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Biological characterization of recombinant vaccinia viruses in mice infected by the respiratory route

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A murine model based on infection by the respiratory route has been used to study the pathogenesis of recombinant vaccinia viruses. The neurovirulent Western Reserve (WR) strain and the Wyeth smallpox vaccine strain were used as vectors. Recombinant viruses were constructed by insertion of the Epstein–Barr virus membrane glycoprotein 340 gene into the thymidine kinase (TK) gene of each vaccinia virus. Intranasal inoculation of DBA/2 mice with 10^6 pock-forming units (pk.f.u.) of the WR strain was lethal but mice survived similar infection with the WR recombinant virus. Each virus was recovered from lung, blood and brain but, unlike wild-type virus, the recombinant virus was subsequently cleared. No deaths occurred after similar infection with the Wyeth strain or the Wyeth recombinant virus. There was limited growth of the Wyeth strain in the respiratory tract, low levels of virus in the blood and only sporadic recovery in brain extracts. The Wyeth recombinant virus was cleared rapidly with little viraemia or detectable infection of the central nervous system. No phenotypic character determined *in vitro* could be related consistently to the virulence of wild-type and recombinant viruses. Although the lethal character of the WR strain was affected by its TK^+ phenotype, mice survived infection by intranasal inoculation with 10^6 pk.f.u. of WR TK^+ recombinant viruses which either expressed the human interleukin 2 gene or had a deficient vaccinia virus growth factor gene.

The development of suitable experimental animal models is essential for the biological characterization of recombinant vaccinia viruses. Such expression vectors have considerable potential as live vaccines but concern about their safety must be recognized (Brown, 1990). The virulence of recombinant vaccinia viruses may be affected by the introduction of foreign genes or by their recombination with other poxviruses (Baxby *et al.*, 1986; Kaplan, 1989). Further, several vaccinia virus genes have been identified recently that are not essential for replication *in vitro* although they could have important functions *in vivo*. The products of such genes include a secretory polypeptide similar to the C4b-binding protein, a control element in the complement cascade (Kotwal & Moss, 1988) and other proteins related to a family of serine protease inhibitors, the 'serpins' (Boursnell *et al.*, 1988; Kotwal & Moss, 1989; Smith *et al.*, 1989). The presence of these protease inhibitors in infected tissues may affect immune responses to the foreign gene products expressed by recombinant vaccinia viruses (Townsend *et al.*, 1989). Studies with another orthopoxvirus have shown a serpin gene is responsible for haemorrhage and the inhibition of inflammatory responses in lesions caused by cowpox virus (Pickup *et al.*, 1986; Palumbo *et al.*, 1989; Chua *et al.*, 1990).

There is no obvious animal host for experimental studies with vaccinia virus because its origins are not known (Fenner *et al.*, 1989). However, natural infections with other orthopoxviruses can occur through the respiratory tract (Fenner *et al.*, 1989). Intranasal inoculation of mice shows laboratory strains of vaccinia virus vary considerably in virulence but only neurovirulent strains appear to be lethal by this route (Briody, 1959; Turner, 1967; Payne, 1980). Similar infectious doses by intravenous, intradermal or intraperitoneal inoculation are not lethal despite levels of viraemia commensurate with those obtained by respiratory infection. Also, intraperitoneal inoculation can result in limited virus replication (Briody, 1959) and the apparently lethal effect of infection with certain vaccinia virus strains following intracerebral inoculation may be due to non-specific toxic factors (Turner, 1967). Interestingly, early Chinese literature from the 16th century A.D. records the insufflation of powdered smallpox scabs by the intranasal route for active immunization against smallpox (Needham, 1980). Administration of other vaccines,

possibly recombinant vaccinia viruses, by this route has recently been suggested for protection against respiratory syncytial virus infection (Murphy *et al.*, 1989).

Intranasal inoculation of mice appears, therefore, to be the most appropriate route of infection for experimental studies of the pathogenesis of recombinant vaccinia virus infections. In this study, the ability of selected wild-type and recombinant vaccinia viruses to infect the respiratory tract, establish viraemia and infect the central nervous system has been compared. Two vectors were used, the Wyeth (New York City Board of Health) smallpox vaccine strain and the neurovirulent Western Reserve (WR) laboratory strain which was derived from the Wyeth strain by repeated passage in mouse brain. Each recombinant virus was constructed by insertion of the Epstein–Barr virus (EBV) membrane glycoprotein gp340 gene into the vaccinia virus thymidine kinase (TK) gene (Mackett & Arrand, 1985); they will be identified in this paper as Wyeth/EB and WR/EB. A TK-deficient variant of Wyeth, WyethTK$^-$, was isolated in TK$^-$ cells maintained in the presence of 5-bromo-2′-deoxyuridine and TK-16 (Weir & Moss, 1983), a TK-deficient variant of WR identified in this paper as WRTK$^-$, was obtained by courtesy of Dr Bernard Moss, National Institutes of Health, Bethesda, Md., U.S.A. A preliminary account of some of this work has been reported elsewhere (Williamson *et al.*, 1988).

In initial experiments, 4- to 5-week-old DBA/2 mice were inoculated intranasally with various virus doses and kept for a total of 3 weeks post-infection (p.i.) before the LD_{50} value was calculated by the Spearman-Kärber method. Purified virus was prepared essentially as described by Joklik (1962) and infectivity titrations were made by pock formation on the chick chorioallantois (McCarthy & Dumbell, 1961). Appropriate infective doses in 20 μl volumes were given to groups of five mice anaesthetized with etomidate (Gomwalk & Healing, 1981). The WR strain had an LD_{50} of $10^{5.3}$ and at a dose of 10^6 pock-forming units (pk.f.u.) or greater all infected mice died within 6 to 7 days p.i. Although there were transient signs of respiratory distress, WR/EB and WRTK$^-$ were less virulent than the wild-type virus with LD_{50} values of $10^{6.5}$ and $10^{6.9}$, respectively. Thus, although WR/EB had a slightly lower LD_{50} value than WRTK$^-$, this was a reproducible distinction (results not presented). Both viruses were attenuated by comparison with the wild-type virus. These results are compatible with other observations on the decreased virulence of TK$^-$ recombinant vaccinia viruses (Buller *et al.* 1985). Wyeth, Wyeth/EB and WyethTK$^-$ did not cause lethal infections even with the highest virus dose, 10^7 pk.f.u., indicating an LD_{50} value $>10^{7.5}$ for these viruses. A similar pattern of susceptibility to infection with these viruses was found with CBA mice (Williamson *et al.*, 1988).

To study the pathogenesis of these infections, 4- to 5-week old DBA/2 mice in groups of 20 were inoculated intranasally with 10^6 pk.f.u. of each virus and two mice from each group were killed at daily intervals. The lungs and brain were removed by dissection to make extracts in 4 mM-phosphate–citrate buffer pH 7·4 by vigorous shaking with glass beads, and blood samples were taken by cardiac puncture.

The amounts of virus recovered from the lungs of WR-infected mice rose steeply from day 1 p.i. to reach titres of 10^9 pk.f.u./g on day 7 p.i. immediately before all remaining mice died (Fig. 1*a*). Titres of about 10^7 pk.f.u./g were maintained in the lungs of mice infected with WR/EB and WRTK$^-$ until day 5 p.i. After that time they declined significantly and there was a 3 to 4 $\log_{10}$ decrease in the titres of WR/EB by 10 days p.i. but at this time infective virus was no longer detectable in the lungs of mice infected with WRTK$^-$ (Fig. 1*a*).

There was extensive viraemia in WR-infected mice from a very early stage in the lethal infection and high titres, about 10^8 pk.f.u./ml, were found on day 7 p.i. shortly before all the mice died (Fig. 1*b*). On the first day virus was detected in the cardiac blood of only one mouse of each pair infected with WR/EB and WRTK$^-$, but from day 2 until day 5 p.i., infective virus was present in the blood of all mice although the maximum titres reached were about 100-fold lower than WR-infected mice. Both WR/EB and WRTK$^-$ were eventually cleared from the blood to become undetectable by day 8 p.i. (Fig. 1*b*).

The rapid systemic infection with WR was accompanied by recovery of virus in brain extracts. Infectivity titres rose steeply from day 1 p.i. to reach $10^{6.4}$ pk.f.u./g shortly before death occurred (Fig. 1*c*). If present, virus was not detected until day 2 p.i. in the brain extracts from mice infected with either WR/EB or WRTK$^-$ and the maximum titres reached at 5 days p.i. were significantly lower than that attained by WR. By day 9 p.i. no virus could be recovered from the brain extracts of mice infected with either WR/EB or WRTK$^-$ (Fig. 1*c*).

Although initial levels of infection were similar, the subsequent titres of Wyeth, Wyeth/EB and WyethTK$^-$ in infected lung extracts were markedly lower than WR, WR/EB or WRTK$^-$ (Fig. 2*a*). On day 1 p.i. infective virus was recovered from both mice infected with Wyeth but from only one mouse in each pair infected with Wyeth/EB or WyethTK$^-$. Infectivity titres were initially maintained between 10^4 and 10^5 pk.f.u./ml in Wyeth-infected mice but despite a 2 to 3 $\log_{10}$ decrease after day 6 p.i., this virus had not been cleared completely by day 10 p.i. However, both Wyeth/TK$^-$ and Wyeth/EB were cleared rapidly from day 1 p.i. to become undetectable by day 5 and day 6 p.i., respectively (Fig. 2*a*).

Low levels of Wyeth with a maximum titre of about

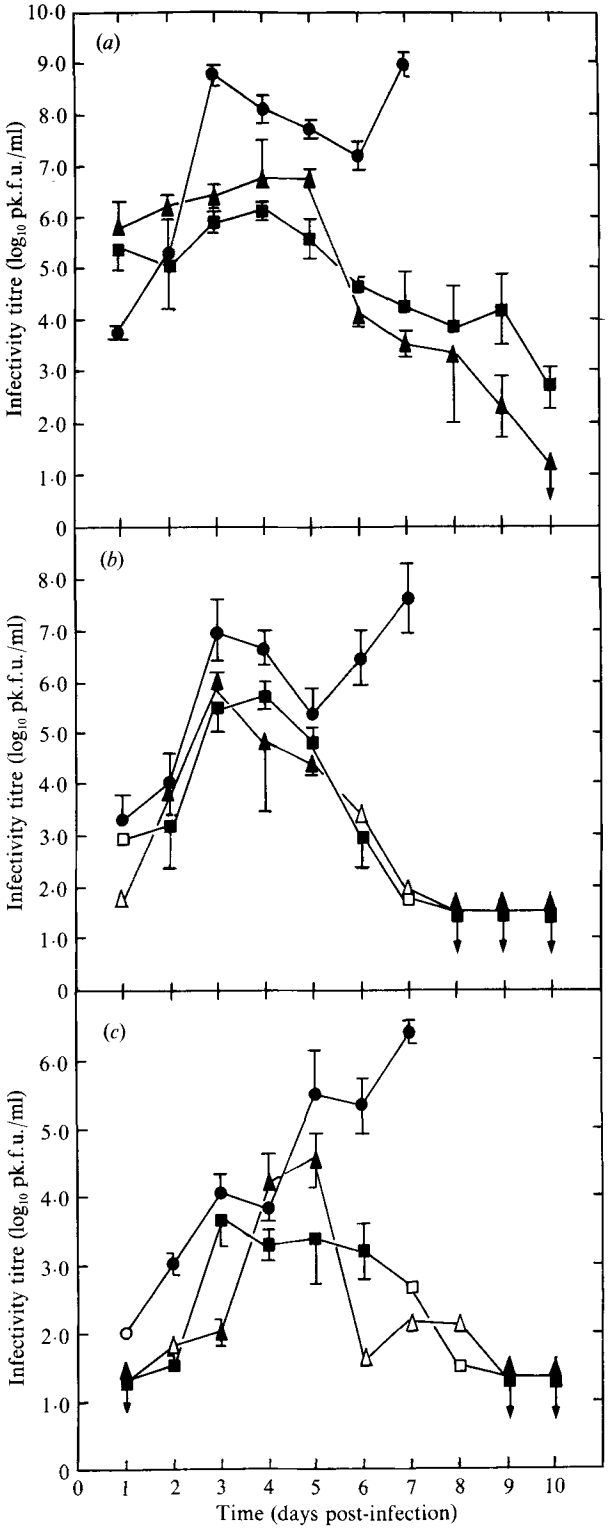

Fig. 1. Respiratory infection of 4- to 5-week-old DBA/2 mice with the neurovirulent WR strain of vaccinia virus (●, ○), recombinant vaccinia virus WR/EB constructed by insertion of the EBV gp340 membrane antigen gene into the TK gene of the WR strain (■, □) and the TK-deficient variant, WRTK$^-$ (▲, △). Infectivity titres of virus recovered from (*a*) lung, (*b*) blood and (*c*) brain of two mice were made at various times after infection. Closed symbols are geometric means obtained from both mice with bars representing ranges; open symbols are titres of infective virus recovered from one mouse only. The symbols with arrows indicate the limits of the sensitivity of the infectivity assay.

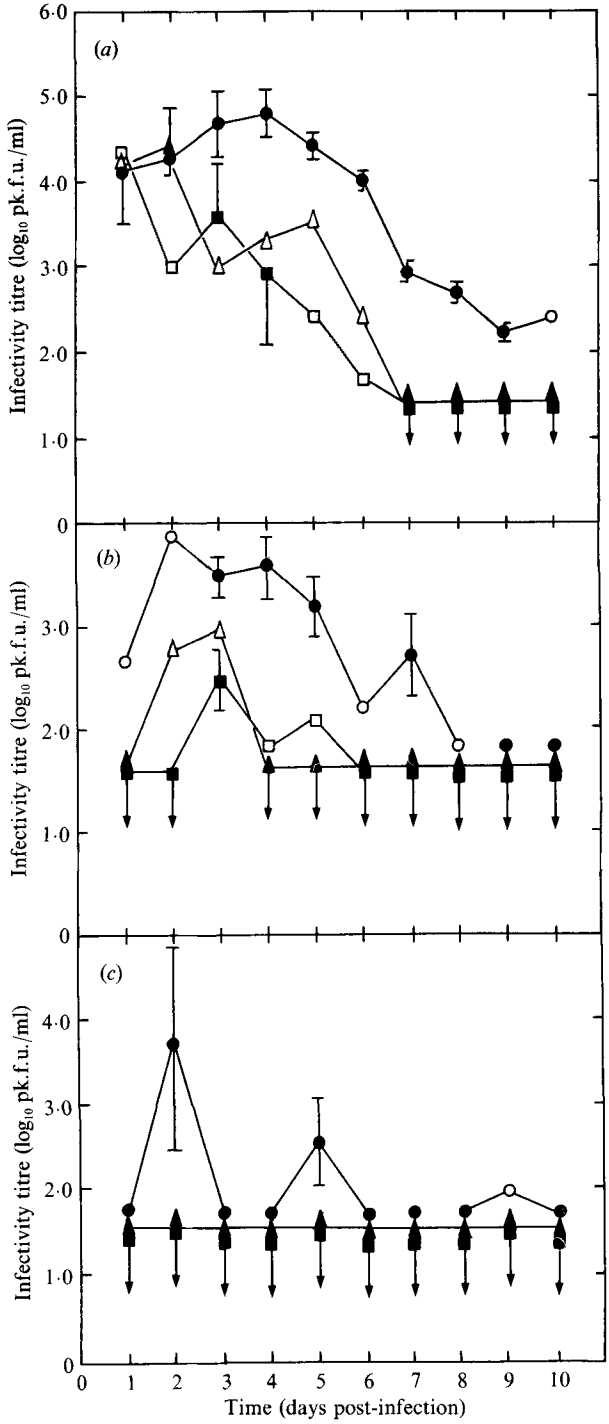

Fig. 2. Respiratory infection of 4- to 5-week-old DBA/2 mice with the Wyeth smallpox vaccine strain of vaccinia virus (●, ○), recombinant vaccinia virus Wyeth/EB constructed by insertion of the EBV gp340 membrane antigen gene into the TK gene of the Wyeth strain (■, □) and the TK-deficient variant, WyethTK$^-$ (▲, △). See legend to Fig. 1 for further details.

$10^{3.5}$ pk.f.u./ml were detected in the blood of some infected mice from day 1 to day 7 p.i. but viraemia did not continue after day 8 p.i. Both Wyeth/EB and WyethTK$^-$ were detected in cardiac blood only between days 2 and 5 p.i. with maximum titres about 10-fold lower than Wyeth (Fig. 2*b*). Low titres, less than 10^3 pk.f.u./g, were detected in brain extracts from Wyeth-infected mice but only at days 2 and 5 p.i. and in one mouse only at day 9 p.i. Brain extracts will inevitably be contaminated with blood released from cerebral, meningeal and other blood vessels in the choroid plexus. However, virus could be detected in the blood of Wyeth-infected mice at times when virus was not detected in brain extracts and at day 9 p.i. virus was detected in the brain only. No virus was detected at any time in the brain extracts of mice infected with Wyeth/EB or WyethTK$^-$ (Fig. 2*c*).

Thus, the pathogenesis of WR after intranasal inoculation appears to be extensive respiratory infection followed by viraemia leading to infection of the central nervous system although spread via the nasal mucosa and cribriform plate cannot be excluded (Turner, 1967). Despite establishment of systemic infections, lower titres were attained by WR/EB and WRTK$^-$ in the affected organs tested. The highest titres of infective virus were found in WR-infected tissues, particularly brain extracts, shortly before mice died at day 7 p.i., whereas from day 5 p.i. virus began to be cleared in mice infected with the recombinant or variant viruses. Lethal infection by WR appears therefore to be determined both quantitatively and qualitatively. Resistance to vaccinia virus infection is effected by cellular immune responses and cytotoxic T cell activity reaches a peak at 5 to 6 days p.i. (Hirsch *et al.*, 1968; Koszinowski & Thomssen, 1975). Such responses could be overwhelmed by the growth of WR and its TK$^+$ phenotype may favour replication in cells of the nervous system (Buller *et al.*, 1985). This is supported by the limited growth of both Wyeth/EB and WyethTK$^-$ together with their rapid clearance from infected mice. However, infection with Wyeth, despite its TK$^+$ phenotype, resulted in a low level of viraemia but only occasional recovery in brain extracts of relatively small amounts of virus. It is apparent that other factors must also affect the neurovirulence of vaccinia virus strains.

The very limited growth of Wyeth/EB demonstrated in this study may be related to its reduced immunogenicity demonstrated in other studies. Vaccination of rabbits with either WR/EB or Wyeth/EB elicited antibodies that recognized EBV gp340 membrane antigen and neutralized EBV but the Wyeth recombinant elicited much lower titres of vaccinia virus-specific antibodies (Mackett & Arrand, 1985). Cottontop tamarins can be protected against EBV-induced lymphoma when immunized by scarification with the WR/EB but not with the Wyeth/EB recombinant viruses (Morgan *et al.*, 1988).

In further laboratory tests, various phenotypic characters were determined which have been shown in previous studies to distinguish between different strains of vaccinia virus. On the chick chorioallantois, neurovirulent strains tend to produce ulcerated pocks with haemorrhagic centres whereas those produced by less virulent strains are not haemorrhagic (Baxby, 1975). WR gave haemorrhagic pocks but the pocks produced by WR/EB and WRTK$^-$ were less haemorrhagic and Wyeth, Wyeth/EB and WyethTK$^-$ pocks were non-haemorrhagic. Pathogenic strains of vaccinia virus also grow more readily at elevated temperatures (Baxby, 1974). In this study, HeLa cell monolayers in 25-well plastic dishes (Sterilin) were infected using 0·1 p.f.u./cell before maintenance in Eagle's MEM supplemented with 2% foetal calf serum and 1% HEPES. Replicate infected cultures were frozen immediately while other infected cell cultures were incubated at a particular temperature by floating in a water-bath fitted with a temperature control unit accurate to 0·1 °C (Grant Instruments). At 48 h p.i. infectivity titrations were carried out by plaque formation in CV1 cells. WR grew at 40·5 °C in HeLa cells whereas the Wyeth strain was inhibited (Table 1). However, WR/EB was indistinguishable from WR although the ceiling temperature of Wyeth/EB was lower than Wyeth.

Vaccinia virus growth in cell cultures results in the production of intracellular virus together with extracellular progeny which differs morphologically in its possession of an outer envelope (Boulter & Appleyard, 1973). To determine total virus yields, replicate cultures of confluent cell monolayers were infected with 5 p.f.u./cell and after virus adsorption for 1 h all infected cultures were washed with Hanks' balanced salts solution before some samples were frozen and further samples were incubated at 36 °C until 24 h post-infection. At this time all infected cell monolayers were disrupted into their maintenance medium by two cycles of freezing and thawing followed by ultrasonic treatment. Infectivity titres were determined by plaque formation in CV1 cells.

It is apparent that the total virus yields in infected cultures at 37 °C were influenced very significantly by the host system (Table 1). In CV1 cells they could be ranked in the order WR > WR/EB > Wyeth > Wyeth/EB but such distinctions were not made in HeLa cells. In MRC-5 fibroblasts both WR and WR/EB gave yields lower than Wyeth but again the lowest yield was obtained with Wyeth/EB. Diploid cells are most likely to respond to contact inhibition by decreased metabolism and host cells with low levels of thymidine metabolism are less able to support replication of the TK$^-$ variants of

Table 1. *Phenotypic characterizaton of WR, WR/EB, Wyeth and Wyeth/EB by laboratory tests* in vitro

Laboratory test	Virus			
	WR	WR/EB	Wyeth	Wyeth/EB
Ceiling temperature*	41	41	40·5	40
Virus production in†:				
CV-1 cells	110 (250)‡	60 (70)	31 (430)	6 (24)
HeLa cells	110 (230)	110 (80)	50 (160)	25 (80)
MRC-5 fibroblasts	26 (100)	13 (40)	57 (164)	2 (30)
Mouse sarcoma 180 cells	129 (ND§)	11 (ND)	0	0

* Temperature is in °C and was measured in HeLa cells.

† Infectivity titres were determined by plaque formation in CV-1 cells.

‡ Total virus yield was measured as p.f.u./cell. The numbers in parentheses represent the ratio of intracellular virus:extracellular virus.

§ ND, Not determined.

herpesviruses (Jamieson *et al.*, 1974). MRC-5 fibroblasts in mitosis infected with Wyeth/EB gave virus yields of 90 p.f.u./cell. Although there was limited replication of WR and WR/EB, there was no growth of either Wyeth or Wyeth/EB in mouse sarcoma 180 cells; this cell line has been shown previously to support growth of the neurovirulent vaccinia virus IHD strain but to be non-permissive for the Lister smallpox virus vaccine strain (Osborn *et al.*, 1984).

The yield of extracellular virus produced by several vaccinia virus strains in infected cell cultures is related to their pathogenicity in mice infected intranasally although WR proved exceptional (Payne, 1980). In this study, extracellular virus was recovered by removing the medium from infected cell cultures (m.o.i. of 1 p.f.u./cell) following incubation at 36 °C for 24 h. The infected medium was centrifuged at 1500 *g* for 5 min and the infectivity titre of the supernatant was determined by plaque formation in CV1 cells. Both WR/EB and Wyeth/EB were attenuated but produced more extracellular virus than the wild-type viruses, particularly in MRC-5 fibroblasts and in CV-1 cells (Table 1). In all tests, WRTK$^-$ and WyethTK$^-$ were indistinguishable from the appropriate recombinant virus (results not presented). Thus, no phenotypic character of the viruses studied could be consistently related to their pathogenisis in mice infected intranasally.

Further experiments made *in vivo* were able to show that host- and virus-specific factors can affect the virulence of vaccinia viruses in this murine model. Recombinant vaccinia virus vCF13 expresses the human interleukin 2 (IL-2) gene (Flexner *et al.*, 1987) and a mutant, vSC20, lacks a functional vaccinia virus growth factor (VGF) gene (Buller *et al.*, 1988). Both recombinant viruses express vaccinia virus TK activity together with prokaryotic β-galactosidase (β-gal) and a third recombinant vTFCLZ, which has a TK$^+$, β-gal$^+$ phenotype, was used as a control. These recombinant viruses were kindly supplied by Dr Bernard Moss. In 4- to 5-week-old DBA/2 mice infected intranasally, the LD_{50} for vTFCLZ (TK$^+$, β-gal$^+$) was $10^{5.5}$ indicating that its virulence was comparable with the vector, WR. An LD_{50} value $> 10^{7.5}$ was obtained for vCF13 (IL-2$^+$, TK$^+$, β-gal$^+$). Such vector-directed expression of IL-2 stimulates natural killer cell responses in nude mice although cytotoxic T cell responses in normal mice were unaffected and other factors may also contribute to such protection (Flexner *et al.*, 1987; Karupiah *et al.*, 1990). Significant attenuation without loss of the TK$^+$ phenotype was also indicated by the LD_{50} value of $10^{6.9}$ obtained with vSC20 (VGF$^-$, TK$^+$, β-gal$^+$). Vaccinia virus VGF is closely related to epidermal growth factor (EGF) and EGF receptors on astrocytes and glial cells may be relevant to the neurovirulence of WR although VGF could also be important for virus replication in resting cells (Buller *et al.*, 1988).

Vector vaccines based on vaccinia virus, despite the attendant problems, continue to engage interest; for example, they have been applied for vesicular stomatitis virus infection in cattle (Mackett *et al.*, 1985), AIDS in man (Zagury *et al.*, 1988), rabies in the fox (Blancou *et al.*, 1986), rinderpest in cattle (Yilma *et al.*, 1988) and Rift valley fever virus infection in man and in other animals (Dalrymple *et al.*, 1989). During the smallpox eradication campaign, however, the rates of complications in vaccinees gave rise to persuasive arguments against compulsory vaccination in countries where the disease was no longer endemic (Dick, 1966; Lane *et al.*, 1969). Post-vaccinial encephalitis was the most serious complication but no single laboratory test was described

that could satisfactorily determine the neurovirulence of vaccinia virus for man (Baxby, 1975). For example, in clinical trials the CV1 smallpox vaccine was shown to be attenuated (Kempe, 1968) but it appeared to be more virulent if inoculated intracerebrally into newborn mice (John, 1969).

There is obviously a complex interaction of viral and host factors in the expression of virulence in vaccinia virus infections. It is important to understand the mechanisms involved in order to develop recombinant vaccinia virus vaccines and infection of mice by the respiratory route is a suitable experimental model for this purpose.

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