18. The radioimmune precipitation assays (RIPA) were done as follows. Radioactively labeled hepatoma lysates 1.5×10^6 count/min of each were reacted with 10 μl of antiserum for 60 minutes. The antigen-antibody complexes were precipitated with Formalin-fixed *S. aureus*. The pellets were washed twice with 500 m*M* LiCl, 100 m*M* tris (*p*H 8.5), and analyzed for radioactivity. Where competition with peptide is indicated, the antiserum was incubated with 100 μg of peptide for 60 minutes prior to the addition of the labeled antigen. All pellets were dissolved in 40 μl of sample buffer, boiled for 3 minutes, centrifuged to remove cell debris, and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis as described in Fig. 2. The gels were dried and autoradiographed.
19. Nitrocellulose strips were developed with a 1:50 dilution of antiserum as described in Fig. 2, with two exceptions: (i) 50 m*M* tris (*p*H 7.6) and 150 m*M* NaCl containing 1 percent bovine serum albumin, 5 percent fetal calf serum, and 0.2 percent Tween 20 was used in place of Blotto in all incubations and washes and (ii) the antigen-bound antibodies were detected with horseradish peroxidase–conjugated goat antibody to rabbit immunoglobulin G, and the complexes were developed with 4-chloro-1-napthol.
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Vaccinia Virus Recombinants: Expression of VSV Genes and Protective Immunization of Mice and Cattle

Abstract. *Vesicular stomatitis virus (VSV) causes a contagious disease of horses, cattle, and pigs. When DNA copies of messenger RNA's for the G or N proteins of VSV were linked to a vaccinia virus promoter and inserted into the vaccinia genome, the recombinants retained infectivity and synthesized VSV polypeptides. After intradermal vaccination with live recombinant virus expressing the G protein, mice produced VSV-neutralizing antibodies and were protected against lethal encephalitis upon intravenous challenge with VSV. In cattle, the degree of protection against intradermalingually injected VSV was correlated with the level of neutralizing antibody produced following vaccination.*

The development of vaccinia virus as an infectious eukaryotic cloning vector (*1–3*) suggests a novel alternative to vaccines derived from whole viruses or viral subunits. Partly because of the role of vaccinia virus as a smallpox vaccine, attention has focused on the construction of recombinant viruses expressing genes of human pathogens (*4–10*). However, the origin of vaccinia virus from cowpox or a related virus and its ability to infect a variety of domesticated animals raise the possibility of veterinary as well as medical uses. Vesicular stomatitis virus (VSV), a member of the rhabdovirus family, causes a contagious disease of horses, cattle, and pigs, characterized by vesicular lesions on the tongue and oral mucosa. Severe outbreaks of the New Jersey serotype of VSV (VSV_{NJ}) have caused substantial economic losses in the United States, Mexico, and parts of Central and South America.

VSV contains a single negative strand of RNA which encodes five messenger RNA's (mRNA's) and five proteins. Complementary DNA copies of mRNA for the G, M, N, and NS proteins of VSV have been cloned and sequenced (*11–13*), and the G and N genes of the Indiana serotype (VSV_I) have been expressed in eukaryotic cells (*14, 15*). To facilitate cloning of the G and N genes into vaccinia virus, a previously used plasmid vector, pGS20 (*3*), was modified (*16*) to contain a single Xho I site downstream of a promoter region that was isolated from a vaccinia virus gene encoding a 7500-dalton (7.5-kD) protein. Into this plasmid, designated pMM34, we placed the G or N gene from VSV_I so that the vaccinia virus transcriptional and VSV translational start sites were juxtaposed. The resulting chimeric gene was flanked by segments of the vaccinia virus thymidine kinase (TK) gene. The G protein gene from VSV_{NJ} was modified (*17*) so that it also could be inserted into the Xho I site of pMM34 as well as into the single Sal I site of another plasmid vector, pLTP1, which contains the promoter region of a vaccinia virus gene encoding a protein of 28 kD (*18*).

Vaccinia virus hybrids containing the chimeric genes were formed by homologous recombination, selected on the basis of their TK^- phenotype and identified by hybridization to a ^{32}P-labeled

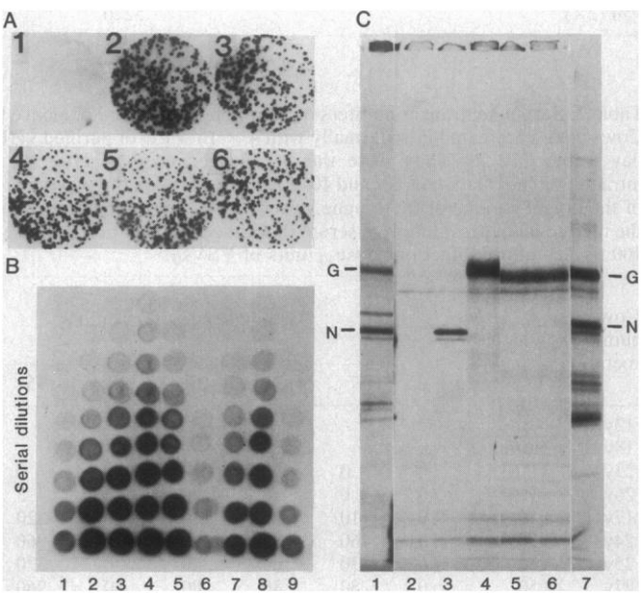

Fig. 1. (A) Antibody binding to recombinant virus plaques. Duplicate monolayers of CV-1 cells contained plaques produced by wild-type vaccinia virus (dishes 1 and 4), recombinant virus v50 (dishes 2 and 5) or v51 (dishes 3 and 6). Binding of antiserum to VSV_{NJ} (dishes 1 to 3) or vaccinia virus (dishes 4 to 6) was followed by incubation with ^{125}I-labeled staphylococcal A protein (*9*). Autoradiographs are shown. (B) Kinetics of expression of chimeric genes. Monolayers of CV-1 cells were infected with 30 PFU per cell of wild-type vaccinia virus or recombinants v50 or v51 in the presence or absence of arabinosylcytosine (Ara-C) at a concentration of 40 μg/ml. Serial twofold dilutions of cytoplasmic extracts at varying times after infection were spotted on nitrocellulose paper and were then incubated with antiserum to VSV_{NJ}, washed, and incubated with ^{125}I-labeled staphylococcal A protein. An autoradiograph is shown. Series 1, wild-type vaccinia virus, 6 hours; series 2, v50, 2 hours; series 3, v50, 6 hours; series 4, v50, 12 hours; series 5, v50, 12 hours, Ara-C; series 6, v51, 2 hours; series 7, v51, 6 hours; series 8, v51, 12 hours; series 9, v51, 12 hours, Ara-C. (C) Characterization of VSV polypeptides made by vaccinia virus recombinants. Monolayers (25 cm^2) of CV-1 cells were infected with 30 PFU per cell of wild-type or recombinant vaccinia virus or with VSV_I or VSV_{NJ} in medium containing 0.01 m*M* methionine. After 2 hours at 37°C, 100 μCi of [^{35}S]methionine (1000 Ci/mmol) was added and the incubation was continued for an additional 10 hours. The preparation of cytoplasmic extracts, immunoprecipitation, and polyacrylamide gel electrophoresis was similar to that described (*4*). Antiserum to VSV_{NJ} was used to precipitate polypeptides from cells infected with VSV_{NJ}, v50 and v51 whereas antiserum to VSV_I was used for polypeptides from cells infected with VSV_I, wild-type vaccinia virus, v37 and v38. Series 1, VSV_I; series 2, wild-type vaccinia virus; series 3, v38; series 4, v37; series 5, v50; series 6, v51; series 7, VSV_{NJ}.

VSV DNA probe and restriction endonuclease analysis (*2*). Vaccinia virus recombinants v37, v38, and v50 contain genes for VSV_I G, VSV_I N, or VSV_{NJ} G proteins, respectively, under control of the vaccinia virus promoter for the 7.5-kD protein gene. Recombinant v51 contains the gene for the VSV_{NJ} G protein under control of the vaccinia virus promoter for the 28-kD protein gene.

Evidence for expression of the VSV genes and an indication of the purity and stability of the recombinant viruses were obtained by binding antibody directly to virus plaques (Fig. 1A). Direct comparisons of stained cell monolayers and autoradiographs indicated that all v50 and v51 plaques bound antibody raised against VSV_{NJ}. Similar results were obtained when v37 and v38 plaques were incubated with the appropriate VSV_I or VSV_{NJ} antibody (*19*).

Antibody binding was used to detect the VSV_{NJ} G protein in lysates of cells infected with v50 and v51. Examination of autoradiographs indicated that G protein accumulated during a 12-hour period (or more) in cells infected with v50 or v51 (Fig. 1B). However, expression was first detected at 2 hours after infection with v50 and at 6 hours after infection with v51. These kinetics are consistent with previous data indicating that the promoter of the 7.5-kD protein gene (used for v50) is active at both early and late times after infection (*3*) whereas the promoter for the 28-kD protein gene (used for v51) is active only at late times (*18*). Also, arabinosylcytosine, an inhibitor of DNA replication and consequently of late protein synthesis, prevented expression of G protein in cells infected with v51 (Fig. 1B).

To characterize the recombinant products further, infected cells were labeled with [^{35}S]methionine. The polypeptides were immunoprecipitated with antiserum to VSV, dissociated with sodium dodecyl sulfate, and resolved by polyacrylamide gel electrophoresis. Fluorographs revealed that v50 and v51 synthesized similar amounts of a polypeptide of approximately 65 kD that comigrated with G protein produced in cells infected with VSV_{NJ} (Fig. 1C). Similarly, v37 and v38 expressed immunoprecipitable polypeptides of approximately 67 kD and 45 kD that comigrated with authentic VSV_I G and N proteins, respectively. The correct sizes of the G proteins produced by the recombinants suggest that the extent of glycosylation is similar to that occurring in cells infected with VSV.

In cells infected with VSV, the G protein is transported through the Golgi apparatus to the cell surface. Immunofluorescence of cells infected with recombinant vaccinia viruses v50 and v51 showed cell surface labeling with VSV antiserum. Internal labeling of the same cells after permeabilization showed strong fluorescence of the Golgi region, which is typical of normal G protein (*14*). In contrast, the VSV antiserum did not stain the surface of cells infected with wild-type vaccinia virus although there was faint nonspecific staining within permeabilized cells.

Of the antibodies to known proteins of VSV, only antibody to G protein has been shown to be protective in mice (*20*). After intradermal inoculation of mice with a vaccinia virus recombinant that expresses the G protein, significant VSV neutralization titers were detected by day 14 and increased over a 42-day period (Table 1). Half of the mice were given a booster vaccination on day 28, which resulted in an increase in serum VSV neutralization titers of about sevenfold over that obtained with a single vaccination.

The challenge was carried out by intravenous injection of VSV, which produces lethal encephalitis in mice within 6 to 12 days. For control purposes, a group of mice was vaccinated with vHBs4 (*4*), a vaccinia virus recombinant that expresses the hepatitis B virus surface antigen gene. When this group was challenged, 7 of 11 animals died of encephalitis. By contrast, only 1 of 15 mice that received a primary vaccination with v50 died and none of 16 that received two vaccinations died (Table 1).

To further evaluate the immunogenicity of the recombinant virus, cattle were vaccinated intradermally (on their backs) with either v50 or vHBs4. The typical pox lesions were confined to the sites of inoculation and, 2 weeks after vaccination, they were covered by dry scabs which eventually fell off. The cattle vaccinated with v50 developed significant VSV neutralization titers which were boosted severalfold by a second vaccination on day 28 (Table 2). On day 44 the cattle were injected intradermalingually with 10^2 and 10^3 plaque-forming units (PFU) of VSV_{NJ}. All unvaccinated cows and control cows vaccinated with a recombinant expressing the hepatitis B

Table 1. Average serum neutralization titers of vaccinated mice and response to challenge with VSV_{NJ}. Mice were vaccinated intradermally with 10^5 PFU of purified vHBs4 or v50 at a single site in the caudal fold of the tail. All mice received a primary vaccination on day 0 and half (designated 2×) received a booster vaccination on day 28. Serum neutralization titers are expressed as the reciprocal of the dilution of serum that gave complete protection against the cytopathic effect of 100 tissue culture infectious $dose_{50}$ units of VSV_{NJ}. Mice were challenged 44 days after the primary vaccination with 10^8 PFU of VSV_{NJ} by intravenous administration in the tail vein.

Group	Titers on day				Mice	
	6	14	28	42	Challenged (No.)	Died (No.)
vHBs4	0	0	0	0	11	7
v50	10	20	420	760	15	1
v50 (2×)				5220	16	0

Table 2. Serum neutralization titers of vaccinated cattle and response to challenge with VSV_{NJ}. Cows were vaccinated intradermally with 4×10^8 PFU of purified vHBs4 or v50 at four sites on day 0 and day 28. They were then challenged 44 days after the primary vaccination by intradermal inoculation of 10^2 and 10^3 PFU of VSV_{NJ} on the two upper and two lower quadrants of the dorsal surface of the tongue, respectively. Serum neutralization titers are expressed as the reciprocal of the dilution of serum that gave complete protection against cytopathic effect of 100 tissue culture infectious $dose_{50}$ units of VSV_{NJ}.

Cow number	Vaccine	Prechallenge titers on day							Vesicular lesion (PFU)	
		0	7	14	21	28	35	44	10^2	10^3
12y	None								+	+
18y	None								+	+
23y	vHBs4	0	0	0	0	0	0	0	+	+
26y	vHBs4	0	0	0	0	5	5	30	+	+
17y	v50	0	10	120	120	160	320	640	+	+
24y	v50	0	160	480	480	320	960	2560	−	+
25y	v50	0	30	160	240	80	320	480	+	+
27y	v50	0	80	80	80	80	960	1920	−	+
28y	v50	0	80	160	240	320	640	1280	−	+
29y	v50	0	160	160	160	160	1280	2560	−	+

virus surface antigen developed typical vesicular lesions on the tongue at the 10^2 PFU and 10^3 PFU VSV injection sites within 2 days (Table 2). In contrast, only two of six cows vaccinated with v50 developed vesicular lesions at the lower challenge dose of VSV. The remaining four cows that were vaccinated with v50 had no lesions at the 10^2 PFU VSV injection sites although they all developed lesions at the 10^3 PFU sites. In one cow (28Y) there was partial protection even against the higher VSV challenge dose since the lesion at the 10^3 PFU site remained localized. In all other cows the lesion spread to the entire surface of the tongue.

A good correlation was observed between antibody titers and protection against 10^2 PFU of VSV. All animals that were protected had neutralizing antibody titers of 1280 or greater whereas those that were sensitive had titers of 640 or lower. The degree of protection obtained is particularly noteworthy in view of the severity of the experimental challenge.

Previous experience with mass production, storage, and administration of smallpox vaccine suggests that vaccinia virus or related poxvirus recombinants would be economically feasible as veterinary vaccines. Moreover, the ability of recombinant vaccinia virus to stimulate a cytotoxic T-cell response specific for a cloned surface glycoprotein (*21*) may provide a significant advantage over subunit or inactivated whole vaccines, which in general do not prime effectively for cell mediated immunity. The large capacity of vaccinia virus for foreign DNA (*22*) also raises the possibility of multivalent vaccines for different serotypes of the same virus or even against entirely different pathogenic agents.

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16. Construction of pMM34. PGS20 (*3*) was cleaved with Xho I and partially digested with Ava I and a DNA fragment of approximately 5 kilobases (kb) was isolated. The ends of the latter were ligated together and used to transform *Escherichia coli* HB101. A resulting plasmid lacking an Xho I site was cleaved with Sma I and ligated to a phosphorylated Xho I linker. This plasmid was then cleaved with Hind III and partially digested with Pvu II. The 5′ overhanging end of the Hind III site was filled in with deoxyribonucleotides by means of the large fragment of DNA polymerase I. A 4.2-kb DNA segment was isolated. The ends of the DNA were ligated together and the resulting plasmid was designated pMM34.
17. Construction of pNJGE-2. The entire sequence coding for the VSV_{NJ} G protein was excised from pNJG6 (*13*) by partial digestion with Pst I, purified by polyacrylamide gel electrophoresis and trimmed with nuclease Bal 31 to remove the GC (G, guanine; C, cytosine) tails. Synthetic DNA linkers containing the Xho I site (CCTCGAGG) (C, cytosine; T, thymine; G, guanine; A, adenine) were then ligated to the trimmed DNA. This DNA was then cloned into the single Xho I site of plasmid JC119 (*15*). One plasmid obtained by this procedure (designated pNJGE-2) was found to have the sequence GGTATG . . . at the 5′ end following the Xho I linker. The ATG is the initiation codon for the VSV_{NJ} G protein.
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Fluorogenic Substrate Detection of Viable Intracellular and Extracellular Pathogenic Protozoa

Abstract. *Viable* Leishmania *promastigotes and amastigotes were detected by epifluorescence microscopy with fluorescein diacetate being used to mark living parasites and the nucleic acid–binding compound ethidium bromide to stain dead cells. This procedure is superior to other assays because it is faster and detects viable intracellular as well as extracellular* Leishmania. *Furthermore, destruction of intracellular pathogens by macrophages is more accurately determined with fluorescein diacetate than with other stains. The procedure may have applications in programs to develop drugs and vaccines against protozoa responsible for human and animal disease.*

Leishmaniasis causes more medical problems than any other protozoal disease except malaria. Phlebotomine sandfly vectors bite and infect man with promastigote stages of 13 *Leishmania* species. Within mononuclear phagocytes, motile promastigotes transform into small, nonmotile, oval amastigotes that induce species-dependent visceral or cutaneous disease. There are no safe, reliable vaccines, and chemotherapy is dangerous and is often ineffective (*1–4*).

Assays of cell viability are frequently required in research with pathogenic protozoa. *Leishmania* viability has been determined by [^{3}H]thymidine uptake (*5*), by parasite transformation or replication in vitro (*6, 7*), and by microscopic procedures with vital stains or dyes (*8–11*). However, radioisotopic procedures are not simple, some isolates grow poorly in vitro (*12*), vital staining may be difficult to detect (*9*), and no procedure rapidly identifies viable intracellular *Leishmania* by their metabolic activity.

We report the use of fluorescein diacetate (FDA) and ethidium bromide (EB) to detect viable intra- and extracellular *Leishmania* in a two-color epifluorescence microscope procedure (*13–16*). Esterases in living cells convert highly permeable, nonfluorescent FDA to fluorescein. Accumulated fluorescein imparts a striking yellow-green cellular fluorescence, termed fluorochromasia, to cells irradiated with light at 419 nm (*13*). Dead or damaged cells do not exhibit fluorochromasia and, unlike living cells, are rapidly penetrated by EB. Complexes of EB and nucleic acids cause dead cells illuminated at 546 nm to fluoresce red (*17*).

Living and dead *Leishmania donovani* promastigotes and intracellular amastigotes were incubated with EB and FDA and photographed on glass slides or in a