early–late 7.5 kDa promoter. Although absolute levels of any foreign gene product will vary depending on mRNA and protein stability, the information available on these various promoters allows the time of expression and approximate level of product to be chosen.

3.2. Bacteriophage Promoters and Prokaryotic Control Elements

Significant yields of around $500\,\mu g^{-1}/l$ and in some cases more, of protein such as HIV-1 gp160, have been achieved using the T7 RNA polymerase system developed by Moss and colleagues (54). Two viruses are used: one expresses the T7 RNA polymerase gene and the other contains the foreign gene under control of a T7 promoter and transcription terminator. Expression of the foreign gene occurs only in cells infected with both viruses. The principle of using two viruses, one expressing the prokaryotic polymerase and the second containing a target gene under control of the appropriate promoter, has been extended to both bacterio-phage SP6 RNA polymerase (67) and bacteriophage T3 RNA polymerase (68) with similar results.

This system has also been used on a large scale to produce HIV-1 gp160. A 40 l fermenter containing 2×10^{11} cells on microcarriers was dually infected with the T7 RNA polymerase recombinant and a recombinant with the HIV-1 gp160 gene under control of the T7 promoter (69). After a four-step purification protocol, 50 mg of gp160 was recovered (an 11% yield).

A further refinement of the system improves the levels of protein produced was achieved by including the ribosome entry sequence of encephalomyocarditis virus (EMCV) in the untranslated leader of the mRNA produced from the T7 promoter (55). This allows cap-independent translation of the mRNA and circumvents the difficulties in translating the uncapped mRNA produced by the promoter (70). In one case the foreign gene product produced accounted for 10% of total cell protein (55). The basic T7 system gives levels of product approximately equal to those derived from the 11 kDa promoter; however, inclusion of the EMCV ribosome entry site improves levels by a factor of between 5 and 7. Several laboratories have reported transfer vectors that simplify the construction of these recombinants (71).

A second type of controlled expression derived from prokaryotes has been transferred to vaccinia. Unlike the T7 system the inducible *lac* repressor/operator system from *E. coli* is incorporated into a single virus (72–74). The advantage of this modification is that it allows inducible expression of the foreign gene. One recombinant virus is generated which expresses the *lac* I repressor and contains the gene of interest under the control of a hybrid *lac* operator/vaccinia promoter. Expression of the foreign gene can be induced by the addition of IPTG to cells infected with both viruses.

A problem that has taken some time to solve is the incorporation of the T7 polymerase gene into a virus that contains a T7 promoter. The reasons for this failure are not clear; however, the problem has recently been solved by placing the polymerase under control of a very weak promoter (75) or by using the lac I inducible system and only inducing T7 polymerase expression when required (76).

A further advantage of using the basic two-virus system is that toxic genes can be expressed. For example, the vesicular stomatitis virus (VSV) M gene could not be expressed using the conventional vaccinia vectors and promoters. Replacing the 7.5 kDa promoter for the T7ϕ10 promoter allowed a recombinant to be made which did not express the M gene. Dual infection of cells with this recombinant and the T7 RNA polymerase recombinant allowed the otherwise toxic VSV M gene to be expressed and analysed (77).

4. TRANSIENT EXPRESSION SYSTEM

A vaccinia virus recombinant expressing the bacteriophage T7 RNA polymerase gene has been used extensively in a transient assay system. Cells infected with a recombinant vaccinia virus expressing the T7 RNA polymerase gene are transfected with a plasmid containing the gene of interest under control of the T7ϕ10 promoter and terminator. The T7 RNA

polymerase transcribes the gene of interest from the input plasmid and terminates at the terminator sequence supplied in the plasmid. Sufficient protein is made from the transcripts to allow a number of biological assays to be undertaken. Analysis of the CD4 domains involved in HIV gp160 interaction was undertaken using this system. CD4 and truncated versions of the molecule were expressed and immunoprecipitated with anti gp160 or anti-CD4 antisera (78). Truncated molecules with the ability to bind gp160 were identified and the binding domain mapped to a region of CD4. Studies on the transport of CD4 (79) and its association with p55^{LCK} have also proved fruitful (80). Vectors are available with or without the EMCV ribosome entry site which improves the translatability of the foreign gene containing transcripts (81).

A number of integral membrane proteins involved in ion transport have also been expressed in the transient system and shown to produce faithfully their biological activity. The cystic fibrosis transmembrane conductance regulator expressed by the T7 system was shown to correct defective chloride channel regulation in cystic fibrosis airway epithelial cells (82). The most impressive result achieved with the system was the production of defective VSV particles by co-transfection of vaccinia T7 RNA polymerase recombinant infected cells with five different T7ϕ10 VSV gene constructs, all of which were required for the particles to be formed (83).

The system also has the advantage that only one recombinant virus is needed, i.e. the T7 RNA polymerase virus. All other manipulations can be undertaken in plasmids and the activity of the mutated genes can be analysed without the time consuming effort of making viruses for all mutants. For example, the protease activity of the NS2B gene of dengue virus type 4 was studied using the transient system on a series of deletion mutants where the assay was self-cleavage of the NS2B-NS3 junction. A 40 amino acid hydrophobic region in the middle of the gene, conserved among flaviviruses, was identified as essential for protease activity (84).

5. ANALYSIS OF THE FUNCTIONAL ACTIVITY OF FOREIGN GENES

5.1. Processing and Transport

Tables II to IV illustrate the large number of foreign genes successfully expressed by vaccinia viruses. A full version of Table II including all the virus genes expressed from all families of viruses instead of the herpesvirus family shown would extend to many pages. Table III gives a flavour of the different types of non-virus gene product that has been expressed.

Foreign proteins made by vaccinia recombinants are in general of

TABLE III Non-virus Origin Genes Expressed by Vaccinia Virus Recombinants

Gene	Refs
Preproenkephalin, prothrombin, proinsulin	235–237
Poliovirus receptor	238
Drosophila shaker K$^+$ channel	103
Nerve growth factor	239
Fish nerve growth factor and brain derived neurotrophic factor	240, 241
Oct 1 transcription factor	242, 243
MHC class 1 Kd	244
p53 wild type and p53 mutants	245
Rab1 and Rab2 Ras related proteins	246
Retinoblastoma protein	247
Carcinoembryonic antigen, 180Kd glycoprotein	248
Acidic FGF1	249
Human retinoic acid receptor γ-1	250
Hybrid insulin receptors	174
Cystic fibrosis transmembrane conductance regulator (CFTR) and deletions/mutants	82
Cytochrome P450s (over 15 papers and many isoforms)	e.g. 251
Cytochrome B5	252
LFA3 adhesion molecule	253
IL-1	152
IL-2	46, 153–155, 254
IL-6	157
GM-CSF	255
Ovine leutenizing hormone	256
Human chorionic gonadotrophin	257
Mycobacterium HSP65	258
Egg sperm receptor protein ZP3	259
Streptococcal M protein	260, 261
T1 secreted glycoprotein (member of Ig superfamily)	262
Myelin proteolipid protein	135, 263
E. coli Lac 1	72–74
Plasmodium falciparum and *P. Bergii* circumsporozoite major surface protein	89, 264, 265
Plasmodium falciparum secreted and anchored S proteins and RESA	159, 266
Human blood clotting factors VIII, IX and mutants and XII with deletions	87, 267–269
von-Willebrand factor	270
γ-Interferon	271
Antibody heavy and light chains	272
p97 glycoprotein expressed on melanoma cells	273–275

TABLE IV Enzymes Expressed by Vaccinia Recombinants

Gene	Refs
E. coli β-galactosidase	36, 37
E. coli guanine–phosphoribosyl transferase	38, 39, 60
E. coli galK	276
Chloramphenicol–acetyl transferase	50, 277
Neomycin resistance gene	40
Hygromycin resistance gene	42
Firefly luciferase	278
72 kDa Gelatinase	279, 280
Phospholipase c and isoforms. Phospholipase A2	281, 282
Furin	86, 236
PC2 protease	236
PC3 protease	236
Steroid 21 hydroxylase	282
Bovine dopamine β-hydroxylase	283
Adenovirus DNA polymerase and pTP	199
Bacteriophage SP6 RNA polymerase	67
Bacteriophage T3 RNA polymerase	68
Bacteriophage T7 RNA polymerase	54, 55

the predicted size and, as a rule, undergo faithfully the authentic post-translational modifications. Glycosylation (85), proteolytic cleavage (84, 86), γ-carboxylation (87), phosphorylation (88), and myristilation (88) of foreign genes have all been reported. For example, Epstein-Barr virus (EBV) gp340 from vaccinia recombinant infected cells and EBV transformed lymphocytes are glycosylated to a similar extent, bind CR2 and are similar molecular weights on polyacrylamide gels (85).

A few exceptions have been reported where modification depended on the cell line that recombinants were grown in or on enzymes produced by the parent organism. For example, the malaria sporozoite stage major surface antigen expressed by vaccinia gave a product of a different size to the authentic antigen, presumably because post-translational modification by the parasite was not carried out (89).

The vast majority of foreign proteins expressed by vaccinia are transported normally and detected at the appropriate subcellular location. Nuclear, cytoplasmic and cell surface transport as well as secretion have all been described. The most striking example of authentic transport in recombinant infected cells was the demonstration that the correct polarity of transport is maintained (90). Influenza A virus haemagglutinin was directed to the apical surface of recombinant infected MDCK cells, whereas murine leukaemia and VSV envelope proteins were directed to the baso-lateral surface of MDCK cells. Protein sequences determining the polarity of expression of particular glycoproteins have also been investigated.

Truncation of the gp70/p15E protein of Friend mink cell focus inducing virus followed by expression of the modified genes in vaccinia revealed that the polarity of expression was determined by the p15E portion of the molecule (*91*).

5.2. Engineered Products

One of the distinct advantages of the vaccinia system is the ability to express genetically engineered genes. Biological activity of variant proteins can then be studied and structural functional relationships established. For example, the effects of mutations in single N-linked glycosylation sites in HIV gp160 were analysed in our laboratory and in others using recombinants expressing mutant forms of gp160. The analysis showed that the absence of a single glycosylation site can diminish or completely abolish the ability of HIV gp160 to fuse CD4 positive cells (M. Carroll, unpublished results) (*92*).

It may also be possible to increase the immunogenicity of a gene product by simple modifications to the primary coding sequence (see Section 7.1.3).

5.3. Interactions between Molecules

Interactions between molecules expressed by vaccinia recombinants can also be studied. If herpes simplex virus (HSV) glycoprotein gH is expressed independently of HSV either in vaccinia recombinants or in other expression systems it is trapped within the cell. It was suspected that another HSV glycoprotein, gL, acted as a "chaperone" promoting the correct folding and transport of gH. By expressing the genes in two different recombinants and co-infecting cells it was shown that this was the case (*93*). The interaction of HIV-1 gp160 with GRP 78-BIP has been investigated and a role for this protein in the transport and folding of gp160 has been proposed (*94*). In this case the BIP chaperone was endogenous and not expressed by a vaccinia recombinant.

Haffar *et al.* (*95*) first showed that HIV-like particles are released from recombinant vaccinia virus infected cells when the virus expresses HIV 1 *gag-pol*. Processed gag p55 protein was the major HIV protein found in cell lysates, although a small amount of p24 was also detected in the culture medium. Protein analysis of virus-like particles purified from the medium revealed the presence of both p55 and p24 proteins and an absence of reverse transcriptase activity. Electron microscopy of the released particles revealed a diameter of 100–120 nm with a cylindrical core structure. Other laboratories have also reported the detection of HIV-like particles released

from recombinant vaccinia-infected cell lines (96–98).

Subsequent to this first report co-expression of HIV *gag-pol* and gp160 from vaccinia recombinants was shown to produce virus-like particles that also contain gp160 (99, 328). These particles are highly immunogenic and would be good vaccine candidates if enough of the material can be produced.

Other virus-like particles have also been generated by vaccinia recombinants. These include HIV-2, [100], human papillomavirus (HPV) 16 [101] and HPV-1 [102]. The requirements for transport and processing as well as interaction between the molecules can all be analysed using site-directed mutagenesis of the expressed genes.

5.4. Receptor Molecules, Neurotransmitters and Ion Channels

One of the features of vaccinia virus vector systems is that they can infect many cell types. As a consequence molecules can be expressed in their natural context, even if highly specialized cell types such as neurones and muscle cells need to be used. Surprisingly, even *Xenopus* oocytes can be infected with vaccinia recombinants (103) and produce biologically active proteins. In recent years a large number of proteins involved in membrane excitability and signalling across membranes have been identified through molecular cloning and some of these have been expressed using vaccinia virus. Voltage-dependent ion channels (104, 105), neurotransmitters (103), G-protein coupled receptors (103) and other excitability proteins (78) have all been expressed. This indicates that it is possible to study membrane proteins that require cell-specific post-translational processing or association with cell specific subunits as well as those proteins that require coupling to endogenous second messenger pathways for full biological activity to be observed.

5.5. Enzymes

Table IV shows the diverse nature of enzymatic activities that have been expressed by vaccinia virus. For example, studies on the adenovirus DNA polymerase and terminal protein pTP are hampered because of the low abundance of the polymerase and pTP as well as their presence in adenovirus infected cells as a complex. The 11 kDa virus promoter over-expressed both the polymerase and pTP separately (30-fold greater than in adenovirus infected cells) and when combined they were fully active for initiation and elongation in an adenovirus DNA replication system (199).

Vaccinia recombinants can also be used to study the effect of enzyme inhibitors, e.g. HIV-1 protease inhibitors. A peptidomimetic proteinase inhibitor, U-81749 (107) was used on cells infected with a HIV-1 gag-pol

recombinant. The protease (part of the pol protein) is responsible for processing the gag precursors primarily into p55 to p24. Without the inhibitor p24 represented 31% of the total immunoreactive protein (using sheep antibody to p24), while 10 μM of the U-81749 reduced the level of p24 to 5% of total immunoreactive proteins (i.e. 84% inhibition). HIV-like particles recovered from inhibitor-treated infected cells revealed almost exclusively p55, with only trace levels of p24.

6. IMMUNOLOGICAL APPLICATIONS

6.1. Generation of Monoclonal Antibodies

Several reports have indicated that it is possible to generate monoclonal antibodies against the foreign gene product by immunization of mice with a vaccinia virus recombinant (108, 109). The main advantages of this approach are the presentation of antigens in a native form and the possibility of inducing immune responses to products normally present only in small amounts, e.g. virus regulatory proteins. Monoclonal antibodies against measles fusion protein (110), VSV nucleoprotein (108), and respiratory syncytial virus (109) have been produced in this fashion.

Human anti-HIV gp160 monoclonal antibodies have also been made by transforming B-cells with EBV from a donor who was vaccinated with a vaccinia recombinant expressing HIV gp160 (111).

A recombinant expressed product can also be used as part of the primary screen for monoclonal antibodies. McClean and colleagues (112) used a bacterial HPV-16 L1 fusion protein to prime mice and immunofluoresence on HPV-16 L1 recombinant infected cells as the primary screen for reactivity. Although rather laborious this method has the advantage of using in the primary screen a procedure for which the antibody was needed. Also, in the case of HPV-16 it would be very difficult to use a natural source of HPV-16 L1 protein.

6.2. Identification of the Targets for Cellular Immunity

6.2.1. Cytotoxic T-cells

Vaccinia recombinants have been valuable in determining the specificity of cytotoxic T-cells (CTLs) raised in natural or experimental infection. Individual genes from multigenic organisms can be expressed by vaccinia and these recombinants used as targets for CTLs in a classical chromium release assay. This has proved to be possible because of the ability of the recombinant viruses to express the foreign antigen on the surface of the

infected cell in conjunction with major histocompatibility complex molecules. These cells may be recognized and lysed by autologous CTLs directed against the foreign antigen (for a review see ref. (*113*)). This obviously requires processing of the foreign antigen which appears to take place in an appropriate manner. Interestingly, cells infected with a vaccinia recombinant expressing a 15 amino-acid peptide from the nucleoprotein of influenza A virus were lysed by CTLs specific for the peptide sequence (*114*). An example of the utility of vaccinia recombinants is seen in the analysis of CTL to influenza A virus. Individual recombinants expressing all 10 genes of influenza A virus (*115*) as well as several engineered genes have been generated and used to analyse CTL responses in animals and humans (*116, 117, 119–121*). These recombinants have helped establish that individuals previously infected with influenza A recognize matrix M1, polymerase PB2 and nucleoprotein in conjunction with MHC class 1 antigens (*88*). Furthermore, transfection protocols and the recombinant approach have indicated that influenza HA-specific CTLs induced in animals are mostly non cross-reactive and nucleoprotein is the major target for anti-influenza cross-reactive CTLs.

Recombinants which express truncated genes can also be used to generate targets for CTLs either using bulk cultures or T-cell clones. Thus specific epitopes within a protein that generate T-cell reactivity can be identified (*122, 123*). In the case of HIV-1 env, gag, vif, nef and reverse transcriptase-specific CTLs have been demonstrated in infected individuals (*17, 122, 124–129*) and a number of the epitopes recognized by T-cell clones mapped by a combination of the use of peptides and vaccinia recombinants expressing truncated genes. Since vaccinia can infect a very broad range of cell types a large variety of infected target cells may be tested in cytotoxicity assays and even studies on HLA restriction can be analysed (*130*).

Vaccinia recombinants can themselves prime and stimulate cell mediated responses to foreign gene products in vaccinated animals, e.g. influenza HA vaccinia recombinants primed CTLs in mice that recognize and lyse cells expressing the same HA subtype (*116*). This type of experiment has been done for a number of different viruses and in general the response to the foreign gene has parallelled the response to that gene in natural infection of the normal host. Some caution is required as it has been shown (*56*) that temporal regulation of the influenza HA expression in vaccinia can affect the immune response. HA produced early and late in infection is recognized by B- and T-cells and can serve as targets for CTLs, whereas HA synthesized only at late times in infection is not recognized by CTLs.

A more topical aspect of this priming was demonstrated when Zagury immunized himself with a vaccinia recombinant expressing the HIV-1 *env* gene. After re-stimulation by vaccination with his own fixed recombinant infected cells he demonstrated that the recombinant had primed a T-cell

mediated response to HIV-1 env (*131*). Other volunteers immunized with
the HIV-1 *env* recombinant and stimulated by several other procedures are
also primed for T-cell responses to HIV env (*11*). T-cells from 13 out of
16 individuals immunized with a vaccinia recombinant expressing the HIV
gp160 gene proliferated in response to one or more HIV gp160 preparation
confirming in humans that these recombinants can stimulate T-cell
responses (*7*).

6.2.2. Antibody-dependent Cellular Cytotoxicity

Several reports (*16, 132–134*) have shown that it is possible to analyse
antibody-dependent cellular cytotoxicity (ADCC) responses using vaccinia
virus recombinants. Both papers use recombinant infected cells as targets
for ADCC in a manner analogous to work done with cytotoxic T-cells.
Recombinant infected cells expressing HIV-1 env or gag were labelled with
chromium, incubated with the sera under test and monitored for lysis
following incubation with the appropriate dilution of effector cells. From
these studies it was shown that HIV-1 env is a target for ADCC, whereas
gag specific antibody-dependent cytotoxicity was not found. The ADCC
titre in the patients examined did not correlate with the stage of their
HIV-related illness. As neutralizing antibody does not have access to
intracellular virus, vaccines which stimulate antibodies against env and
stimulate ADCC may be important for protection against cell-associated
virus. Vaccinia recombinants are thus useful reagents in determining the
antigenic specificity of ADCC.

6.3. Models for Autoimmunity and Allergens

Recently, several recombinants have been described (*135, 136*) which
have been designed to express antigens which are the target of autoimmune
reactions. Thus animals vaccinated with the virus develop an auto-
immunity. This technology may be very valuable in the design and study
of possible therapeutic intervention strategies. It is also conceivable that
antigens from allergens could be expressed in vaccinia and used in the
design of experimental models of allergy.

In some cases the recombinants generated are a potential safety risk and
one would wish to evaluate carefully the possibility of autoimmune or
allergenic reaction in personnel handling the viruses and preferably use a
parent virus that cannot replicate in human cells.

TABLE V Protection Afforded by Immunization with Recombinant Vaccinia Viruses

Gene expressed	Experimental animal	Virus*	Protection**	Refs
HSV1 gB	Mouse	HSV1	High	183, 187
HSV1 gC	Mouse	HSV1	High	188
HSV1 gD	Mouse	HSV1	High	144, 191
		HSV2	High	
		HSV1	2/3	
MCMV IEpp89	Mouse	MCMV	High	284
MCMV IEpp89 peptide linked to HBcAg	Mouse	MCMV	High	285
Polyoma small T, middle T, large T	Fischer rat	$2 + 10^4$ tumour cells	O-High	286
Bovine leukemia virus envelope	Sheep	BLV	4/4	287
Pseudorabies virus gp50, gII and gIII	Mice and swine	PRV	High	51
Anthrax PA	Guinea pigs and mice	Anthrax	Partial	288
Japanese encephalitis virus NS1, NS2, E and pRM	Mice	JEV	High	289
Hantavirus N, G1 and G2	Mongolian gerbils	Hantavirus	Partial–High	290
Yellow fever virus pRM and E	Mice	YFV	High	291
LCMV 2 or 3 linked CTL epitopes	Mice	LCMV	High	292
Rinderpest HA, F	Cow	Rinderpest	High	167
Rinderpest HA	Rabbit	Rinderpest	High	168
FIPV Spike	Kittens	FIPV	Early death	293
VSV G	Mouse	VSV	High	294
	Cow	VSV	Low	294
Rabies G	Mouse	Rabies	High	295
	Fox	Rabies	High	21, 24
	Raccoon	Rabies	High	296
	Skunk	Rabies	High	23

*HSV1, herpes simplex virus type 1; VSV, vesicular stomatitis virus; BLV, bovine leukaemia virus; PRV, pseudo-rabies virus; JEV, Japanese encephalitis virus; RSV, respiratory syncytial virus; HTLV, human T-cell lymphocytic virus type 1; YFN, yellow fever virus; LCMV, lymphocytic chorio-meningitis virus; MCMV, murine cytomegalovirus; FIPV, feline infectious peritonitis virus.

**Many examples of protection achieved with vaccinia recombinants have been omitted. Those shown here illustrate the diversity of experimental systems used in protection experiments. For more detail the reader is referred to the specific reference quoted.

TABLE VI Immunization of Primates with Recombinant Vaccinia Virus

Gene	"Experimental animal"	Immune response		Outcome	Ref.
		Humoral	Cellular		
Epstein–Barr virus (EBV) gp340	Cotton top tamarins	0	ND	3/4 protected	221
	Cotton top tamarins	0	ND	No protection	221
Hepatitis B virus HbsAg	Chimpanzees	0	ND	High* level of protection	298
Parainfluenza virus HN,F	Patas monkeys	Virus neutralizing antibody	ND	High** level of protection	299
Dengue 2 virus C, M, e	Chimpanzees	0	ND	No protection	300
Simian type D retrovirus envelope glycoproteins	Macaques	+ Virus neutralizing antibody	ND	Protects against both SRV-1 and Mason–Pfizer monkey virus (SRV-3)	301
Respiratory syncytial virus surface glycoproteins	Chimpanzees	Moderate levels of poorly neutralizing antibody	ND	Poor protective efficacy (on challenge antibodies boosted to high levels)	302
Lassa virus glycoprotein	Rhesus monkeys	2/4 developed antibody	ND	4/4 protected 2/4 limited virus replication	303
Human IL-2 and influenza HA	Patas monkeys	+	ND	Attenuation of virus	156
Parainfluenza virus F or G	5 separate species	4/5 species developed neutralizing antibody at high level	ND	Owl monkeys protected	304

Construct	Animal model	Antibody		Comments	Ref.
Plasmodium falciparum RESA, MS1, MS2 and AMA1	Saimiri monkeys	No significant antibody	+	Failure to protect. On challenge antibody boosted to high level	264
HIV gp160	Chimpanzee	+	+	0†	305
HIV gp160	Macaques	+	+	–	306
HIV gp160 + IL-2	Macaques	+	ND	IL-2 co-expression appears to improve antibody response	307
Rabies virus glycoprotein	Squirrel monkeys, chimpanzees	+	ND	8/11 develop rabies virus neutralizing antibody	308
Simian type D retrovirus envelope glycoprotein	Macaques	+	+	Simian retrovirus neutralizing antibody, ADCC. 4/4 protected	309
HIV gp160, p25 or *nef* (some protocols include IL-2 in the recombinant)	Chimpanzee	+	+	Transient and weak antibody and T-cell reactivity even if IL-2 present	13
SIV gp160 and a boost with baculovirus derived gp160	Macaques	+	+	4/4 protected from infection as determined by PCR	327
SIV gp130 and boost with virus derived gp130	Rhesus macaques	+	+	Difference in B- and T-cell response to different vaccination regimes (see ref.)	119
SIV gp130 and a boost with gp130 protein	Rhesus macaques	Transient non-neutralizing	–	No protection	310

HIV-1, human immunodeficiency virus type 1; SIV, simian immunodeficiency virus; ND, not determined.

*Chimpanzees were protected against overt disease despite limited replication of the challenge virus.

**Virus replication was reduced in the upper and lower respiratory tract. Duration of virus shedding was also reduced.

†HIV was recovered from lymphocytes despite some reduction in lymphadenopathy.

TABLE VII Human Vaccination with Poxvirus Recombinants

| Virus/recombinant gene | Immunization procedure | Immune response | | | Outcome | Ref. |
		Humoral	Cell mediated			
Vaccinia strain Tian Tan expressing EBV gp340 from 11 kDa late structural promoter	All scarified. 11 vaccinia and EBV positive adults(A) 108 p.f.u./ ml^{-1}, 6 EBV positive vaccinia negative schoolchildren (B), 9 children under 3 (C) (B) and (C) 107 p.f.u./ml^{-1}	(A) no response to gp340 (B) anti-gp340 titres boosted (C) children develop anti gp-340 titres	ND		Partial efficacy (reported at meetings not in the reference)	312
Vaccinia WR expressing HIV gp160 from 7.5 kDa promoter	Scarified followed by boosting with fixed autologous vaccinia gp160 recombinant infected cells	Neutralizing antibody	CTL activity		Antibody and T-cell response	11
Vaccinia expressing HIV-1 gp160 from the 7.5 kDa promoter	(See detail in ref., boost with vaccinia gp160 infected autologous PBLs)	Transient and weak	Long-term memory response		2-year follow-up indicates CTLs produced	164
Vaccinia Wyeth expressing HIV-1 gp160 from the 7.5 kDa promoter	See ref. for details	2/31	13/16 proliferate to soluble gp160		Weak T-cell specific responses	7

As above, boosted by purified gp160	See ref. for details	Neutralizing antibody in 7/13	Good proliferative responses to gp160	Sustained high levels of antibody in 75% of recipients for > 18 months	8
As above, boosted by 640 μg baculovirus derived gp160	See ref. for details all vaccinia naive individuals	Neutralizing antibody in 8/12 (3/12 cross-neutralizing)	ND	High levels of antibody (higher than either vaccinia recombinant or protein on its own)	9
Vaccinia Wyeth expressing HIV gp160 from the 7.5 kDa promoter	See ref. for details	+	ND	Magnitude of antibody response similar to naturally infected laboratory workers with a qualitative difference	12
Canarypox expressing the rabies virus glycoprotein	See ref. for details	+	ND	9/9 high dose recipients produced good levels of rabies virus neutralizing antibody	166

TABLE VIII Immunization with Avipoxvirus Recombinants

Virus	Recombinant gene	Challenge virus	Outcome	Refs
Fowlpox	Infectious bursal disease virus (IBDV) VP2-β-galactosidase fusion	IBDV	Chickens protected from mortality not from damage to the bursa	313
	Avian influenza HA	Pathogenic avian influenza	Wing web vaccinated totally protected, comb scarified partially protected from lethal challenge	314–316
	Newcastle disease virus (NDV) Haemagglutinin-nueraminidase gene	NDV	Protection of chickens	317–319
	Fusion	NDV	Protection of chickens	320–322
	Mareks disease virus (MDV) gB or gB and pp38	MDV	Protection of chickens	323, 324
	Avian reticuloendotheliosis retroirus (ARR) envelope glycoprotein	ARR	Decrease in viraemia	326
	Measles virus fusion protein	Measles virus	Protection of mice from fatal encephalitis	325
Pigeonpox	NDV fusion	NDV	Protection of chickens	330
Canarypox	Feline leukaemia virus	FeLV envelope glycoprotein	Protection of cats	331
	Measles virus F and HA	Measles virus	Protection of mice from fatal encephalitis	332
	Rabies virus glycoprotein	–	9/9 high dose recipients produced good levels of virus neutralizing antibody	166

7. POTENTIAL VACCINES

7.1. Human Vaccines

The notion that recombinant vaccinia viruses might have value as live vaccines is based on the use of vaccinia virus in the smallpox-eradication campaign. A further impetus to the evolution of this approach to vaccination has been given by recombinant vaccinia viruses which induce neutralizing antibodies and protect animals against challenge with the appropriate pathogen. Tables V to VIII show a selection of the different recombinant viruses that have been used in protection experiments and the outcome of the protocols. Vaccinia recombinants have several advantages as vaccines, including the cheapness of the vaccine to manufacture, its ease of administration, stability without refrigeration, potency as a single inoculation, and its ability to stimulate humoral and cell mediated immunity. A further advantage stems from the flexible packaging capabilities of the vaccinia particle. Over 25 kbp of foreign DNA can be accommodated without compensating deletions in its genome (137). Consequently, it may be possible to express 10–20 different antigens and protect against more than one disease. It has already been shown that viruses expressing three different genes can be constructed and that antibody to all three foreign proteins was induced in vaccinated animals (138). It is also possible to elicit responses to engineered gene products with improved antigenicity (see Section 5.2).

Table VII summarizes the studies using poxvirus recombinants in humans and the various immunization schedules used. Although there is good reason to be optimistic about the long-term use of these recombinants it can be seen that the immunity induced by recombinants alone is probably not sufficient to confer immunity to HIV. The combination schedules look more promising. Some of the alternative approaches to improving immunogenicity are described in Section 7.1.3.

7.1.1. Obstacles to the Use of Vaccinia Recombinants as Vaccines

7.1.1.1. Complication Rates

Despite these advantages there is considerable resistance to the use of vaccinia recombinants, primarily because of low levels of well-documented adverse reactions to vaccination with vaccinia. In a study of 14.2 million vaccinations in the U.S.A. in 1968, there were 572 hospitalizations, nine deaths and many less severe complications attributable to vaccinia (139). The problems encountered ranged from abnormal skin eruptions to very rare disorders affecting the central nervous system. Severe adverse reaction

of 1 in 50,000 vaccinations are clearly unacceptable compared with other live attenuated virus vaccines such as polio and measles where complication rates are of the order of 1 in 1,000,000. Concern has also been expressed over the use of all live vaccines in countries where there is a high rate of immune suppression due to HIV infection. The fear is that disseminated vaccinia would be life-threatening as has been reported in a military recruit (140) who was vaccinated with vaccinia and subsequently died after it was shown he was HIV-positive. Several deaths in AIDS patients taking part in study using fixed vaccinia recombinant infected cells were probably due to disseminated vaccinia from incompletely inactivated virus (141, 142).

Autoimmunity may also occur unexpectedly. Syrian hamsters inoculated with a vaccinia virus expressing the rubella virus E1-E2 glycoprotein develop anti-pituitary antibodies (136).

7.1.1.2. Pre-existing Immunity

Another reservation that can complicate the use of vaccinia recombinants is the existence of immunity within the target population. This can be considered both as pre-existing immunity to vaccinia as well as to the expressed antigen. Replication of a vaccinia recombinant expressing the HSV type-1 glycoprotein gD and the influenza virus haemagglutinin was not inhibited by pre-existing immunity to either HSV or influenza (143). This suggests that pre-existing immunity to the foreign gene expressed by a recombinant is unlikely to be a problem. However, existing immunity to vaccinia is likely to decrease replication of any recombinant used for immunization and hence decrease its efficacy. Recently, acquired immunity to vaccinia has been shown to abolish efficacy in mice challenged with HSV of a recombinant expressing HSV glycoprotein gD (144). Recombinants expressing the major envelope glycoprotein of (EBV) (gp340) were used in volunteers in China. It was shown that those individuals previously immunized with vaccinia failed to produce antibody to gp340 (145). Similarly, in volunteer studies (7) in both the U.S.A. and France individuals previously immunized with vaccinia did not mount an antibody response to the HIV gp160 expressed by a vaccinia recombinant. The vaccinia naive individuals were primed to HIV as evidenced by the development of T-cell proliferative responses and serum antibody to gp160. Thus, although prior vaccination is a problem for adults, it is less of a problem for younger age groups as routine vaccination ceased over 15 years ago.

7.1.1.3. Levels of Immunity

Generally, in the experimental protocols used in small animals (see Table V) large doses of virus are used in a manner that gives the best

immune response possible. Although this establishes the principle that vaccination with the vaccinia recombinant can work, it is difficult to translate this into practice in humans. The lower efficacy rates reported in primates (see Table VI) is not just due to the value of primates and investigators needing to publish most experiments in primates. It reflects a real difficulty in generating protective responses possibly due to the fact that smaller doses of virus per kilogram of body weight have to be administered.

7.1.2. Improving Attenuation

Reservations over the use of vaccinia virus recombinants as a vaccine have provided an impetus to study possible ways of attenuating the virus and genes involved in virus pathogenicity.

7.1.2.1. Currently Available Attenuated Strains

During the smallpox eradication campaign there was sufficient concern over complications to encourage the development of attenuated vaccinia strains. In 1975, LC16m8, a derivative of the Lister vaccine strain, was licensed for use in Japan (*146*). This virus has been used to express hepatitis B virus surface antigen with similar results to recombinants based on the parent strain (*147*). One strain, CV1-78, was tested in 3000 eczematous children with no adverse effects (*311*). (Eczma is an acknowledged contraindication for vaccination with vaccinia.) MVA is a particularly interesting strain (*148*), it is multiply attenuated containing six major deletions totalling 31 kbp and will not replicate in human cells to give infectious virus. It has been used as a vaccine in 120,000 people with no side effects reported and has recently been used to generate recombinants (*149*).

7.1.2.2. Engineered Attenuation

In the past, attenuated strains were derived by long-term passage in tissue culture. Consequently the genes responsible for this attenuation, as in the case of MVA, have not been identified. It is possible to engineer specific deletions or insertions in the virus genome and hence provide a more rational approach to attenuation. It has been shown that inactivation of either the TK gene (*150*) or the 19 kDa epidermal growth factor (EGF) homologue gene (*47*) results in a marked reduction in pathogenicity. Although 19 kDa viruses grow well in tissue culture, they give poor yields in fertile hens eggs and are probably too attenuated to be used as vaccines. Many TK-minus viruses based on the laboratory strain WR have been used

in protection experiments (Tables V to VIII); however, when vaccine strains have been used the TK minus phenotype seems more drastic and fairly poor immune responses to foreign genes have been reported (Table VII).

A highly attenuated vaccinia virus mutant described by Esteban and colleagues (151) has several lesions, a deletion at the left hand end of the genome and point mutations in a gene controlling plaque size. This virus is perfectly viable and is able to induce antibody responses against a foreign gene expressed by the virus. A multiply attenuated strain (NYVAC) has been described (145) and shown to have some potential as a vaccine. However, the disadvantage of all these attenuated viruses is that they tend to compromise the immune response to the foreign gene and a long-lasting protective response will probably require more than one immunization. It is therefore necessary to balance the advantages of attenuation and possible benefits of a reduction in complication rates against a potential decrease in efficacy.

Another suggestion has been the use of self-inactivating vaccinia viruses that have host range genes deleted. These genes allow replication in human cells and viruses lacking these genes would only be capable of infecting cells and would not produce further infectious virus. Whether these viruses would be effective vaccines remains to be seen.

7.1.2.3. Incorporation of Lymphokine Genes

One possible way round this dilemma is to express genes which improve the immune response or decrease the complication rates. Several groups have generated recombinants which express IL-1 (152), IL-2 (46, 153–156), IL-6 (157) and γ-interferon (158). Under some circumstances the IL-1 recombinants induced better immune responses than their parent virus. The IL-2 recombinants were highly attenuated with respect to their parent viruses when used to vaccinate immune suppressed mice. 100 p.f.u. of wild-type virus will kill a nude mouse, whereas these mice survive inoculation with 100,000,000 p.f.u. of IL-2-expressing recombinant virus. Vaccination lesions in rhesus and squirrel monkeys vaccinated with an IL-2-containing recombinant were much smaller than the equivalent dose of wild-type virus (156). In general, co-expression of IL-2 had little effect on the immunogenicity of the foreign gene. This study suggests that inclusion of IL-2 or other lymphokines may make it possible to use vaccinia recombinants in immune compromised individuals. Even if protective immunity is not elicited, there should be less risk of complication from vaccinia itself.

7.1.3. Improved Immunogenicity of Foreign Genes

It is possible under some circumstances to improve directly the immunogenicity of the expressed protein. For example, HIV-1 gp160 is cleaved by

proteases to give gp120 and gp41. When expressed in vaccinia virus gp120 is much less immunogenic than a modified gp160 molecule that has been engineered to delete the protease cleavage sites (18). This is probably due to gp160 being anchored in the membrane of the infected cell and hence being presented more efficiently to the immune system of the infected animal.

Another example of an improved immune response to an engineered product expressed by a vaccinia recombinant is that of anchoring a secreted *Plasmodium falciparum* gene in the plasma membrane. The immune response to the gene when anchored was significantly greater than when the gene product was secreted (159). A further example of analysis of antigenicity of engineered proteins is the fusion of foot and mouth disease virus (FMDV) major neutralizing epitope to the hepatitis B virus core antigen. When expressed using a vaccinia recombinant the fusion protein was purified and found to assemble into highly immunogenic particles. Animals vaccinated with these particles were protected against challenge with FMDV.

Other avenues of investigation might also prove beneficial in improving both immunogenicity and attenuating the virus. For example, attenuation by insertion into genes such as the complement control protein homologue (35), the serine protease inhibitor genes (160) or the 13 kDa virokine gene (161) may have a positive effect on the immune response to vaccinia and the protective immunogen. Indeed, insertion into the 13 kDa gene attenuates the virus dramatically but has little effect on the immunogenicity of the virus (161), while insertion into the serine protease inhibitor genes improves immune responses to the foreign gene (160).

Fusion of protective epitopes with vaccinia virus structural proteins may produce vaccinia particles which incorporate the fusion gene into their structure. This appears to have been achieved with β-galactosidase fusions (162) as well as 14 kDa fusions (163) and may prove valuable in improving immune responses to the foreign gene. Other strategies along these lines may incorporate the foreign gene as a hybrid protein into the extracelluar form of the virus. This form is important for the generation of virus neutralizing antibody *in vivo*.

7.1.3.1. Priming with Vaccinia Recombinants

Recently several studies have used vaccinia recombinants expressing HIV gp160 to prime individuals to gp160 followed by boosting either with paraformaldehyde-fixed, recombinant-infected PBLs (141, 164) or purified gp160 (8) (see Table VII). In both cases it was found that improved levels of circulating antibodies resulted and T-cell responses were achieved. This experimental design was based on the observation that mice vaccinated with

a vaccinia recombinant expressing HIV gp160 followed by purified gp160 gave better responses than when the recombinant or purified protein were used on their own (165). A priming–boosting protocol may well overcome the limited immunity generated by both subunit HIV gp160 vaccines and vaccinia recombinants when used on their own.

7.1.4. Alternative Poxviruses

The use of avipoxviruses as alternative vectors to vaccinia for both human and animal vaccination is the most developed of the approaches using poxviruses other than vaccinia for vaccination. They combine the advantage of a live vaccine in infecting the cell and presenting peptides via class-1 major histocompatibility complex and the safety advantages of a killed vaccine in that they cannot undergo full replication in non-avian species. Vaccination of poultry using fowlpox or pigeonpox recombinants may well be a viable proposition and in a number of instances they have been shown to protect against disease (Table VIII). Canarypox recombinants expressing rabies virus G protein have been used in a phase-1 trial in humans (166) and nine out of nine individuals receiving the higher dose had good levels of rabies virus neutralizing antibody.

7.2. Animal Vaccines

7.2.1. Vaccinia-based Vectors

When considering immunization of animals the debate on the use of vaccinia virus as a vaccine shifts from problems associated with the side-effects of vaccination to issues such as the spread of genetically engineered viruses in the environment. Currently two vaccinia based animal vaccines hold the most potential, one to protect cattle against rinderpest virus (167–170), the other to protect wildlife against rabies virus (24). The closest to widespread use is the vaccinia recombinant expressing the rabies virus glycoprotein. It has been shown to be effective in the field situation (25). Attempts to set up similar trials in the U.S.A. were initially thwarted by the environmental lobby. Before this is taken up on a bigger scale, issues such as spread of the virus to the human population, recombination with other poxviruses in nature and detrimental effects on non-target animals will have to be carefully assessed.

7.2.2. Other Poxviruses

It has been suggested that other members of the poxvirus family might make suitable vectors for animal vaccines. Attenuated vaccines for sheeppox and goatpox, for fowlpox (see Section 7.1.4), canarypox (see Section 7.1.4) and for orf virus (a poxvirus of sheep) have been used to

protect against pathogenic strains of the respective viruses. All these attenuated viruses have a narrow host range and, therefore, have the advantage that they will not spread widely in the environment. The physical organization of the genome of many of these viruses have been determined and some functions mapped. Other less well characterized orthopoxviruses might also be used as vectors for foreign genes, for example raccoonpox. In this case the attraction is that raccoonpox is endemic in some areas of the U.S.A. so rather than use a vaccinia virus recombinant expressing the rabies virus glycoprotein to vaccinate wildlife it is suggested that a raccoonpoxvirus–rabies glycoprotein recombinant (171) may be more efficacious in vaccinating raccoons in the wild.

8. CONCLUSIONS

Although poxviruses have been used as vaccines for nearly 200, years it is clear that their potential as vaccines is far from exhausted. Recombinant vaccinia viruses are valuable laboratory reagents for a variety of purposes and it is likely that they will be used as such for many years to come. Trials in humans and other primates have been only partially successful and attempts to boost specific immunity have had to be taken. This coupled with concern over complication rates and immune suppression due to HIV are likely to limit the use of vaccinia recombinants in humans. However, engineered attenuation, the expression of lymphokines and the fact that effective HIV vaccines have yet to be developed may well mean the approach is pursued vigorously either with vaccinia or canarypox recombinants.

Other concerns, such as the spread of genetically engineered viruses in the environment, could limit the use of poxvirus based vaccines in animals, although there is good reason to be optimistic about their efficacy. In the final analysis it is likely that a complex set of factors such as political, economic and sociological considerations will play a large role in the decision as to whether poxvirus recombinants will be used as vaccines on a large scale.

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