analyzed the genome of the human A431 carcinoma cell line, which contains a highly amplified number of copies of the gene encoding the EGF-r (*4, 10, 11*). To determine whether the *neu* gene was coamplified in this cell line, a set of nitrocellulose filters was prepared containing Eco RI–digested DNA isolated from A431 cells and from HeLa cells; the DNA from HeLa cells, another human tumor line, was used as a control. When this filter was hybridized with the EGF-r cDNA subclone 64-1, the A431 cell line was observed to contain amplified DNA segments homologous to the probe (Fig. 2A). We, and other investigators (*4, 10*), have found that segments homologous to other domains of c-*erb*B are also amplified. In initial studies, we hybridized a duplicate filter with a 4.0-kb Bam HI genomic subclone of the *neu* gene and found that this segment hybridized preferentially to *neu*. The human genome contained a 7.2- and 16-kb Eco RI segment homologous to this *neu* probe; neither of these segments was amplified in the A431 cell line (Fig. 2A). Thus, *neu* has not been coamplified with c-*erb*B in A431 cells, suggesting that the two genes are separate and nonoverlapping.

We determined the chromosomal position of *neu* in order to further define its relationship to *erb*B. The human *erb*B gene encoding the EGF-r is located on chromosome 7, region 7p11-p13 (*12*). We first ascertained the chromosomal location of the human *neu* gene by in situ hybridization of the 4.0-kb Bam HI segment to metaphase chromosome preparations. In situ hybridization (Fig. 3) resulted in 217 silver grains on 110 metaphase cells, 36 (16.6 percent) of which were over a specific chromosomal region, 17q11.2-q22. No other sites were labeled above background. Southern blot analysis of DNA's prepared from ten human × rodent somatic cell hybrids confirmed the localization of *neu* to chromosome 17. The hybrids carrying human chromosome 17 all contain the 7.2-kb Eco RI segment (Fig. 2B) that is specific for the human *neu* gene (the 16-kb human segment being visible after longer exposure). The intense 8.0-kb band seen in Fig. 2B (lanes 3 and 6 to 12) is the hamster homolog of *neu*, which reacts strongly with the probe because of its rodent origin.

Chromosome 17 is the only chromosome with perfect concordant segregation, and other chromosomes could be excluded as possible sites for *neu* by at least two discordances. Moreover, in a mouse × human hybrid containing only the q21-qter region of chromosome 17, the 7.2-kb human *neu* Eco RI segment was also detected (Fig. 2B, lane 4). We conclude that the human *neu* gene is located on q21 of chromosome 17, in contrast to the gene encoding the EGF-r, which is found on chromosome 7.

The localization of *neu* coincides with the map position of the human c-*erb*A1 locus at 17p11-q21 (*13*). The cellular *erb*A and *erb*B genes of chicken have both been incorporated into the genome of avian erythroblastosis virus (*14*). The significance of the coincidental mapping of the *erb*-related *neu* and c-*erb*A1 genes is obscure at present.

While the *neu* and EGF-r genes are distinct and unlinked, they are closely related in at least one region, that which encodes the tyrosine kinase domain. Thus, the *neu* gene, like *erb*B, is a member of the family of genes encoding tyrosine kinase domains. Moreover, the present data suggest that *neu* and *erb*B are more closely related to one another than to other members of this family. Because of the strong similarity in structure of the *neu*- and c-*erb*B-encoded proteins, we believe that the p185 protein, like its relative, is a receptor for a cellular growth factor and mediates similar biological effects.

Although an activated *neu* gene has been isolated from rat neuroglioblastomas, it is noteworthy that the human *neu* gene maps to a chromosomal band that is often involved in a nonrandom reciprocal translocation t(15;17)(q22:q21) in acute promyelocytic leukemia (*15*). The relationship between *neu* and human malignancy is under investigation.

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20. We thank C. Bargmann for helpful discussions. Supported by fellowships from NIH (A.L.S.) and the Cancer Research Institute (M.C.H.) and by grants from the American Business Cancer Foundation NCI (CA 39964-01) (R.W.), and NIH (GM 26105) (U.F.).

17 April 1985; accepted 13 June 1985

Isolation and Propagation of a Human Enteric Coronavirus

Abstract. *Coronavirus-like particles were found by electron microscopy in stools from infants with necrotizing enterocolitis. Stool samples from these infants as well as control specimens were passaged in cultures of human fetal intestinal organs. Two samples yielded virus-like particles and these have now been passaged 14 times (HEC 14). Gradient-purified HEC 14 strains had typical coronavirus morphology on electron microscopy and contained five major proteins with molecular sizes ranging from 190 to 23 kilodaltons. Infants with necrotizing enterocolitis developed specific antibody to the viral antigens between the acute and convalescent stages of the disease, as shown by examining serum specimens by single radial hemolysis, immunoenzymatic assay, and Western immunoblotting. No cross-reactivity was shown with other coronavirus strains tested, or with the newly isolated viruses of the Breda-Berne group, responsible for calf or horse diarrhea.*

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The genus *Coronavirus* contains medium-sized (80 to 150 nm), rounded or polymorphic particles with club-shaped surface projections and a positive-stranded RNA genome. These viruses are widely distributed among various animal species (*1–5*). Only two coronaviruses, the well-characterized OC43 and 229E strains (*6–8*), have been found to cause disease in humans, both viruses producing the common cold. However, coronavirus strains have been associated with diarrheal diseases in lower animals, and there is evidence that these viruses may be involved in human enteric diseases (*9–12*). Most of the data in support of the latter hypothesis result from electron microscopic observations of coro-

navirus-like particles in stool samples obtained from patients with acute gastroenteritis or necrotizing enterocolitis (NEC) (*13–23*). Attempts to cultivate these particles for antigenic or biochemical analysis have been unrewarding to date.

An epidemic of NEC occurred in a hospital special care nursery in Dallas, Texas in 1982–83. All of the patients showed the established criteria for NEC (*24*)—intolerance to food, abdominal distension, occult or gross blood in stool, and radiologic evidence of pneumatosis intestinalis. Stool samples from patients revealed coronavirus-like particles. During the epidemic, stool specimens and sera were obtained from controls and from patients with NEC at acute and convalescent stages of disease. We report the isolation and serial passage of a human enteric coronavirus (HEC) from stools of two of the patients.

All stool specimens were screened for the presence of coronavirus-like particles by electron microscopy before inoculation in cultures of human fetal intestinal organs. The cultures were prepared as described (*25–28*). Briefly, the intestines were opened longitudinally with microscissors, and pieces 2 by 2 mm, with the intestinal villi oriented upward, were placed in tissue culture dishes. At each passage, the organ cultures were incubated in Leibovitz L-15 medium (*p*H 6.8) supplemented with antibiotics in a humidified environment at 37°C with a 5 percent CO_2 atmosphere. Uninoculated organ cultures, prepared as controls, were maintained under the same conditions.

Seven stool samples showing positive results by electron microscopy and eight showing negative results were cultured. None of the negative inocula resulted in the growth of coronavirus-like particles after being passaged five times and assayed by electron microscopy and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Two of seven positive specimens led to the isolation and propagation of coronavirus-like particles and these have been maintained for 14 passages (HEC A14 and HEC C14). After the first blind passages (3 to 5), the two strains produced destruction of the brush border of the intestinal epithelium and degeneration of the villi. For passages 8 to 14, trypsin, which is thought to have an enhancing effect on coronavirus replication, was added to the growth medium at 5 μg/ml. Cultures infected in the absence of trypsin were maintained in parallel. Treatment of the inocula with chloroform or heat (56°C for 15 minutes) nullified the effect of the inocula on the organ cultures—that is, there was no cytopathic effect, virus-like particles were not seen on electron microscopy, and the characteristic protein band profile was not revealed by SDS-PAGE. Control infected cultures not treated with chloroform or heat continued to show evidence of the presence of HEC. Filtration of the inocula through filters of 0.45- and 0.22-μm pore did not prevent infection of the organ cultures.

The supernatants and tissue extracts of the infected cultures from passages 6 to 14 were purified on a glycerol–potassium tartrate gradient (*7, 29*). The peak of spectrophotometric activity at 280-nm wavelength corresponded to a density of 1.18 g/cm^3. Observation of the band collected from the gradient revealed particles with morphology typical of coronaviruses—namely, club-shaped spikes, a diameter of 100 to 150 nm, a pleomorphic appearance, and an erythrocyte-like profile (Fig. 1). The purified particles were tested for hemagglutination with goose, chicken, rat, guinea pig, rabbit, and human O erythrocytes at 4°, 25°, and 37°C. No hemagglutinating activity was observed.

During single radial hemolysis (SRH) assay (*30*), the purified material reacted with convalescent-stage sera from six patients with NEC; four of the patients showed seroconversion. Sera from con-

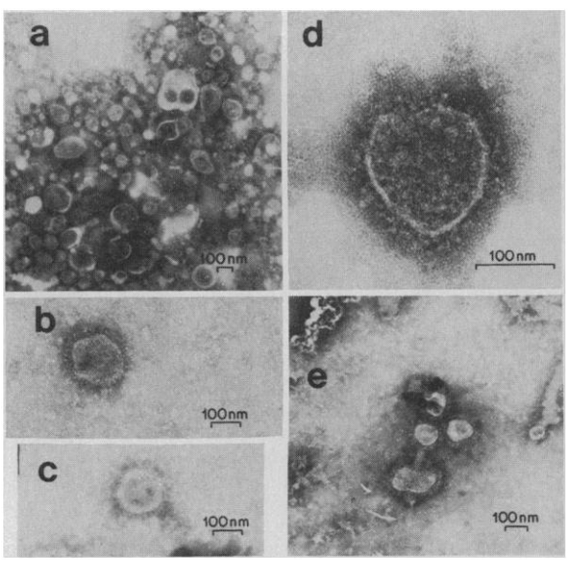


Fig. 1. Electromicrographs showing coronavirus-like particles. (a) Stool sample from a patient with NEC. (b to d) Particles from the passage 14 after gradient purification. (e) Purified particles observed by immunoelectronmicroscopy. The particles were incubated with convalescent-stage serum from an infant with NEC. The particles were suspended in the serum (final dilution 1:20), incubated for 1 hour at room temperature, and then stained for immunoelectronmicroscopy. The stain used was 2 percent phosphotungstic acid at *p*H 7.2.

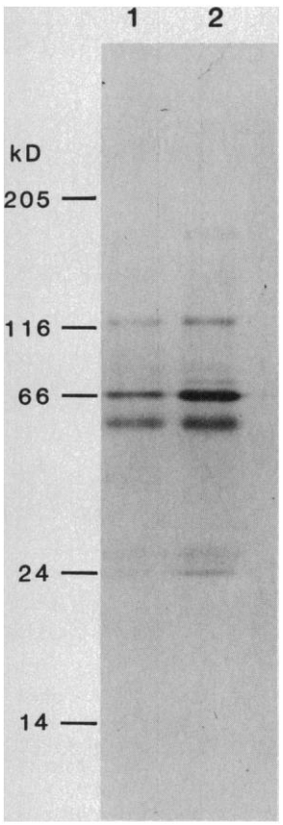
Table 1. Single radial hemolysis (SRH) assay, performed according to a previously described technique (*30*). Before being used, all sera were adsorbed overnight (4°C) with human fetal intestinal homogenate. All sera were tested after heating at 56°C for 30 minutes (1, first serum; 2, second serum). A serum was considered positive when the reaction caused a halo of hemolysis at least 3 mm in diameter (well diameter, 1 mm). The first or acute-stage serum was collected within 2 weeks of the onset of illness, and the second or convalescent-stage serum was collected 4 to 8 weeks after the onset of illness. Controls were infants in the nursery without necrotizing enterocolitis or diarrhea. A6, A9, A14 and C6, C9, C14 designate the passage level of the isolates. Abbreviations: NCS, newborn calf serum; FCS, fetal calf serum; BSA, bovine serum albumin; SRBC, sheep red blood cells; ND, not done.

Patients	Diameter of HEC sample (mm)						Controls	Diameter of HEC sample (mm)					
	A6	A9	A14	C6	C9	C14		A6	A9	A14	C6	C9	C14
H-1	0	0	0	0	0	0	R	0	0	0	0	0	0
H-2	4.3	3.4	4.5*	4.2	3.5	4.0*	McM-1	0	0	0	0	0	0
M-1	0	0	0	0	0	0	McM-2	0	0	0	0	0	0
McC-1	0	0	0*	0	0	0*	P	0	0	0	0	0	0
McC-2	0	0	5.0*	0	0	4.8*	Pe	0	0	0	0	0	0
E-2	ND	ND	3.5	ND	ND	3.5	T	0	0	0	0	0	0
D-1	0	0	0*	0	0	0*	Lo	0	0	0	0	0	0
D-2	3.5	3.5	4.2*	3.5	3.5	4.0*	Hi	0	0	0	0	0	0
Hn-1	0	0	0*	0	0	0*	NCS	0	0	0	0	0	0
Hn-2	3.0	3.5	4.5*	3.0	3.0	4.3*	FCS	0	0	0	0	0	0
Mo-1	0	3.5	3.5*	0	3.5	3.5*	Anti-OC43	0	0	0	0	0	0
Mo-2	4.0	4.0	4.5*	3.8	4.0	4.3*	Anti-229E	0	0	0	0	0	0
							Anti-BSA	0	0	0	0	0	0
							Anti-SRBC	10	10	10	10	10	10

*Sample diluted 1:5; all other sera were used undiluted except the NCS, FCS, anti-BSA, and anti-SRBC, which were diluted 1:10.

trol infants showed no reactivity against the antigens (Table 1). Neither the purified HEC antigens nor the infants' sera revealed cross-reactivity with antisera to OC43 and 229E and with OC43 and 229E antigens, respectively.

Antigens (purified HEC A14 and C14, OC43, 229E, and human fetal intestinal homogenates) were tested by enzyme-linked immunosorbent assay (ELISA) against the infants' sera and against antisera to OC43, 229E, MHV-A59, and Breda 1 and 2 viruses (*31–34*) by standard techniques (*35, 36*). The working dilutions of serum were 1:10 and 1:100, and each determination was made in triplicate. Appropriate controls for all of the reagents used were included in each assay. All of the sera were adsorbed overnight (4°C) with human fetal intestinal homogenates before being used. Briefly, both the infants' sera and the other antisera were assayed by binding the antigen directly to the wells of the microtiter plates. After incubation of the test serum samples, alkaline phosphatase–conjugated immunoglobulins (anti-human, anti-rabbit, and anti–guinea pig) were incubated in the wells. The enzyme substrate (*p*-nitrophenyl phosphate in diethanolamine buffer) was added after washing, and the reaction was stopped and evaluated for absorbance (AB) in a spectrophotometer. Antisera to OC43, 229E, MHV-A59, and Breda 1 and 2 strains were also assayed by means of a capturing antibody-coated well assay, with specific guinea pig antisera to HEC A14 and C14 used as coating immunoglobulin. Purified HEC A14 and C14, OC43, and human intestinal homogenates, test antisera, conjugated immunoglobulins, and enzyme substrate were consecutively incubated in the wells. Each incubation step was followed by washing three times with standard buffer solutions. The AB value of each serum–control antigen reaction was subtracted from the corresponding AB value of the serum–viral antigen reaction to obtain the value of the test sample. A threshold cutoff point was determined on the basis of the highest values obtained on a group of negative controls. Positive sera always had an AB value greater than 3 standard deviations above the mean of a group of negative control sera. The ELISA test confirmed the results obtained by the SRH assay. Convalescent-stage sera from five of the infants with NEC showed titers of 1:100 or more; two infants showed seroconversion; and six control sera had titers less than 1:10. The infants' sera did not react with OC43 and 229E antigens. No reactions were demonstrable between A14 or C14 antigens and antisera to OC43, 229E, MHV-A59, and Breda 1 and 2 viruses.



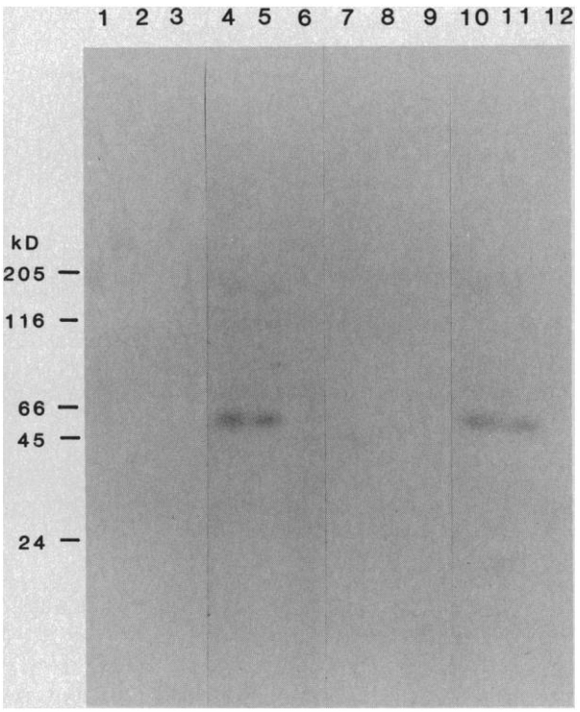


Fig. 2 (left). Analysis of viral proteins with SDS-PAGE. Gradient polyacrylamide slab gels (0.1 percent of SDS by volume) were prepared from acrylamide and *N,N'*-methylene bisacrylamide (5 percent to 17 percent). The protein samples were diluted in Laemmli sample buffer and heated for 5 minutes at 100°C. The electrophoresis was performed overnight at room temperature in a tris-glycine-SDS buffer (*p*H 8.3) at 7.5 mA per gel. After electrophoresis, the proteins were stained with Coomassie brilliant blue. The standard molecular weight proteins were MW-SDS-70 and MW-SDS-6A (Sigma). The virus strains were purified on a glycerol–potassium tartrate gradient. (Lane 1) HEC C14 and (lane 2) HEC A14. Fig. 3 (right). Transfer of proteins from SDS-PAGE gels to nitrocellulose paper was performed according to a published method (*36, 37*). The electroelution was performed overnight at room temperature at 200 mA in tris-glycine-methanol buffer (*p*H 8.3). After the transfer was completed, the nitrocellulose paper was soaked for 1 hour at 37°C in blocking buffer (tris-HCl, 5 percent BSA, and 0.2 percent Nonidet P-40 at *p*H 7.4). The nitrocellulose paper was cut into strips and incubated with a serum sample for 2.5 hours in a rotator at room temperature. Each serum sample was diluted 1:100 in blocking buffer. The strips were then washed three times (10 minutes each) in tris-HCl, 0.2 percent NP-40, 0.1 percent SDS, and 0.25 percent sodium deoxycholate (*p*H 7.4). Lactoperoxidase ^{125}I-labeled (*31*) antibody to human immunoglobulin G (H and L chains) (Cappel) (10^6 cpm/ml) were incubated with each strip for 45 minutes. The strips were washed as before, and the final rinse was in distilled water. Dried nitrocellulose paper strips were then autoradiographed with Kodak X-Omat and intensifying screen (DuPont Cronex Lightning Plus) at −70°C for 3 to 6 hours and then developed. The virus strains were purified on a glycerol–potassium tartrate gradient. Acute- and convalescent-stage sera from two patients with NEC were treated with various antigens. (Lanes 1, 2, and 3) Reaction of acute-stage serum (H-1) with A14, C14, and OC43 antigens, respectively; (lanes 4, 5, and 6) reaction of convalescent-stage serum (H-2) with A14, C14, and OC43 antigens; (lanes 7, 8, and 9) reaction of acute-stage serum (McC-1) with A14, C14, and OC43 antigens; (lanes 10, 11, and 12) reaction of convalescent-stage serum (McC-2) with A14, C14, and OC43 antigens.

Gradient (5 to 17 percent) SDS-PAGE of the purified particles revealed the presence of at least five major bands corresponding to molecular sizes ranging from 190 to 23 kilodaltons (Fig. 2). Electrophoretically separated proteins (HEC A14 and C14, OC43 and 229E viruses, and human fetal intestinal homogenates) were blotted onto nitrocellulose paper for Western immunoblotting (see Fig. 3 for details on the technique used) (*37, 38*). The blotted proteins were allowed to react with dilutions (1:50 or 1:100) of the acute- and convalescent-stage sera from patients and serum samples from controls obtained during the Dallas NEC epidemic. Seven of seven convalescent-stage sera and two of 11 control sera reacted against HEC A14 and C14. Two patients showed seroconversion. The convalescent-stage sera did not react against OC43 and 229E viruses. Reactions occurred mainly with proteins corresponding to molecular sizes of 190, 120, and 50 kD. A reaction was seen with the 23-kD protein for some of the samples. The reaction to these antigens of acute- and convalescent-stage sera from

two patients with NEC is shown in Fig. 3.

Several in vitro systems, such as primary human embryonic kidney cells, human embryonic lung fibroblasts, HEP-2, Vero, and BHK cells, did not support the growth of the viral particles. Although attempts have been made to adapt the virus to a cellular substrate that can be more easily managed, human fetal intestinal organ culture appears to be the only reproducible system at present. Treatment of the cultures with trypsin appeared to facilitate the infection, since the treated cultures gave rise to higher yields of viral particles, as seen on electron microscopy, than did untrypsinized cultures.

In tests to date, the two strains isolated appear to be identical. Immunologic tests with specific antisera should allow verification of this finding and enable the establishment of possible antigenic relationships with other coronaviruses. Other workers have reported an association between coronaviruses and NEC or with serious gastrointestinal disease in nursery infants. These reports were based on observations of virus particles in stools by electron microscopy or immune electron microscopy (*19–21*). However, attempts to cultivate these particles were not successful. Our results lend further credence to the hypothesis of the existence of a human enteric coronavirus and suggest an association between these virus particles and cases of NEC observed in the Dallas epidemic. Further study is needed to substantiate this association, and a complete epidemiological investigation of the outbreak will be reported later.

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39. We thank L. Casey for invaluable cooperation, J. Hierholzer for providing the OC43 and 229E antigens and antisera, G. N. Woode for providing the Breda 1 and 2 specific antisera, and K. V. Holmes for providing the MHV antigen, the corresponding antiserum, and other reagents.

17 April 1985; accepted 16 July 1985

Recombinant Vaccinia Virus: Immunization Against Multiple Pathogens

Abstract. *The coding sequences for the hepatitis B virus surface antigen, the herpes simplex virus glycoprotein D, and the influenza virus hemagglutinin were inserted into a single vaccinia virus genome. Rabbits inoculated intravenously or intradermally with this polyvalent vaccinia virus recombinant produced antibodies reactive to all three authentic foreign antigens. In addition, the feasibility of multiple rounds of vaccination with recombinant vaccinia virus was demonstrated.*

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The ability to introduce endogenous inactive subgenomic fragments of vaccinia virus into infectious progeny virus by marker rescue techniques (*1, 2*) suggested that extensions of these protocols might allow for the insertion of foreign genetic material into vaccinia virus. This was initially demonstrated by the insertion and expression of DNA sequences derived from herpes simplex virus encoding thymidine kinase (*3, 4*). One of the notable uses of vaccinia virus expressing foreign genes is the potential generic approach for the production of live recombinant vaccines directed against heterologous pathogens. Examples of recombinant vaccinia viruses expressing the hepatitis B virus surface antigen (HBsAg) (*5, 6*), the herpes simplex virus glycoprotein D (HSVgD) (*5, 7, 8*), the influenza virus hemagglutinin (InfHA) (*9, 10*), the rabies glycoprotein (*11, 12*), the *Plasmodium knowlesi* sporozoite antigen (*13*), and the vesicular stomatitis virus G protein (*14*) have been described. In all cases, expression of the foreign gene in vitro was, by all biochemical and biophysical criteria applied, similar to, if not identical with, the gene product synthesized under native conditions. Vaccination of laboratory animals with recombinant vaccinia produced antibodies capable of neutralizing the infectivity of correlate viruses (*5, 7, 9, 12, 14*), induced specific cytotoxic T-lymphocytes (*12, 15*), and, significantly, protected laboratory animals on subsequent challenge with the correlate pathogen (*5, 7, 10, 12, 14, 16*).

An advantage of vaccinia for vaccine construction is the potential for the insertion of multiple foreign genetic elements within a single vaccinia virus genome. Such a polyvalent vaccine could elicit immunity to a number of heterologous infectious diseases with a single inoculation. We report here the construction of vaccinia virus recombinants expressing multiple foreign genes. The immunological responses to inoculation obtained with these polyvalent recombinant vaccinia viruses as well as data obtained from multiple vaccinations of laboratory animals are presented.

The modification of a spontaneously occurring viable deletion mutant (*17*) of vaccinia virus to express the 1780–base pair (bp) complementary DNA (cDNA) of the RNA segment encoding the InfHA has been detailed (*9*). This recombinant virus, vP53, was used as a substrate for insertion of the HSVgD coding sequence (*18*). The recombinant vaccinia virus,