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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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,

vP124, containing both the InfHA and the HSVgD was used in turn as substrate for insertion of the HBsAg coding sequence, thus generating the triple vaccinia virus recombinant vP168 (*19*). All three foreign coding sequences were inserted at nonessential sites in the vaccinia genome, and transcription of the inserted genes was right to left relative to the vaccinia genome. The HSVgD, the HBsAg, and the InfHA sequences were inserted 23.9, 25.1, and 34.1 kilobases (kb), respectively, from the left terminus of the vaccinia genome, as referenced, to the L variant vaccinia genotype (*17*) (Fig. 1A).

The recombinant vaccinia virus, vP168, was plaque-purified, amplified to high titer, and highly purified on sucrose gradients (*20*). The DNA was extracted and cleaved with Hind III or Bam HI restriction endonucleases. The DNA fragments were separated on agarose, blotted onto GeneScreen Plus (DuPont) by the Southern method (*21*), and probed with ^{32}P-labeled nick-translated sequences homologous to the foreign genes (Fig. 1B). Hind III digestion released a 1090-bp fragment (Fig. 1B, lane 2) equivalent in size to a purified HBsAg sequence (lane 1) and a 1338-bp fragment (lane 2) equivalent in size to a purified HSVgD sequence (lane 3). The third band in lane 2 represents the internal vaccinia Hind III F fragment harboring the InfHA sequences inserted at the unique Bam HI site (*9*) (Fig. 1A). The InfHA sequences, which were excised from the recombinant vaccinia genome by Bam HI digestion (lane 4), migrated to a size of 1780 bp, equivalent to the size of purified HA sequences (Fig. 1B, lane 5). With Bam HI digestion the HSVgD sequences reside within a larger vaccinia fragment of approximately 4 kb. HBsAg sequences are contained in the same 4-kb fragment as well as in a larger 9-kb fragment (Fig. 1A). The results with a mixed HSVgD, HBsAg, and InfHA probe (Fig. 1B, lanes 1 to 5) were confirmed by the use of individual HBsAg, HSVgD, and InfHA probes (Fig. 1B, lanes 6 to 8, respectively). The presence of additional faint bands containing HSVgD sequences (Fig. 1B, lanes 4 and 7) are due to minor rearrangements of the vaccinia genome.

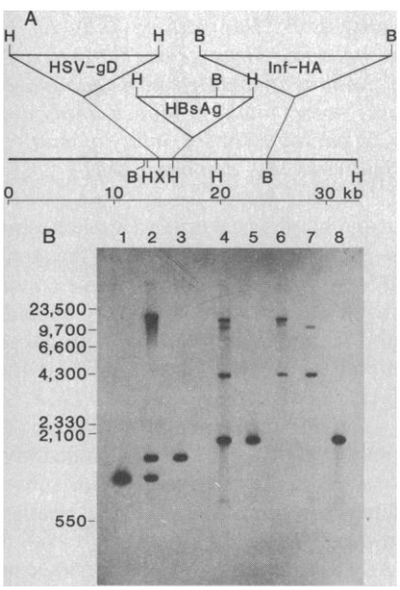

Fig. 1. (A) Structure of the vaccinia virus recombinant harboring the HSVgD, HBsAg, and InfHA coding sequences. The leftmost 30 kb of the vaccinia S variant genome (*17*) encompassing the terminal Hind III F, M, K, and internal F fragments are shown. The restriction sites identified in Fig. 1A are B (Bam HI) and H (Hind III). The X denotes the deletion of approximately 0.6 kb of vaccinia sequences described in the construction of pMP62-15 (*18*). (B) Southern blot hybridization analysis of the vaccinia virus recombinant vP168. DNA was extracted from purified vaccinia virus, digested with restriction endonucleases, and blotted to GeneScreen Plus after electrophoretic separation of the DNA fragments on agarose. vP168 DNA was digested with Hind III (lane 2) or with Bam HI (lane 4 and lanes 6 to 8) and probed with a mixture (lanes 1 to 5) or single (lanes 6 to 8) ^{32}P-labeled nick-translated HBsAg, HSVgD, and InfHA sequences. Purified DNA fragments containing the coding sequences for HBsAg (lane 1), HSVgD (lane 3), and InfHA (lane 5) were run as standards. Sizes of the fragments were obtained with Hind III–cleaved λ DNA as markers.

The expression in vitro of the InfHA, HSVgD, and HBsAg genes by the vaccinia virus recombinant vP168 was detected by means of serological tests (Fig. 2). BSC-40 cells infected with vP53, containing the InfHA and its engineered offspring vP124 and vP168, were shown to express the InfHA by radioimmunoassay. No radioimmunological reactivity with antiserum to InfHA was observed in the cells infected with wild-type (thymidine-kinase–deficient) vaccinia virus, VTK$^-$79. Similar radioimmunoassays with antiserum to herpes simplex virus gave positive signals of HSVgD expression when BSC-40 cells were infected with the recombinant vP124 and its engineered offspring vP168. No serological reactivity was observed when the cells were infected with either VTK$^-$79 or vP53. When a commercially available radioimmunoassay was used to detect HBsAg expression, positive results were obtained with the vP168 recombinant, whereas no serological reactivity was demonstrated with cells infected with its progenitors vP124 and vP53 or with VTK$^-$79.

Rabbits were inoculated either intravenously or intradermally with the vaccinia virus recombinant vP168. Serum samples were obtained weekly and screened for antibodies to the foreign antigens (Table 1). Both rabbits responded by making antibodies to all three of the foreign antigens synthesized under vaccinia virus regulation. The intravenous route of inoculation also provides a criterion for the absence of toxicity in the preparation of the vaccinia recombinant.

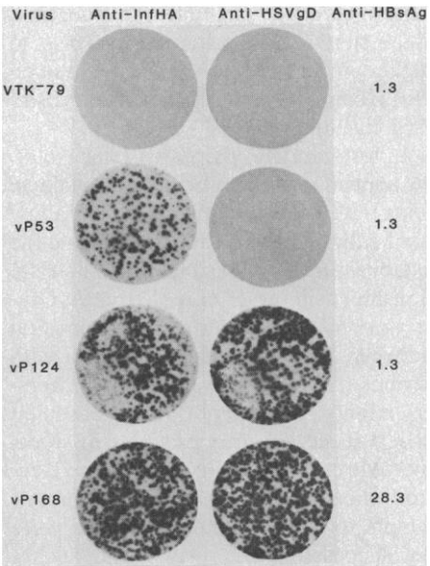

Fig. 2. Detection of InfHA, HSVgD, and HBsAg expression by vaccinia virus recombinants. BSC-40 cells were infected with either wild-type vaccinia virus, VTK$^-$79, or the vaccinia virus recombinants containing coding sequences for the InfHA (vP53), the InfHA and HSVgD (vP124), or the InfHA, HSVgD, and HBsAg (vP168). Two days after infection, the plaques were visualized by neutral red staining. The monolayers were exposed to antiserum to InfHA or to HSV at room temperature for 1 hour. Unreacted antibody was removed by washing in phosphate-buffered saline, and ^{125}I-labeled protein A from *Staphylococcus aureus* was added as previously described (*9*). Unreacted ^{125}I-labeled protein A was removed by washing in saline. The monolayers were imprinted on nitrocellulose filters, dried, and radioautographed. Film densities indicate presence of antibody-bound ^{125}I-labeled protein A. Unlike the InfHA and the HSVgD, which are localized on the infected cell membrane, the HBsAg synthesized by vaccinia virus is excreted from the infected cells (*5*). Therefore, BSC-40–infected cell extracts were prepared by three cycles of freezing and thawing, and the presence of HBsAg was detected with a commercially available radioimmunoassay kit AUSRIA II (Abbott). The numbers represent the positive-to-negative ratios (P/N) as defined by the manufacturer. The horizontal columns represent BSC-40 cells infected with the wild-type VTK$^-$79 or the vP53, vP124, or vP168 recombinant vaccinia viruses. The vertical columns represent radioimmunoassays of unfixed, infected BSC-40 monolayers in which antiserum to InfHA or to HSVgD was used, or the P/N ratio as detected with the AUSRIA II kit.

To determine whether a primary vaccination precludes revaccination with vaccinia virus recombinants engineered to express novel foreign antigens, we used two approaches. A rabbit inoculated with a vaccinia virus recombinant expressing the HBsAg retained high titers of antibody to HBsAg for more than 1 year (Table 2). Revaccination with a vaccinia recombinant expressing the same foreign antigen led to increased levels of antibodies reactive with the HBsAg (Table 2, rabbit 488). Revaccination also induced increased antibody against vaccinia virus. Since the recombinant vaccinia harbors the genetic information encoding HBsAg, this anamnestic response is compatible with the interpretation that additional HBsAg was synthesized during replication of the recombinant vaccinia virus on revaccination. Direct evidence for the induction of antibodies reactive to a foreign antigen on revaccination of a previously immune animal was obtained as follows. A rabbit was inoculated initially with a vaccinia virus recombinant expressing the HBsAg (Table 2, rabbit 517). Antibodies to HBsAg persisted at high titer for a year beyond the initial inoculation, at which time the same rabbit was revaccinated with a vaccinia virus recombinant expressing the InfHA instead of the HBsAg. As expected, this revaccination had essentially no effect on the titer of antibodies to HBsAg. However, InfHA antibodies were detectable in the serum after the rabbit was revaccinated with the novel vaccinia virus recombinant expressing the InfHA.

Genetically engineered vaccinia virus harboring foreign genes encoding pertinent vaccine antigens can be used to immunize against a variety of foreign pathogens. Recombinant vaccinia virus can be used in a revaccination schedule either to give a booster effect or to elicit an immunological response to a different foreign antigen, even when there is already an immune response to vaccinia. In addition, one may elicit immunity against multiple foreign pathogens by constructing engineered vaccinia virus harboring coding sequences of multiple foreign antigens. Thus, a single vaccination would serve to immunize against various heterologous pathogens. The ability of the virus to accept large quantities of foreign DNA makes the construction of polyvalent recombinant vaccinia viruses possible. Indeed, as much as 20 (*9*) to 25 kb (*22*) of foreign DNA can be stably inserted into vaccinia virus vectors. Additional space for the insertion of foreign DNA can be obtained by using defined viable deletion mutants of the virus. One can readily think of polyvalent vaccinia virus vaccines genetically engineered to express foreign genetic information encoding several dozen antigens. Whether the immunological system could respond adequately to such an intrusion remains to be established. Nevertheless, as we have shown in this report, animals respond to at least three novel foreign antigens even in the presence of the several hundred endogenous vaccinia antigens that are produced on vaccinia infection. Polyvalent vaccinia-vectored vaccines would be useful in circumstances in which multiple serotypes of a given pathogen need to be considered—for example, in the construction of a veterinary vaccine directed against foot-and-mouth disease. The use of polyvalent recombinant vaccinia virus

Table 1. Immunological response to vaccination with a recombinant vaccinia expressing multiple foreign genes. Rabbits were inoculated with 5×10^7 plaque-forming units in 0.5 ml either intravenously (rabbit 257) or intradermally (rabbit 288) with a vaccinia recombinant expressing the HBsAg, HSVgD, and InfHA genes, and the immunological response was followed. Sera were tested for antibodies to HBsAg with the commercially available AUSAB radioimmunoassay (RIA) kit (Abbott); titers are expressed in RIA units per milliliter of serum as defined by the manufacturer. Antibodies to InfHA were measured by hemagglutination inhibition tests performed with chicken erythrocytes and four hemagglutinin units. The reciprocal of serum dilution is indicated. Plaque-reduction assays monitoring reduction of HSV or vaccinia virus infectivity were performed on CV-1 cells. The reciprocal of serum dilution giving greater than 50 percent reduction in plaque number is shown.

Weeks after inoculation	Anti-HBsAg (RIA units)	Anti-InfHA	Anti-HSV	Anti-vaccinia
		Rabbit 257		
1	160	160	640	6,400
2	160	160	5,120	6,400
3	160	320	2,560	12,800
4	670	320	2,560	12,800
5	1,840	640	1,280	12,800
		Rabbit 288		
1	80	20	<40	<40
2	160	80	320	400
3	360	40	160	400
4	760	80	320	400
5	760	80	160	400

Table 2. Revaccination of rabbits with recombinant vaccinia virus. Rabbits inoculated intradermally with 1.8×10^7 plaque-forming units of a vaccinia virus recombinant expressing the HBsAg were revaccinated at 56 weeks with an equivalent dose of a vaccinia virus recombinant expressing HBsAg (rabbit 488) or revaccinated at 54 weeks with a novel vaccinia virus recombinant expressing the InfHA (rabbit 517). Anti-HBsAg antibody levels were determined using a commercially available RIA kit from Abbott Laboratories and are noted in RIA units ($\times 10^{-3}$) per milliliter of serum as defined by the manufacturer. For plaque-reduction assays, vaccinia virus was mixed with dilutions of antisera and kept at 4°C overnight until they were titrated on CV-1 monolayers. The percent reduction of plaques obtained on CV-1 monolayers are indicated as the reciprocal of the serum dilution. Antibody to InfHA was determined by standard hemagglutination inhibition tests with chicken erythrocytes and four HA units. Reciprocal of serum dilution is shown.

Weeks after inoculation	Anti-HBsAg (RIA units)	Vaccinia virus plaque reduction (%)		Anti-InfHA
		1600	6400	
		Rabbit 488		
1	0.1	63	12	
3	0.4	69	35	
6	18.4	82	12	
54	6.8	41	0	
57	32.7	85	4	
62	24.3	90	67	
		Rabbit 517		
3	0.2			<10
6	16.0			<10
9	72.0			<10
40	128.0			<10
54	72.0			<10
55	72.0			40
56	63.0			160
57	43.0			80
58	63.0			160
59	63.0			320
60	63.0			640

vaccines would also skew the benefit-risk ratio of vaccination to the benefit side since the benefit to be derived would be that of immunity against the total number of heterologous pathogens, whereas the risk would be that of the single vaccination.

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18. A 1.8-kb fragment derived from vaccinia Hind III M was directionally cloned into the Hind III–Sal I region of pBR322, generating pMP62. An 800-bp Sph I–Bgl II vaccinia fragment isolated from pMP62 was subjected to partial Rsa I digestion and a 200-bp Rsa I–Bgl II fragment containing an early promoter upstream from the Rsa I site was isolated. The Rsa I terminus and the Sph I site of pMP62 were modified to contain Hind III sites. The Hind III–Bgl II fragment containing an early vaccinia promoter was then ligated into the modified Sph I–Bgl II region in pMP62, generating pMP62-15 and resulting in a deletion of 0.6 kb of vaccinia sequences. The 2.5-kb Hind III–Sst I fragment containing the HSVgD (*5*) coding sequence was trimmed by Nru I digestion (*23*). The 1330-bp fragment was modified by linker addition, and the Hind III–terminated HSVgD coding sequences were inserted into the Hind III site within the vaccinia sequences of pMP62-15, generating plasmid pLP2. This plasmid was used as donor DNA for in vivo recombination (*3*) with vP53, and a novel recombinant vaccinia virus, vP124, containing the HSVgD sequences in addition to the InfHA sequences, was isolated by replica filter plating (*3*).
19. A 2.7-kb Bgl II fragment spanning the vaccinia Hind III M-K junction and containing an internal Bgl II site was inserted into the Bam HI site of pBR322H$^-$, a pBR322 derivative having a deleted Hind III site; this generated pMP18. A 360-bp Hind III–Bam HI fragment containing an early vaccinia virus promoter derived from Hind III fragment I was directionally cloned at the single Hind III–Bgl II site of pMP18, eliminating 0.1 kb of endogenous vaccinia sequences; this generated a novel plasmid pMP18PP. A Hind III–terminated HBsAg coding sequence of 1090 bp (*5*) was inserted at the single Hind III site of pMP18PP in proper orientation with the vaccinia promoter. This donor plasmid pPP18sAg was used as donor DNA for in vivo recombination with vP124 and a novel recombinant vaccinia virus, vP168, containing the HBsAg sequences in addition to the InfHA and HSVgD sequences was isolated by replica filter plating. Plasmid constructions as well as recombinant vaccinia viruses were confirmed by restriction analysis.
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Growth Regulation of Human Melanocytes: Mitogenic Factors in Extracts of Melanoma, Astrocytoma, and Fibroblast Cell Lines

Abstract. *Melanocytes derived from fetal or adult skin do not propagate in vitro unless cultured in the presence of factors such as 12-O-tetradecanoylphorbol 13-acetate (TPA). In a search for physiological factors regulating the growth of melanocytes, extracts of various cultured cell types were tested. Factors produced by melanoma and astrocytoma cell lines support continued proliferation of melanocytes in the absence of TPA. WI-38, a fibroblast cell line derived from human embryonic lung, was the most active source of melanocyte growth factors. No melanocyte growth-promoting activity was found in extracts of cultured neuroblastoma, renal cancer, normal keratinocytes, or renal epithelium. Nerve growth factor, epidermal growth factor, melanocyte-stimulating hormone, transforming growth factor-β, and platelet-derived growth factor did not have growth-promoting activity for melanocytes. The presence of melanocyte growth factors and TPA together resulted in the strongest mitogenic activity for melanocytes, permitting the recovery (at 20 days) of 4 to 20 times as many cells as in growth factor or TPA alone.*

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Melanocytes are the melanin pigment–producing cells of the body, which in normal human skin represent a minor cell population that undergoes mitosis only rarely (*1*). Little is known about melanocyte growth regulation, owing mainly to difficulties in obtaining sufficient numbers of melanocytes for studies in vitro. We showed earlier (*2*) that 12-*O*-tetradecanoylphorbol 13-acetate (TPA) fosters replication of melanocytes in vitro by permitting the preferential attachment of melanocytes from skin cell suspensions and stimulating them to grow. This mitogenic activity of TPA can be potentiated by cholera toxin (*2*) and isobutylmethylxanthine (*3*). In contrast to melanocytes, human melanoma cells generally grow vigorously in vitro in the absence of TPA, suggesting that their independent growth might be associated with the production of melanocyte growth factor or factors. To pursue this idea, we began a search for factors produced by melanoma and other cell types that would permit growth of melanocytes in the absence of TPA.

Melanocytes were isolated and cultured as described (*2*). To avoid fibroblast contamination, we trypsinized the cultures and purified them by immune rosetting and Percoll gradients (*4*), or we treated them with geneticin (*3*). Cultures used for experiments were free of contaminating fibroblasts as shown by leucine-aminopeptidase staining (*5*). Nonpigmented melanoma cell lines were selected for study to avoid the toxic effects of intermediate metabolites in melanin synthesis. In preliminary studies, we evaluated melanocyte growth-stimulating activity in the supernatant fluids of four melanoma cell lines. Since little or no activity was detected, we turned to cell extracts.

Figure 1 illustrates tests with extracts derived from two cell lines of neuroectodermal origin—namely, SK-MEL-131 melanoma and AO_2V_4 astrocytoma—and from the embryonic lung fibroblast line WI-38. Melanocytes cultured in the absence of TPA showed minimal [^{3}H]thymidine incorporation and no cell growth. In the presence of SK-MEL-131, AO_2V_4, or WI-38 extracts, melanocytes showed active [^{3}H]thymidine incorporation and repeated rounds of cell division. Extracts of SK-MEL-131 or AO_2V_4 resulted in a sixfold increase in [^{3}H]thymidine incorporation (as compared to melanocytes grown in the absence of added extract or TPA). Extracts of WI-38 resulted in a 20-fold increase in [^{3}H]thymidine incorporation. With regard to cell growth and division, melanocytes cultured in the absence of TPA generally round up and detach, and after 20 days cultures are lost. The effect of cell extracts on melanocyte growth parallels their effect on [^{3}H]thymidine incorporation, with extracts of WI-38 exerting the strongest mitogenic activity for melanocytes. Titration experiments showed that extracts diluted 1:500 to 1:1000 gave optimal stimulation of melanocyte growth; activity could still be detected at dilutions of 1:10,000. Growth inhibition, most likely nonspecific, was found with extracts diluted less than 1:100. Eighty percent of the growth-stimulating activity of WI-38 extracts was removed