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Molecular Attenuation of Vaccinia Virus: Mutant Generation and Animal Characterization

MOON SOO LEE, J. MICHAEL ROOS, LAURA C. McGUIGAN, KENNETH A. SMITH, NANCY CORMIER, LAWRENCE K. COHEN, BRYAN E. ROBERTS, AND LENDON G. PAYNE†*

Applied bioTechnology, Inc., 80 Rogers Street, Cambridge, Massachusetts 02142

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These studies demonstrated that the inbred BALB/c mouse strain can be optimized for the assessment of vaccinia virus virulence, growth, and spread from the site of inoculation and immune protection from a lethal vaccinia virus challenge. The studies established that manipulation of the vaccinia virus genome generated mutants exhibiting a wide range of attenuated phenotypes. The nine NYCBH vaccinia virus mutants had intracranial 50% lethal doses that ranged from 2 to >7 $\log_{10}$ units. The decreased neurovirulence was due to decreased replication in brain tissue. Three mutants had a decreased ability to disseminate to the lungs, brains, livers, and spleens of mice after intranasal infection. One mutant had a decreased transmission from mice infected by tail scarification to naive cage mates. Although the mutants, with one exception, grew to wild-type titers in cell culture, they showed a growth potential on the scarified skin of mice that was dramatically different from that of the wild-type virus. Consequently, all of the mutants had significantly compromised immunogenicities at low virus immunization doses compared with that of the wild-type virus. Conversely, at high immunization doses most mutants could induce an immune response similar to that of the wild-type virus. Three Wyeth vaccine strain mutants were also studied. Whereas the thymidine kinase, ribonucleotide reductase, and hemagglutinin mutants had a reduced virulence (50% lethal dose), only the thymidine kinase mutant retained its immunogenicity.

The advent of molecular biology has led to a rudimentary understanding of the organization and expression of the vaccinia virus genome and a delineation of structures generated by intraviral recombination. This understanding has also allowed the easy manipulation of specific vaccinia virus sequences to introduce foreign sequences into the viral genome, resulting in the formation of recombinant virus. The manipulation of the viral genome has thus been used to insert and express a number of foreign genes (17, 21) with elicitation of a protective immune response in animals against pathogenic viruses (16). This seminal finding demonstrated the feasibility of constructing vaccinia virus recombinants expressing any immunogenic foreign antigen.

Recombinant vaccinia virus-based vaccines represent a significant technological advance, with a number of advantages over traditional vaccines. Acceptance of such vaccines by regulatory agencies will, however, hinge on achieving a significant reduction in the complications (15) associated with vaccinia virus. The traditional means of decreasing vaccinia virus virulence, i.e., attenuation, involved serial passage in animals. The NYCBH vaccine strain was brought from England to the United States in 1856 and maintained as a dermotropic virus by rabbit and calf passage with humanization three times a year (26). Extensive intracerebral mouse passage generated the neurotropic strains WR and IHD, which were therefore unsuitable as vaccines. In contrast, passage in minced chicken embryo resulted in the generation of the highly attenuated CVI strain but at the sacrifice of vaccine potency (13). The attenuation achieved by the long-term passage of virus resulted in the accumulation of undefined genomic alterations that contributed to virulence reduction, but these changes were, nevertheless, insufficient to eliminate all vaccine-associated complications.

A more recent approach to attenuation is the application of molecular biological methods to the construction of mutants with specific gene inactivations (1, 2, 4, 5, 7, 14, 19, 22). These alterations obviate reversion and attempt to dissociate functions that are exclusively related to viral virulence from those responsible for the induction of protective immunity. The generation of data on the effects of diverse mutations on a number of virulence and immunogenicity parameters will increase our understanding of vaccinia virus pathogenesis and attenuation. The present report characterizes, by using a mouse model, nine vaccinia virus mutants generated in the NYCBH strain.

MATERIALS AND METHODS

Cells and virus. RK-13 and BSC40 cells were cultivated in Dulbecco minimal essential medium with 5% fetal calf serum. Vaccinia virus stock preparations were propagated in BSC40 cell cultures and purified in sucrose gradients (12). The NYCBH and WR laboratory strains of vaccinia virus were obtained from the American Type Culture Collection, Rockville, Md., and plaque purified. Two different Wyeth (Dryvax) vaccinia virus preparations were used in these studies. One was from a vaccine bottle (Wyeth-CDC) provided by the Centers for Disease Control, Atlanta, Ga., and the other was a plaque isolate (Wyeth-M) from a seed stock at Wyeth Laboratories, Philadelphia, Pa., provided by Bernard Moss, National Institutes of Health, Bethesda, Md. Virus infectivity was assayed on monolayers of RK-13 or BSC40 cells under 0.6% agarose. Virus mutants were generated by recombination in cells infected with parental virus and a plasmid containing altered vaccinia virus sequences as

* Corresponding author.
† Present address: Virus Research Institute, 61 Moulton Street, Cambridge, MA 02140.

described elsewhere (25). A molecular description of the mutants is provided in the Results section.

Assessment of virulence parameters. The infectious dose that was lethal to 50% of the BALB/c mice (LD_{50}) was determined by infecting 2- to 3-week-old immunocompetent mice. The mice were inoculated by the intracranial (50 μl) and intranasal (10 μl) routes with 10-fold dilutions (seven mice per dilution) of each vaccinia virus mutant. The LD_{50} was determined on mice that succumbed between 2 and 12 days postinoculation (p.i.) by calculating the 50% end point by the Reed-Muench method.

The ability of mutants to propagate on mouse skin was assessed by first denuding the dorsum of 7-week-old female BALB/c mice with Nair hair remover. Then the mice were anesthetized and the denuded dorsum was scarified with 20 scratches of a 28-gauge needle; 10^7 PFU of each mutant was applied to the scarified skin of three mice. Three days after inoculation each animal was sacrificed in a CO_2 chamber, the inoculated area was extirpated, the skin flap was ground with a mortar and pestle, and the plaque titer of the homogenate was determined in cell culture. Other mice were infected in the same way, but instead of assaying for virus growth we determined the pock-forming ability of the virus on day 7 p.i.

The mutants were assessed for their capacity to replicate in mouse brain after direct intracranial inoculation. Two- to three-week-old mice were inoculated intracranially with 10^4 PFU in 50 μl. Two mice per virus were sacrificed daily, and the brains were ground with a mortar and pestle before plaque titration.

The ability of the mutants to disseminate infectious virus to various organs of the mouse was assessed after intranasal inoculation. Groups of 12 normal 7-week-old BALB/c mice were inoculated with 10^6 PFU of each mutant by nasal instillation of 10 μl of a virus suspension. Mice were sacrificed daily in a CO_2 chamber, and the lungs, livers, spleens, and brains were removed aseptically and homogenized with a mortar and pestle before plaque titration.

Assessment of the immune response. The ability of the mutants to induce an immune response to vaccinia virus was assayed in the following ways. The scarified tails of five 8-week-old female BALB/c mice were infected with 10 μl of a virus suspension. Three weeks later, mice were bled by the retroorbital route and challenged by intranasal inoculation with 100 LD_{50} of NYCBH vaccinia virus. Mouse mortality was determined between 2 and 12 days after the vaccinia virus challenge. The level of protection was calculated as the immunization dose (in PFU) that protected 50% of the mice (IPD_{50}). In other experiments, infected mice were bled weekly for determination of vaccinia virus antibody levels. The enzyme-linked immunosorbent assay (ELISA) titer of each serum was determined on twofold dilutions by finding the dilution that was equal to the half-maximal absorbance of a positive antibody standard. The group ELISA titers were calculated by taking the mean of the logarithms (base 10) of the individual serum titers.

RESULTS

Derivation of vaccinia virus mutants. The genome of the Copenhagen strain of vaccinia virus is 191,636 bp long and contains 198 major coding regions. The transcription units are defined by using the convention of Goebel et al. (10). The transcripts are named according to their location within specific *Hin*dIII restriction enzyme fragments, numbered consecutively, and the reading orientation is denoted. For example, the thymidine kinase gene is the second rightward-reading transcript in the *Hin*dIII J fragment, designated J2R. Genes located to the left of C10R vary from strain to strain and therefore are not designated in this way. We have used the Copenhagen gene order when referring to genes in NYCBH, even though there is no formal proof that the gene arrangements in the left-hand end of the *Hin*dIII C fragment are the same for the two virus strains. It is, however, noteworthy that the *Hin*dIII map of NYCBH is the same as that described for the Copenhagen strain. Mutant viruses described here are defined by the transcripts that have been subjected to deletional or insertional mutagenesis and named numerically, e.g., the thymidine kinase mutant vAbT213.

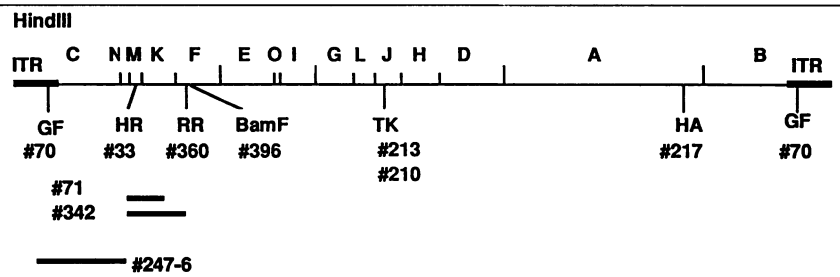
The molecular characteristics of the vaccinia virus mutants are shown in Table 1. Single-gene mutations were generated in NYCBH either by inactivation of targeted genes through deletion or by insertion mutagenesis. Except for the hemagglutinin A56R gene, the targeted genes were nonstructural components of the virus. The relevant sequences were deleted and replaced with the β-galactosidase gene (3, 20) regulated by vaccinia virus promoters designated 7.5K (265 bp), F7L BamF (780 bp), or the fowlpox virus C1 (242 bp) promoter. Alternatively, the β-galactosidase gene was simply inserted into the coding sequence to disrupt the targeted gene. Mutants were visualized and plaque purified by the expression of the β-galactosidase gene activity. An exception to this regimen was the deletion of sequences in the thymidase kinase gene and selection in bromodeoxyuridine. In summary, the J2R thymidine kinase (vAbT210, vAbT213), F4L small subunit of the ribonucleotide reductase (vAbT360), and A56R hemagglutinin (vAbT217) genes were inactivated by deletion of internal coding sequences. The F7L *Bam*F gene (vAbT396) and the vaccinia virus growth factor genes within the terminal repetition (vAbT70) were interrupted by simple insertion mutagenesis.

Multiple gene deletions were made in NYCBH by the elimination of sequences in *Hin*dIII-M and *Hin*dIII-K, resulting in the construction of two host range deletion mutants. The short-host-range mutant (vAbT33) was deleted for the M2L (30-kDa) and K1L (29-kDa host range) genes (30K and 29K genes, respectively) in *Hin*dIII-M, as described in more detail elsewhere (24). The M2L, K1L, K2L, and K3L genes were deleted in the long-host-range mutant (vAbT71). Deletion of the 29K gene eliminates the ability to grow in RK-13 cells, while growth in BSC40 cells is unchanged. In both mutants the deleted sequences were replaced by the β-galactosidase gene under the regulation of the vaccinia virus BamF promoter.

The formation of mutants by the reintroduction of a vaccinia virus promoter regulating β-galactosidase results in the insertion of an additional copy of the promoter sequence within the viral genome. The native and the ectopic promoter copies can occur as either direct or inverted repeats. It is known that direct sequence repeats within the vaccinia virus genome are unstable and can recombine to reconstitute one copy of the repeated sequence with the deletion of all the intervening sequences. The production of a viable mutant, however, requires that the deleted sequences not be essential for growth in cell culture.

The host range mutant (vAbT33) has an ectopic BamF promoter inserted within *Hin*dIII-M, forming a direct repeat with the sequence of the native BamF promoter located 11-kb to the right in *Hin*dIII-F. The appearance of a small-plaque variant was noted in stocks of this short-host-range mutant, and upon plaque purification the progeny virus maintained this phenotype. Southern blot analysis revealed

TABLE 1. Location and nature of alterations in vaccinia virus mutants



Parent virus	Mutant vAbT	Site	Deletion (bases)	Insertion (bases)	Inserted gene
NYCBH	33	Host range (HR)	790	3,900	BamF *lacZ*
	70	Growth factor (GF)	0	2 (3,900)	BamF *lacZ*
	71	*Hin*dIII-MK	3,460	3,900	BamF *lacZ*
	342	*Hin*dIII-MKF	11,000	3,900	BamF *lacZ*
	247-6	*Hin*dIII-CNM	20,000	0	
	213	Thymidine kinase (TK)	420	0	
	217	Hemagglutinin (HA)	630	3,400	7.5K *lacZ*
	360	Ribonucleotide reductase (RR)	180	3,300	*C1 lacZ*
	396	*Bam*F	0	3,100	*lacZ*
WR	210	Thymidine kinase (TK)	420	0	

that the BamF promoters had condensed to a single element with the deletion of the intervening sequences, resulting in the creation of a 11-kb deletion mutant (vAbT342). This mutant was deleted for 15 transcripts between and including M2L and F7L. Appreciation of this phenomenon leads to a general approach for generating viable deletions along the vaccinia virus genome. These deletions have a dual utility: definition of viral sequences not essential for growth in cell culture and delineation of the importance of these sequences in attenuation and immune protection.

Another large deletion mutant (vAbT247-6) was generated when a 7.5K gene promoter sequence was inserted into the *Hin*dIII M fragment of the plaque-purified vAbT33 mutant. This ectopic 7.5K promoter is a direct repeat of the native 7.5K promoter located approximately 20 kb to the left in *Hin*dIII-C. Intergenomic recombination between the ectopic and native promoters deleted the intervening 20 kb of sequences. The number of transcripts involved cannot be determined from the sequence data of Goebel et al. (10) since rearrangements and duplications are common at the vaccinia virus termini.

Characterization of the NYCBH mutants in cell culture. The growth characteristics of each mutant were determined in cell culture since efficient growth is important for both the execution of the proposed experiments and the manufacture of high-titer stocks of a vaccine candidate. The growth of the mutants on confluent monolayers of BSC40 cells was examined at an input multiplicity of 10 PFU per cell. The growth kinetics and viral yields of the mutants and NYCBH at this multiplicity differed within experimental error and were therefore interpreted to mean that the growth potentials of the recombinants in cell culture are essentially the same as that of the parental virus. This indicates that the functions inactivated or deleted, within the limits of these studies, are not essential for efficient growth in cell culture.

Although all mutants had similar growth kinetics, characteristic changes in the plaque size, morphology, and cytopathology were noted in 3 of the 11 mutants studied. The vaccinia virus growth factor-negative mutant (vAbT70) did not clear cells from the infectious center, nor did it show the outer ring of cell proliferation, both of which are characteristics of the parental virus. Deletion of the vaccinia virus hemagglutinin (vAbT217) from *Hin*dIII-A did not alter plaque size; however, the cytopathology of the cells within the infectious center was altered by the fusion of the typically rounded cells to form multinucleated syncytia. Similarly, the deletion of 3.4 kb (vAbT342) in *Hin*dIII MK resulted in the fusion phenotype. The deletion of 11 kb in *Hin*dIII MKF, including the host range 29K gene (vAbT342), resulted in a mutant that generated a small plaque of densely packed cells lacking any central clearing.

Establishment of a mouse model for evaluation of vaccinia virus mutants. Assessing the impact of gene alterations on virulence and immune protection of a mutant requires an understanding of the interactions between the infecting virus and the host animal. Important parameters to be studied are the site of inoculation, the growth of virus, its further dissemination, and the resultant effects on host organs. The efforts of the host to contain and eliminate the invading virus can be studied by examining the antibody- and cell-mediated immune responses. A host that survives this primary infection can then be challenged with a lethal dose to determine whether a state of immune protection was induced. The following experiments were preliminary studies to establish a data base for understanding the pathogenesis of the virulent NYCBH virus for later comparison with and evaluation of mutant viruses.

Infection of BALB/c mice by the intraperitoneal and intranasal routes resulted in approximately equal mortality, but both routes were less sensitive than intracranial infection. In contrast, infection by tail scarification, subcutaneous, and oral routes were not lethal at even the highest attainable dose. Virus infection by the intranasal and subcutaneous routes resulted in approximately the same level of viral replication at the site of inoculation. However, following intranasal infection, virus was detectable in the brain, liver, and spleen on day 2 and in the kidneys on day 3. This is in sharp contrast to the virtual lack of virus dissemination

TABLE 2. Characterization of vaccinia virus mutants in a mouse model

Virus	Mutation	Log_{10} LD_{50}[a]		Skin titer[b] (PFU)	Pock formation[c]	Log_{10} IPD_{50}[d]	Log_{10} ELISA titer[e]
		IC	IN				
NYCBH	None	1.3	4.4	6.9×10^7	Large	2.5	3.26 + 0.14
vAbT396	$BamF^-$	2.2	5.0	6.3×10^7	Medium	2.5	3.30 + 0.18
vAbT213	TK^-	3.0	>7	2.6×10^7	Large	2.4	3.43 + 0.14
vAbT71	Δ3.4 kb	3.1	6.6	3.4×10^7	Medium	2.6	3.24 + 0.35
vAbT33	HR^-	3.5	7.1	2.9×10^7	Medium	3.8	3.11 + 0.46
vAbT360	RR^-	4.2	>7	1.2×10^7	Small	2.8	3.13 + 0.23
vAbT217	HA^-	5.2	>8	1.1×10^7	Medium	3.5	3.14 + 0.24
Wyeth(CDC)	None	5.4	>7	1.9×10^7	Medium	3.6	
vAbT70	GF^-	6.7	>8	1.1×10^7	Medium	3.4	2.73 + 0.45
vAbT247-6	Δ20 kb	6.6	>7	2.7×10^7	Minimal	3.5	2.46 + 0.16
vAbT342	Δ11 kb	6.4	>7	5.3×10^4	None	3.5	1.99 + 0.44
Wyeth(M)	None	7.0	>7	3.8×10^6	Small	4.2	2.79 + 0.47
WR	None	1.4	4.8	1.1×10^8	Large		
vAbT210	TK^-	4.5	>8	1.5×10^8	Large		

[a] Mice were infected by the intranasal (IN) or intracranial (IC) route with 10-fold virus dilutions. The LD_{50} was calculated by the Reed-Muench method.
[b] Scarified skin of mice was inoculated with 10^7 PFU, and the infected skin was harvested on day 3 p.i. for determination of viral content.
[c] Mice were infected as described in footnote *b*. The pocks were photographed on day 7 p.i.
[d] Mice were immunized by tail scarification with 10-fold virus dilutions and challenged 3 weeks later by the intranasal route with 100 LD_{50} of NYCBH vaccinia.
[e] Mice were immunized by tail scarification with 10^7 PFU. Six weeks later the mice were bled and the serum anti-vaccinia virus ELISA titers were determined.

following subcutaneous inoculation. The viral growth kinetics in the skin of scarified mice showed peak viral titers on day 3 p.i., with virus persisting to day 7 p.i. Taken together, these studies established that the BALB/c mouse infected by the intracranial and intranasal routes for LD_{50} determinations, by the intranasal route for dissemination studies, and by skin scarification for viral replication analyses is a sensitive and highly reproducible animal system for assessing vaccinia virus virulence.

Mice infected with 10^6 or 10^4 PFU by the intranasal and skin scarification routes showed detectable ELISA titers by the end of week 2 but were protected from a lethal intranasal vaccinia virus challenge dose (100 LD_{50}) by the end of week 1, when no antibody was detectable. A 10^2-PFU dose elicited no detectable ELISA antibodies, and these mice showed incomplete protection when challenged. Subcutaneous inoculation with 10^6 or 10^4 PFU induced no detectable ELISA antibodies, but these mice were protected against the challenge dose. Therefore, the immune response to vaccinia virus was analyzed by inoculating the mice by tail scarification, collecting serum samples 3 weeks later for antibody titration, and then administering an intranasal challenge of 100 LD_{50} to determine the IPD_{50}.

Virulence of NYCBH mutants. Having garnered at least a basic understanding of some of the parameters of vaccinia virus virulence, we assayed the parental and mutant viruses for six virulence parameters in BALB/c mice. Seven 3-week-old mice per dilution were inoculated intracranially with 50 μl of virus or intranasally with 10 μl to determine an LD_{50} by using a Reed-Muench calculation. The backs of 8-week-old mice were scarified in a 1-cm-diameter area and inoculated with 10^7 PFU in 10 μl to determine viral growth and pock-forming ability. Formation of infectious virus was determined by extirpating the infected area and performing plaque titration in cell culture. Pock formation on mouse skin was recorded by photographing the infected area on day 3 or 7 p.i. The immune response was assayed by determining the IPD_{50} for the mice against a 100-LD_{50} intranasal challenge with virulent NYCBH and by measuring the ELISA antibody response in mice after infection with 10^7 PFU. The following paragraphs describe an analysis of one plaque isolate from each of 10 mutants.

(i) Thymidine kinase mutants. The TK (J2R) gene of vaccinia virus (WR) was the first gene targeted for insertional mutagenesis by other workers. In general, perturbation of this gene in the WR strain resulted in 4- to 5-log_{10} reductions in virulence by the intracranial route (2). Table 2 shows the results of a 417-bp deletion of TK coding sequences in the WR and NYCBH strains. TK deletion in NYCBH (vAbT213) and WR (vAbT210) caused intracranial LD_{50} virulence reductions of 1.7 and 3.2 log_{10} units, respectively. The difference in virulence reduction may be due to either virus strain-specific differences or clonal selection. On the other hand, TK deletion in strains WR and NYCBH did not affect viral replication titers in the skin of infected mice or the extent of pock formation compared with the parental virus. This shows that reductions in intracranial virulence are not necessarily accompanied by reductions in viral propagation or abnormalities in the skin of infected mice.

(ii) Host range mutants. The parental NYCBH strain replicates equally well on BSC40 and RK-13 cells. A 793-bp deletion of the M2L (30K) gene and sequences encoding the carboxy-terminal 29 amino acids (10% of the protein) of the K1L (29K) gene with insertion of BamF *lacZ* results in a virus (vAbT33) that replicates on BSC40 cells with virus yields equal to the yields of the parent virus. However, plaque formation by this mutant on RK-13 cells was reduced by approximately 5 log_{10} units compared with NYCBH. The host range (HR) function was mapped to the 29K gene by others (8). Deletion of these genes and the additional two rightward genes produced a virus (vAbT71) with the same replication pattern on BSC40 and RK-13 cells as vAbT33. Although both these viruses are attenuated by 2 and 3 log_{10} units in intracranial and intranasal LD_{50} assays, respectively, and show reduced pock formation, they produce titers in the scarified skin of mice approximately equal to that of the parent virus.

(iii) Hemagglutinin mutant. A hemagglutinin (A56R) deletion mutant (vAbT217) was generated by the elimination of 630 bp of coding sequence and the insertion of 7.5K *lacZ*. This mutant was attenuated by approximately 4 log_{10} units in intracranial and intranasal LD_{50} assays. This was accompanied by a concomitant near disappearance of pock formation on the skin of scarified mice despite near-wild-type viral

replication. During the course of the present work, it was reported by others (7, 22) that alterations in the hemagglutinin gene result in a great attenuation of the WR vaccinia virus strain.

(iv) Growth factor mutant. The vaccinia virus growth factor (VGF) gene is present in two copies, one in each of the inverted terminal repeats. Formation of a VGF^- mutant requires two recombination events. The first recombination takes place between the plasmid containing a *lacZ* cassette flanked by VGF sequences and one copy of the VGF gene in the viral genome. The second recombination converts the single-copy insertion to a double-copy insertion by inter- or intramolecular recombination between recombinants. vAbT70 was generated by the double insertion of a BamF *lacZ* cassette into the two terminal VGF genes of NYCBH. This mutant was highly attenuated by both the intracranial and intranasal routes of inoculation and grew to wild-type titers but caused medium-sized pocks when inoculated into the scarified skin of mice. A similar interruption of the VGF genes in the WR strain was reported by others (1) to give a similar reduction in intracranial virulence and reduction in pock formation in mice.

(v) BamF mutant. A 3,100-bp *lacZ* cassette was inserted into the unique *Bam*HI (F7L) site located in the *Hin*dIII F fragment of NYCBH to give vAbT396. The *lacZ* gene is promoted by the native BamF promoter located immediately upstream of the insertion. The BamF alteration in vAbT396 may cause a small reduction in the intracranial and intranasal virulence and pock formation compared with that of the parental NYCBH. vAbT396 growth in mouse skin was, however, equal to NYCBH growth.

(vi) Ribonucleotide reductase mutant. A 180-bp deletion in the small subunit of the ribonucleotide reductase (F4L) gene was generated in NYCBH to give vAbT360. The deleted RR sequences were replaced by a fowlpox virus Clb promoter *lacZ* cassette. Both the intracranial and intranasal LD_{50}s of vAbT360 were reduced by approximately 3 $\log_{10}$ units. Although replication in mouse skin was roughly equivalent to that of the parent virus, there was, nevertheless, a reduction in pock formation.

(vii) Large deletion mutants. Large deletions in the left-hand end of the vaccinia virus genome were generated by the ectopic insertion of vaccinia virus promoter sequences in *Hin*dIII-M (24). The resultant duplication of sequences as direct repeats caused an intramolecular recombination that eliminated from the genome all the DNA located between the promoters.

The ectopic insertion of the BamF promoter in *Hin*dIII-M of NYCBH vaccinia virus resulted in a sequence condensation between the native and ectopic BamF promoters with the loss of 11 kb of DNA to give vAbT342. This mutant formed very small plaques in cell culture and was highly attenuated in intracranial and intranasal LD_{50} assays. vAbT342 was greatly reduced in its capacity for growth in mouse skin and did not cause pock formation.

Insertion of a 7.5K promoter sequence into *Hin*dIII-M of vAbT33 duplicated the native 7.5K promoter in *Hin*dIII-C to give vAbT247. Condensation between the native and ectopic 7.5K sequences resulted in elimination of 20 kb of intervening DNA (24). The resultant mutant, vAbT247-6, formed normal-sized plaques in cell culture but was highly attenuated in LD_{50} assays. Although vAbT247-6 grew to near wild-type titers in mouse skin, pock formation was greatly reduced.

Therefore, one or more of the 15 transcripts in the 11-kb deletion to the right of the 29K gene (vAbT342) are, separately or together, responsible for such diverse phenotypes as small plaque and reductions of LD_{50}, growth in skin, and pock formation. On the other hand, the loss of as many as 26 transcripts in the 20-kb deletion to the left of the 29K gene (vAbT247-6) had no effect on growth in cell culture or mouse skin but did reduce the LD_{50} and pock formation.

Mutant growth in mouse brain. The replication of virus in infected mouse brains was studied to determine whether there is a correlation between the intracranial LD_{50}s and the kinetics of virus growth and final virus titers. Ten weanling female BALB/c mice were injected intracranially with 10^4 PFU of each mutant. Two mice per virus strain were sacrificed daily, the intracranial contents were macerated, and the plaque titer of the homogenates on BSC40 cells was determined to find the total PFU content.

The results of this experiment are shown in Fig. 1. The NYCBH strain grew rapidly in the brain, with maximum yields of ca. 7×10^7 PFU per brain appearing by days 3 and 4 p.i. and death ensuing on day 5. The Wyeth-M strain reached peak titers that were 3.5 $\log_{10}$ units lower than that of NYCBH by day 4 and declined on day 5. A 20-kb deletion mutant and the mutants having single-gene deletions in the TK, HA, and RR genes attained peak titers on day 4 that were intermediate between those of the parental NYCBH and Wyeth strains. These four mutants attained different levels of infectivity in the brain, with the TK^- and 20-kb deletion mutants yielding the highest and lowest titers, respectively. Figure 1 also shows the weanling mouse intracranial LD_{50}s for each virus. There is an apparent positive correlation between decreased intracranial LD_{50}s and decreased virus titers in the brain. Thus, decreased intracranial virulence probably results from decreased propagation in the brain.

Mutant dissemination in the mouse after intranasal infection. The dissemination of the parental NYCBH strain and selected mutants (TK^-, HA^-, and RR^-) was studied to determine the effects of specific mutations on the ability of virus to spread in infected mice (Fig. 2). Seven-week-old mice were inoculated intranasally with 10^6 PFU. Two mice were sacrificed each day for 6 days. The lungs, brain, liver, and spleen were macerated, and the infectious virus content was assayed by plaque titration on BSC40 cells. The NYCBH strain was detected in the lungs, brain, and spleen 24 h p.i. but was not detected in the liver until day 4 p.i. In general, peak virus titers were found by day 4 to 5 p.i. More virus could be found in the lungs and brain than in the liver and spleen. Virus appeared later and at ca. 2 to 3 $\log_{10}$ lower levels in mice infected with mutants than in those infected with NYCBH. Except for one mouse infected with the TK mutant, the mutants did not disseminate to and grow in the liver. The RR mutant was not detected in the brain, whereas sporadic infectivity was found in the brains of mice infected with the HA mutant (one mouse) and the TK^- mutant (three mice). All three mutants were found on multiple occasions in the spleen. This finding may reflect the sieving function of the spleen in addition to its potential as a substrate for vaccinia virus replication. The data in Fig. 2 indicate that the virus titers generally appear to be lower in the organs of mice infected with the mutants than in those infected with NYCBH. Compared with NYCBH, the TK^- mutant clearly has a reduced ability to disseminate to and grow in mouse organs. Furthermore, the data demonstrate that the HA^- and RR^- mutants are even more highly attenuated in this regard than the TK^- mutant.

Mutant transmission to naive cage mates. The effect of genetic alterations on the transmission of virus between

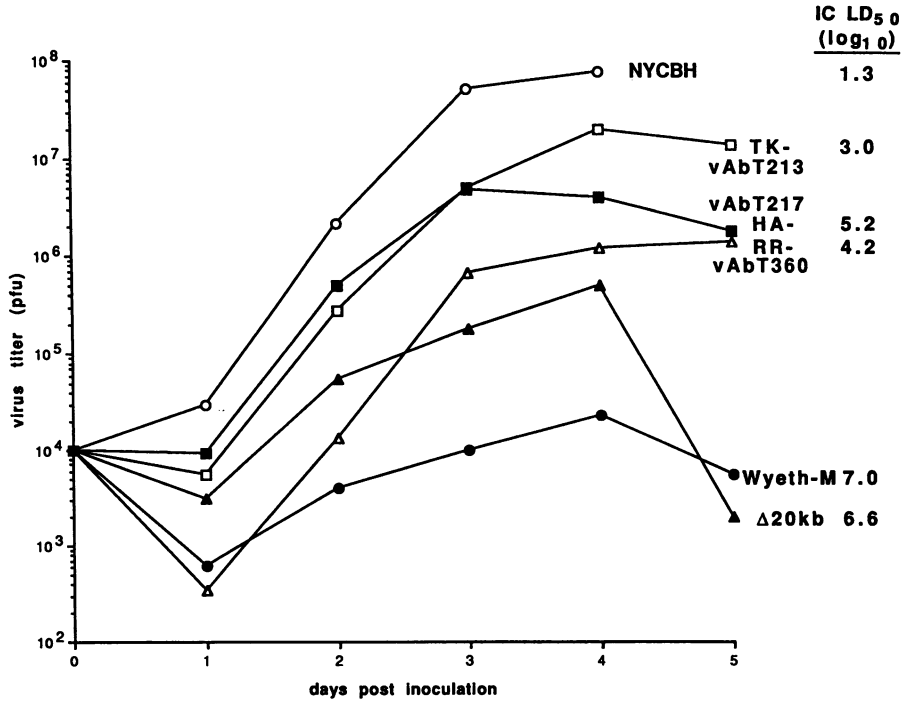


FIG. 1. Vaccinia virus growth kinetics in mouse brain. BALB/c mice (2 to 3 weeks old) were inoculated intracranially with 50 μl containing 10^4 PFU of virus. Mice were sacrificed daily, the intracranial contents were macerated with a mortar and pestle, the material was resuspended in PBS (10%, wt/vol), and the concentration of infectious virus was determined by plaque assay on BSC40 cell monolayers. Each point on the curves represents the average of two mouse brains. The intracranial (IC) LD_{50}s of each virus are indicated to the right of the virus.

infected mice and naive cage mates was examined. Two 7-week-old mice were inoculated with the NYCBH strain and selected mutants at a dose of 10^7 PFU by tail scarification into the skin. Each mouse was immediately housed alone in a cage for 24 h. One mouse was inoculated with phosphate-buffered saline (PBS) for a control. At the end of this period, five unvaccinated naive 7-week-old mice were introduced into each cage. The primary infected mouse and the five naive mice were bled 4.5 weeks p.i. and challenged by the intranasal route with 100 LD_{50} of virulent NYCBH. The mice were observed throughout the experiment (2 weeks) for signs of infection (Fig. 3). Pocks appeared on the primary infected mice, except for the 11-kb deletion mutant (vAbT342), during the first week. Pocks appeared 1 week later on the cage mates of NYCBH, TK^-, HA^-, and 20-kb deletion mutant-infected mice. No symptoms were evident in cage mates of the RR^- and 11-kb deletion mutant-infected mice.

Table 3 shows the results of this experiment. All the primary infected mice had detectable ELISA titers prior to challenge. The highest titers were observed in NYCBH- and TK^- mutant-infected mice and lowest in mice infected with the 11-kb deletion mutant (vAbT342). There is a tendency, as previously shown, for decreased virulence to be correlated to decreased antibody titers. All of the naive mice exposed to the NYCBH-infected mouse developed ELISA titers and were protected from illness and death after challenge. Seven of 10 cage mates of the TK^- mutant-infected mouse developed ELISA titers and were protected from illness and death after challenge. The three cage mates that failed to show detectable ELISA titers were, nevertheless, protected from illness and death after challenge, indicating that the TK^- virus had been transmitted to these mice from the primary infected animal. Very similar results were observed for cage mates of mice infected with the HA^- and 20-kb deletion mutants, indicating that these mutants were transmitted to the naive mice.

The RR^- and 11-kb deletion mutants induced an immune response in the primary infected mice that was detectable in the ELISA assay. Nevertheless, cage mates of the mutant-infected primary mice failed to develop a detectable ELISA titer or to be protected against illness and death after challenge. Although some of the cage mates survived the challenge, all of them became very ill, whereas the cage mates of mice infected with the NYCBH strain or the TK^-, HA^-, and 20-kb deletion mutants all remained free from any sign of illness. Thus, in contrast to the evidence of transmission of the NYCBH strain and the TK^-, HA^-, and 20-kb deletion mutants, the RR^- and 11-kb deletion mutants do not appear to be transmitted from primary infected mice to cage mates. This is perhaps not so surprising for the 11-kb deletion mutant since this mutant grows in skin to a titer that is 2.5 $\log_{10}$ units lower than all the other viruses and is unable to cause the formation of a pock in inoculated skin. More unexpected was the finding that the RR mutant, which grows to near wild-type titers in the skin and forms small pocks, was not transmitted to cage mates. It is therefore surprising that the 20-kb deletion mutant, which forms only minimal pocks in the skin, was nevertheless efficiently transmitted to all of the cage mates.

There is one more observation that derives from the data. It is noteworthy that not all ELISA titers of less than 1:10 are equivalent. This is evident since cage mates of mice infected with the TK^-, HA^-, and 20-kb deletion mutants that had a less than 1:10 titer were protected from challenge whereas cage mates of mice infected with the RR^- and 11-kb deletion

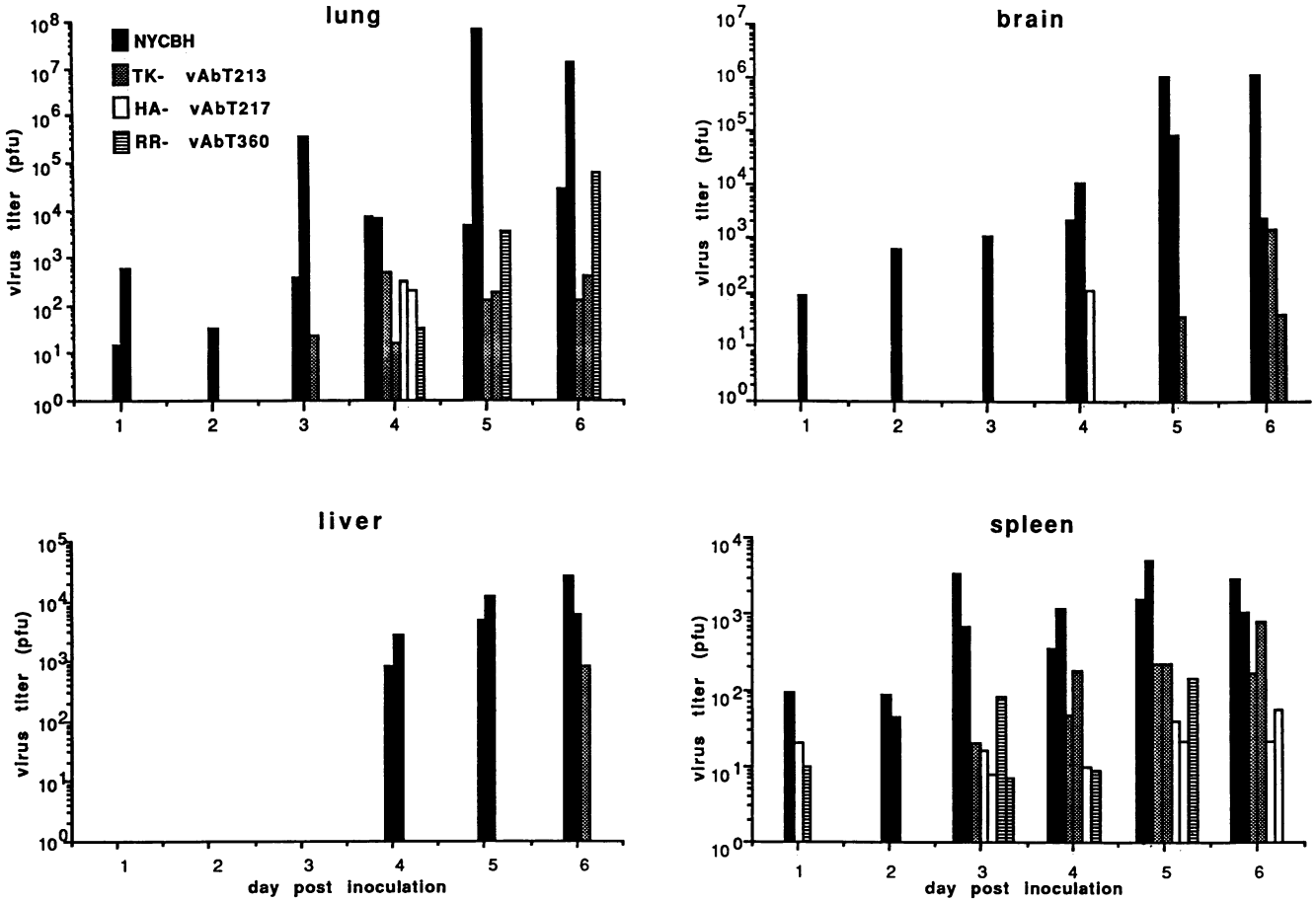


FIG. 2. Dissemination of vaccinia virus after intranasal inoculation. BALB/c mice (7 weeks old) were inoculated intranasally with 10 μl containing 10^6 PFU of virus. Mice were sacrificed daily, the organs (lungs, liver, spleen, and brain) were excised and macerated with a mortar and pestle, and the concentration of infectious virus was determined by plaque titration on monolayers of BSC40 cells. Each bar represents the total virus content of one organ. Organs containing fewer than 10 PFU were regarded as negative.

mutants that had a similar ELISA titer were not protected from challenge. Thus, a challenge assay is more sensitive than ELISA titer determination for the detection of antibodies.

Immune response elicited by mutants. The ability of the mutants to protect against a lethal intranasal vaccinia virus challenge (100 LD_{50}) is shown in Table 2. Mice were immunized by tail scarification with 10-fold dilutions of a virus suspension. The protection provided is expressed as the IPD_{50}. The IPD_{50}s ranged from 2 to 4 $\log_{10}$ units. This is a much narrower spectrum of values than was observed for the LD_{50}s of the same mutant collection. For mutants whose intracranial LD_{50}s are greater than 5.2 $\log_{10}$ units, approximately 10 times as much virus was required in the immunization dose to protect mice than was observed for NYCBH. Table 2 also shows the 6-week ELISA titers in mice immunized by tail scarification with 10^7 PFU. These mice were not challenged. For mutants with intracranial LD_{50}s of 5.2 $\log_{10}$ units or less, the ELISA antibody titers varied less than twofold from the NYCBH ELISA titers. In contrast, mutants with LD_{50}s greater than 5.2 $\log_{10}$ units induced ELISA titers that were reduced compared with the NYCBH titers. The greatest reduction was noted for the 11-kb deletion mutant that was also significantly compromised in its capacity to grow in mouse skin. We conclude that within the limits of the immunoassays used, mutants with LD_{50}s of 5.2 $\log_{10}$ units or less are indistinguishable from the parent NYCBH whereas mutants with LD_{50}s greater than 5.2 $\log_{10}$ units are compromised in their ability to induce an immune response.

The ELISA data imply that mutants having as much as 4 $\log_{10}$ reductions in intracranial virulence can evoke an immune response comparable to that of the virulent wild-type virus. We examined this further by determining whether the mutants and the parental virus demonstrated a similar dose-dependent elicitation of the immune response. Figure 4 shows that mice immunized with 10^6 or 10^4 PFU of NYCBH have similar antibody response kinetics, with plateau titers apparent at 2 weeks p.i. The plateau titers usually ranged between 1,000 and 10,000 which agrees with the 6-week titer of 1,920 (Table 2) elicited by 10^7 PFU of NYCBH. Therefore, the virulent NYCBH strain did not show a dose-dependent immune response over the dose range of 10^4 to 10^7 PFU. Figure 5 shows the 3-week ELISA titers resulting from tail scarification of mice with 10-fold dilutions of mutant and parental virus suspensions. The NYCBH strain demonstrated a clear dose-dependent response when 10^2 and 10^3 PFU were used to infect mice, with the latter dose evoking titers described in Table 2 and Fig. 4. The mutants as a group also showed a clear dose-dependent response, but the doses needed to elicit responses were 10-

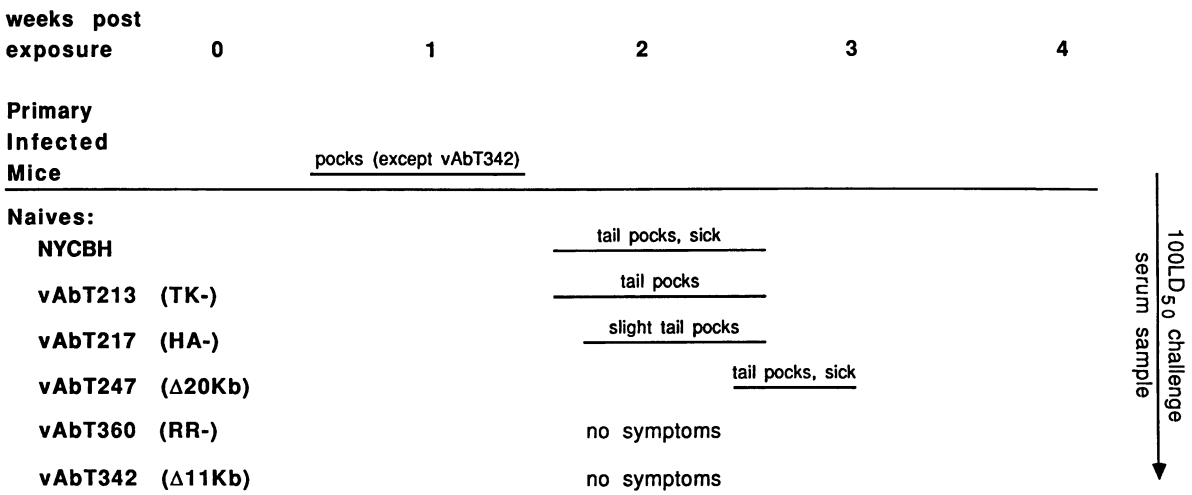


FIG. 3. Virus transmission after tail scarification; clinical development. BALB/c mice (7 weeks old) were infected by tail scarification with 10^7 PFU of virus (two mice per virus). These primary infected mice were housed one mouse per cage for 24 h, when five naive mice of the same age were introduced into each cage. All of the mice were inspected daily for pock formation on the tails and overt signs of illness such as rough fur, hunched back, conjunctivitis, and lack of mobility. At 4.5 weeks after the start of the experiment the mice were bled by the retroorbital route and then challenged by the intranasal route with 100 LD_{50}s of virulent NYCBH vaccinia virus. The serological and mortality data are presented in Table 3.

to 1,000-fold greater than for NYCBH. Thus, the mutants are compromised in their capacity to induce an immune response. Furthermore, it is apparent from a comparison of the virulence and immunogenicity data that an increase in intracranial LD_{50} is correlated with a requirement for increased input virus to stimulate an immune response.

Dose-dependent growth of mutants in mouse skin. The data in Table 2 indicated that the growth of the mutants in mouse skin after inoculation of 10^7 PFU was of the same order of magnitude (with the exception of vAbT342) as that of wild-type NYCBH. Nevertheless, the demonstration that the immune response elicited by the mutants had a markedly different dose-dependent requirement from that of the wild type presented the possibility that the compromised immunogenicity of the mutants was due to a compromised capacity for growth in mouse skin. This possibility was examined by determining the relationship of the size of the inoculation dose to the virus titers obtained from infected skin (Fig. 6).

TABLE 3. Virus transmission from infected mice to naive cage mates: antibody response and challenge

Virus	Cage	ELISA titer in:		Survival (100 LD_{50})[a]
		Infected mouse[b]	Cage mates	
NYCBH	a	3.58	3.27 ± 0.44	5ns/5
	b	3.58	3.39 ± 0.54	5ns/5
vAbT213 (TK^-)	a	3.89	2.91 ± 0.21	5ns/5
	b	3.28	1.15 ± 1.42 (3 neg[c])	5ns/5
vAbT217 (HA^-)	a	2.38	1.39 ± 1.40 (2 neg)	5ns/5
	b	3.28	2.06 ± 1.09 (1 neg)	4ns/4
vAbT247 (Δ20 kb)	a	2.38	0.98 ± 1.24 (3 neg)	5ns/5
	b	2.81	2.83 ± 0.14	5ns/5
vAbT360 (RR^-)	a	3.11	neg	2vs/5
	b	2.68	neg	0/5
vAbT342 (Δ11 kb)	a	1.90	neg	4vs/5
	b	1.90	neg	0/5
None (PBS control)		neg	neg	1vs/6

[a] ns, not sick; vs, very sick (having one or more of the following serious symptoms: tail pocks, rough fur, hunched back, conjunctivitis, lack of mobility).

[b] The primary infected mice were all protected from lethal challenge.

[c] neg, no ELISA titer.

First, the growth kinetics of the wild-type NYCBH and the HA mutant (vAbT217) were ascertained after infection of scarified mice with three inoculum doses (Fig. 6a). The peak virus titers for the wild-type virus appeared on day 4 p.i. and differed by less than 0.5 $\log_{10}$ unit for inoculum doses of 10^6, 10^5, and 10^4 PFU. The propagation of vAbT217 showed, in contrast, a clear dependence on virus inoculum size, with input doses of 10^6 and 10^5 PFU producing peak titers on day 4 p.i. that were ca. 1 $\log_{10}$ unit lower than that observed for wild-type virus. The most marked difference was seen in mice inoculated with 10^4 PFU of vAbT217; five of the six inoculated mice showed no replication of the virus at all. A curious all-or-nothing growth of virus was noted for both the wild-type and mutant viruses. Nevertheless, it is quite clear that the mutant is compromised in its ability to replicate in the skin of infected mice compared with the wild-type virus.

The results of the above-described experiment prompted a reevaluation of the replicative capacity of all of the mutants to determine whether mutation of the vaccinia virus genome was generally detrimental to virus growth. The scarified skin of mice was inoculated with 10^4 PFU of each virus mutant, and the virus titers were assessed on day 4 p.i. (Fig. 6b). The NYCBH wild-type virus grew to high titers in the skin of both inoculated mice. The mutants, on the other hand, showed either no growth in the two mice or growth in only one mouse (vAbT213, vAbT71, and vAbT360). There does not seem to be any overt correlation between the capacity of a mutant for skin growth and its intracranial LD_{50}. Even the minimally attenuated vAbT396 was unable to replicate at the inoculum dose of 10^4 PFU. These results clearly demonstrate that all of the mutants are markedly compromised in their capacity for growth in mouse skin. It is noteworthy that both of the accepted vaccine strains from Wyeth and Connaught Laboratories are similarly compromised.

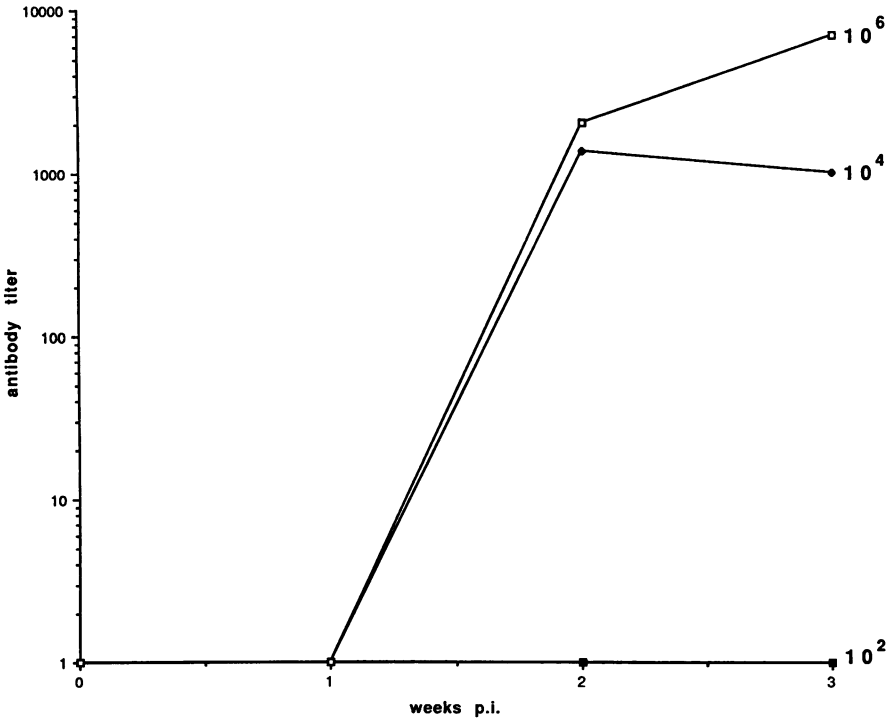


FIG. 4. Antibody response to NYCBH vaccinia virus. BALB/c mice (7 weeks old; five mice per group) were inoculated by tail scarification with 10 μl of the indicated virus concentrations. The mice were bled weekly by the retroorbital route, and the vaccinia virus antibody titers determined by ELISA.

The observed all-or-nothing feature of virus growth in scarified mouse skin needs some explanation. Of all of the many steps involved in these kinds of experiments, from removal of the fur to the final counting of the plaques in BSC40 cells, it is the scarification step that is fraught with the greatest variability. Although the number of scratches is the same for all scarifications, the depth of each scratch, even if made with an eye for this detail, probably varies considerably. Nevertheless, the scarification procedure used for the past 2 years only rarely results in no growth of the wild-type virus. Therefore, the high frequency of no growth of the mutants must be interpreted as a feature of the mutants and not experimental error. The all-or-nothing growth feature may simply reflect the greater dependence of the mutants on having access to deeper cell populations than is required by the wild-type virus.

Virulence and immunogenicity of Wyeth strain mutants. We have studied the effects of mutations on the NYCBH strain because its virulence and immunogenicity could be altered incrementally over a wide range of values. Nevertheless, the Wyeth strain will most probably be the accepted substrate for generation of vaccinia virus-based recombinant vaccines. We have therefore studied the effect of mutations on this vaccine strain. The insertion of human immunodeficiency virus *gag pol/env* sequences (24) into the 3′ noncoding region of the K1L (29K) gene of Wyeth-M vaccinia virus (vAbT4680) had no discernible effect on virulence or immunogenicity. vAbT4680 was therefore used to determine the effect of mutations on the virulence and immunogenicity of a recombinant Wyeth virus (Table 4). The TK^- (vAbT514), RR^- (vAbT515), and HA^- (vAbT516) mutations were introduced into the Wyeth-M strain by using the same plasmids employed to generate these mutations into the NYCBH strain, as described in an earlier section. In the Wyeth-M strain, the RR deletion decreased intracranial virulence (LD_{50}), growth in mouse skin, and immunogenicity as measured both by the ELISA titers and the number of responding mice. Compared with the Wyeth-M parent strain the HA^- deletion mutant had a 0.7 $\log_{10}$ unit reduction in intracranial virulence but its ability to grow in mouse skin was unaltered. This mutant had a decreased immunogenicity at lower immunization doses but elicited ELISA titers at an input dose of 10^7 PFU that were comparable to the parent virus. The TK^- deletion mutant, on the other hand, had phenotypic features that would recommend it as a potential vaccine substrate. This virus had an intracranial virulence that was below detection levels (>8.0 $\log_{10}$ unit), but it was able to grow in mouse skin and elicit an immunity similar to that of the Wyeth-M strain.

DISCUSSION

The development of safe, efficacious vaccinia virus-based recombinant vaccines will revolutionize the immunization against infectious agents for several reasons. It would take advantage of the properties inherent in vaccinia virus that made the smallpox eradication program such a success. A vaccinia virus vaccine is simple to manufacture, easy to test, and very stable in the freeze-dried state, and it induces a strong lifelong immunity and is a proven live virus vaccine. As a live virus recombinant vaccine, vaccinia virus has the additional important feature of expressing high protein levels. These properties will permit an inexpensive means of combating infectious diseases in the developed and the developing worlds.

The utility of vaccinia virus as a recombinant vaccine

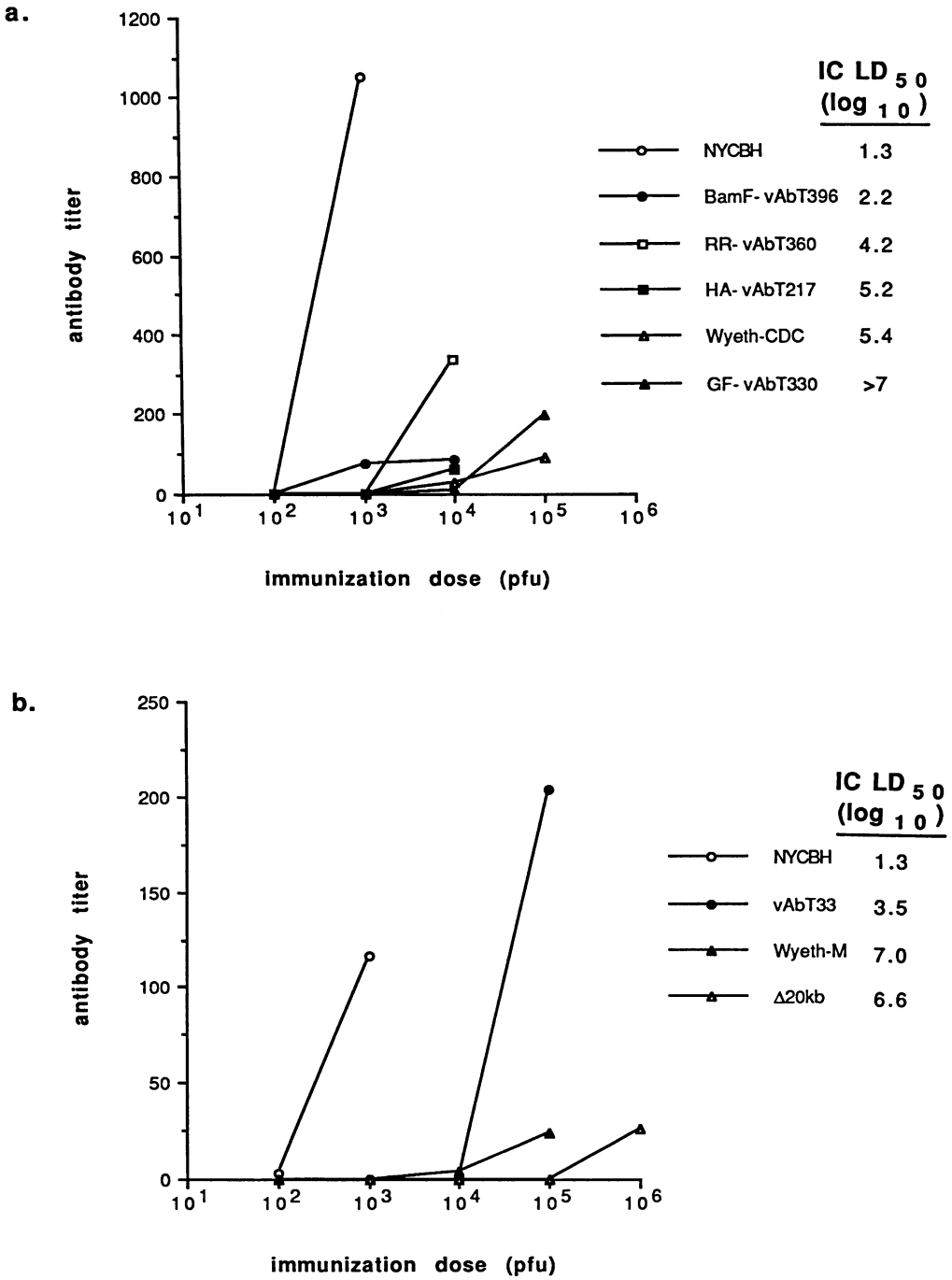


FIG. 5. Antibody response to vaccinia virus mutants. BALB/c mice (8 weeks old; five mice per group) were inoculated by tail scarification with 10 μl of the indicated virus concentrations. The mice were bled 3 weeks p.i. by the retroorbital route, and the vaccinia virus antibody titers were determined by ELISA. The intracranial (IC) LD_{50} for each virus is indicated to the right.

depends ultimately on achieving both efficacy and safety. Therefore, the problem to be addressed is one of retaining the positive features of vaccinia virus replication, antigen expression, and presentation while decreasing the negative features of multifactorial virulence. Previous studies have examined the effects of genetic alterations on the virulence of single vaccinia virus mutants. Such single-mutant studies do not allow one to discover trends and generalities in the mutation of vaccinia virus. We have examined a collection of nine mutants to allow some general statements to be made regarding the interrelationship of various virulence parameters in vaccinia virus and the effect of their reduction on immunogenicity. We chose to study these parameters in a virulent strain of the NYCBH virus since this permitted the relative evaluation of different genomic perturbations over a broad range of intracranial LD_{50}s.

The TK^-, HA^-, VGF^-, and RR^- deletion mutants were derived in our laboratory in the NYCBH strain, whereas

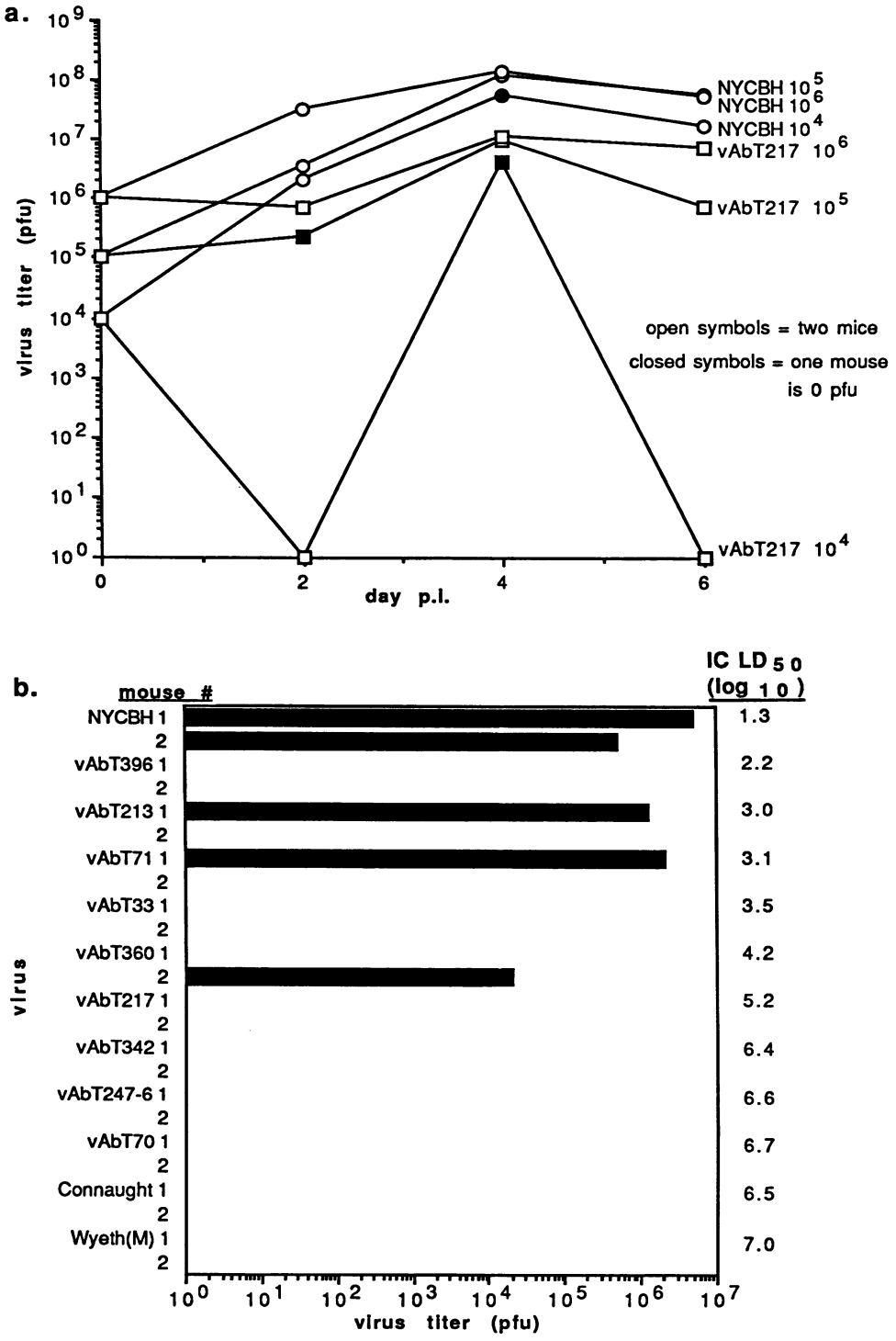


FIG. 6. Relationship of inoculum size and viral growth in mouse skin. The dorsa of 7-week-old BALB/c mice were denuded with Nair hair remover, scarified, and infected with 10 μl of virus inoculum. The infected area was extirpated and macerated with a mortar and pestle, and the virus concentration was determined by plaque assay on monolayers of BSC40 cells. (a) Two mice per time point were inoculated with either wild-type NYCBH vaccinia virus or the HA^- vAbT217 mutant at the virus concentration indicated on the ordinate and to the right of the virus name. The infected skin was collected from two mice on days 2, 4, and 6 p.i. Open symbols represent the average virus titer from two mice, whereas solid symbols represent the virus titer from one mouse with the other mouse having no detectable virus. (b) Two mice per virus were inoculated with 10^4 PFU, and the infected skin was collected on day 4 p.i. Each bar represents the virus titer from a single mouse. The viruses are identified on the left, and their intracranial (IC) LD_{50}s are shown on the right.

TABLE 4. Effect of mutations on the virulence and immunogenicity of Wyeth vaccinia virus

Virus	Mutation	Virulence (log_{10} IC LD_{50})[a]	Skin titer (PFU)[b]	Immunogenicity[c]		
				Immunization dose (PFU)	No. of responders/ total no.	ELISA titer
vAbT4680[d]	None	6.8	$(9.5 \pm 7.0) \times 10^5$	10^4	0/5	neg
				10^5	1/5	0.36 ± 0.71
				10^6	5/5	1.91 ± 0.36
				10^7	5/5	1.91 ± 0.65
vAbT514	TK^-	>8	$(7.4 \pm 4.6) \times 10^5$	10^4	1/5	0.24 ± 0.47
				10^5	3/5	1.12 ± 0.92
				10^6	3/5	1.18 ± 0.99
				10^7	5/5	2.16 ± 0.47
vAbT515	RR^-	>8	$(1.0 \pm 0.4) \times 10^5$	10^4	0/5	neg
				10^5	3/5	0.78 ± 0.67
				10^6	1/5	0.20 ± 0.40
				10^7	2/5	0.71 ± 0.87
vAbT516	HA^-	7.5	$(6.8 \pm 7.9) \times 10^5$	10^4	0/5	neg
				10^5	0/5	neg
				10^6	2/5	0.89 ± 1.10
				10^7	5/5	1.77 ± 0.43

[a] The intracranial LD_{50} of virus was determined in 3-week-old mice.

[b] The replication of virus in scarified dorsal skin of three 6-week-old mice infected with 10^7 PFU of virus was assayed by dry maceration of infected skin harvested at day 3 p.i. followed by plaque titration.

[c] The immunogenicity of virus was determined by ELISA 4 weeks after inoculation by tail scarification and infection with various doses. Five mice were inoculated per group. Mice having anti-vaccinia virus titers of >1/10 were counted as responders.

[d] vAbT4680 is a Wyeth-M recombinant harboring HIV *gag*, *pol*, and *env* genes inserted into the 3′ noncoding region of the K1L gene (24). The TK^-, RR^-, and HA^- mutants were derived from vAbT4680.

other laboratories have generated these mutants in the WR strain. All the mutants have been examined for diverse pathogenic properties, with the only common parameter being the intracranial LD_{50}. The HA mutation in both the NYCBH and WR (7, 22) strains reduced the LD_{50} by approximately 4.0 log_{10} units. Our inactivation of the vaccinia virus GF genes in NYCBH reduced the LD_{50} by 5.4 log_{10} units, but a similar mutation in the WR gene (1) decreased intracranial virulence by only 3.0 log_{10} units. Deletion of the gene coding for the small subunit of the RR in NYCBH decreased the LD_{50} by 2.9 log_{10} units, whereas deletion of the RR large subunit (4) reduced the LD_{50} by only 1.2 log_{10} units. The apparent tendency of LD_{50}s for NYCBH deletion mutants to be equal to or greater than those for similar deletions in WR is not seen in the case of the TK gene. Our deletion of the TK gene in the NYCBH and WR strains reduced intracranial virulence by 1.7 and 3.2 log_{10} units, respectively. Previous studies have shown that this deletion in WR (2, 4, 7) decreased the LD_{50} by about 4.0 log_{10} units. Thus, the quantitative effect of a particular genetic alteration appears to depend on which virus strain is being altered. Furthermore, it is not yet possible to predict the magnitude or direction of an effect caused by an alteration to a vaccinia virus strain.

The nine mutants demonstrated a spectrum of intracranial virulence reductions ranging from as little as 1 log_{10} to as much as 5 log_{10} reduction. Furthermore, the decreased intracranial virulence is due to decreased growth of virus in the brain. Although this correlation is not a surprising finding, it differs from what has been described for the growth of a herpes simplex virus mutant, which has been shown to retain its ability to grow to wild-type titers in the brain but, nevertheless, to have lost its neurovirulence (11). Other HSV mutants show the same positive correlation between LD_{50} and brain titers as we have seen for vaccinia virus. All mutants except one (vAbT342) grew to wild-type titers when 10^7 PFU was inoculated into mouse skin, demonstrating that attenuation by the intracranial route does not necessarily result in reduced growth in the skin. Pock formation in the skin can be reduced without concomitant decreased viral replication at the site of inoculation. Although reductions in intracranial virulence were paralleled by reductions in intranasal virulence, the latter route of inoculation was not as sensitive as the former and could not differentiate among most of the mutants. However, the mutants could be distinguished after intranasal infection, when the ability to disseminate to and grow in various target organs was evaluated.

Although the data demonstrate that the genes reported here can be deleted, with resultant virulence reductions, at least in the mouse model, the indiscriminant deletion of "nonessential" genes, however, is to be cautioned against. Deletion of the E3 gene in adenovirus type 5 changed the pathogenesis of this virus in infected cotton rats from a mild pneumonia to a lethal pneumonia with a profound pulmonary infiltration (9). Although loss of this gene had no effect on the titers of virus produced in the mice, it eliminated the ameliorative role of the gene product on the disease process.

The effect of virulence reductions on immunogenicity is a central issue in attempts to derive an attenuated vaccine. When a high immunization dose (10^7 PFU) was used, the three most highly attenuated mutants reported here elicited an immune response that was decreased as much as 10-fold compared with wild-type infection. On the other hand, immunization of mice with a high dose of mutant virus having intracranial LD_{50} reductions of less than 5 log_{10} units elicited antibody titers similar to those induced by the wild-type virus. Previous work has also shown that the immune stimulation provided at high immunization doses by mutants could not be differentiated from that produced by

the parental virus (2, 6, 22, 23). These results could be interpreted as evidence that both sets of mutants are not significantly compromised in their ability to induce an immune response. A very recent report (14) showed that a vaccinia virus mutant with a large deletion at the left-hand end of the genome could stimulate an immune response equal to that of the wild type at high immunization doses but not at low doses. We have confirmed this finding by demonstrating with dose-response studies that all of the mutants reported here have a compromised immunogenicity at low input doses. Furthermore, there appears to be a clear correlation between decreased intracranial virulence and decreased immunogenicity. It is not clear why this correlation exists.

It is now clear that immunogenicity at low doses is correlated to the replicative properties of the virus in infected scarified skin. Although the skin growth and immunogenicity at low inoculum doses are compromised for all mutants, this deficit can be largely compensated for by inoculation of 10^7 PFU of mutants having intracranial LD_{50}s of less than 6 $\log_{10}$ units. High-dose inoculation, however, is unable to compensate for the decreased immunogenicity of viruses having intracranial LD_{50}s greater than 6 $\log_{10}$ units. This group of viruses includes not only three mutants but also, more importantly, the Wyeth strain. Therefore, the utility of a recombinant Wyeth vaccine having any gene disruption may be very limited.

Immunization against smallpox was performed by the inoculation of scarified skin with the vaccinia virus vaccine. The formation of a pustule was assumed to be a sign that sufficient virus replication had occurred to elicit an antiviral immune response. Furthermore, the formation of a lasting scar was taken as a permanent record of that immune response. In the days of mass immunization against smallpox, the formation of a pustule and a scar were therefore considered by health authorities to be a positive feature of vaccination. However, the virus-containing pustule was no doubt the major source of virus that, in 1 of 300 vaccinees (15), would spread to other areas of the skin and to mucous membranes. In addition, pustular material could also spread to naive individuals in the environment of the vaccinee. Some of the most vocal opponents of the use of vaccinia virus as a live recombinant vaccine vector invoke the cutaneous side effects and the transmission of virus to unvaccinated individuals. The age of vaccination against smallpox is fortunately history, and so should be the requirement that vaccinia virus produce a pustule as a sign that it has evoked an appropriate immune response.

It was therefore of interest to determine the cutaneous manifestations of the mutants in mice inoculated by scarification. The NYCBH strain causes large pocks to form on mouse skin, whereas pocks caused by the mutants range from large to nonexistent. Although we still do not have an assay for the spread of virus on the skin of an individual mouse, we have developed a means of determining the transmission of virus to unvaccinated mice.

The neurotropic WR vaccinia virus strain has been used almost exclusively in the generation of recombinants. Foreign genes were inserted into the vaccinia virus thymidine kinase (TK) gene, resulting in a 4 to 5 $\log_{10}$ reduction in virulence as measured by intracranial LD_{50}. Nevertheless, this recombinant had only slightly diminished abilities to grow in the brain after intracranial inoculation and to spread to the brain after intraperitoneal inoculation (19). These residual virulence properties render the WR strain an inappropriate parent for constructing recombinant vaccines.

The animal analysis of the NYCBH mutants has revealed that the RR^- deletion mutant possesses a number of favorable characteristics that have made it a potential substrate for future vaccine development. The RR^- mutant has an intracranial virulence that is 3 $\log_{10}$ units lower than that of the parent strain, disseminates very inefficiently, grows to near wild-type titers in mouse skin while forming only small pocks, does not transmit to cage mates, and yet induces an immune response that is only slightly lower than that of the parental virus.

The Wyeth vaccinia virus strain was the accepted vaccine virus in the United States during the eradication of smallpox. In this role, the Wyeth strain performed very well by eliciting a protective immunity directed against several antigens. On the other hand, the success of the Wyeth strain as a recombinant vaccine expressing a single foreign gene is less certain. Indeed, it has been shown (18) that primates immunized with a Wyeth vaccinia virus thymidine kinase insertion recombinant expressing the Epstein-Barr virus gp340 glycoprotein were not protected against development of lymphoma after Epstein-Barr virus challenge. A similar recombinant in the virulent WR strain was protective. As demonstrated here, the immunogenicity of the NYCBH strain is orders of magnitude greater than that of the Wyeth strain, and the difference in immunogenicity is related to the virulence of the virus. Thus, researchers developing vaccinia virus recombinant vaccines and regulatory agencies overseeing the development must determine which vaccinia virus strain shall serve as the substrate for recombinant vaccines. The choice lies among (i) a virulent laboratory strain that is attenuated to the desired level of virulence and immunogenicity by methods similar to those described here, (ii) a vaccine strain that is more virulent than the Wyeth strain, such as Lister, and (iii) the Wyeth strain without further attenuation. Regardless of which substrate is finally chosen, it is important to maintain the inherent immunogenicity of the virus by inserting the foreign gene(s) at a site that does not disrupt vaccinia virus coding sequences as described elsewhere (24).

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