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Immunology

A vaccinia virus double recombinant expressing the F and H genes of rinderpest virus protects cattle against rinderpest and causes no pock lesions

(insertional inactivation of vaccinia genes/attenuation/vaccine development)

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ABSTRACT **Rinderpest is a highly contagious viral disease of ruminants with >95% morbidity and mortality. We have constructed an infectious vaccinia virus recombinant that expresses both the fusion (F) gene and the hemagglutinin (H) gene of rinderpest virus. The Wyeth strain of vaccinia virus was used for the construction of the recombinant. Cattle vaccinated with the recombinant virus were 100% protected from challenge inoculation with >1000 times the lethal dose of rinderpest virus. No transmission of recombinant vaccinia virus from vaccinated animals to contact animals was observed. The lyophilized form of vaccinia virus is thermostable and allows circumvention of the logistical problems associated with the distribution and administration of vaccines in the arid and hot regions of Asia and Africa. The insertional inactivation of both the thymidine kinase and the hemagglutinin genes of vaccinia virus led to increased attenuation of the virus; this was manifested by the lack of detectable pock lesions in vaccinated animals. This approach may have wide application in the development of safe and efficacious recombinant vaccines for humans and animals. This becomes quite relevant with the concern of the use of vaccinia virus in a population with high incidence of the human immunodeficiency virus.**

Rinderpest is a highly contagious viral disease of cattle, buffalo, and other ruminants in Africa and Asia. It is characterized by high fever, profuse bloody diarrhea, and >95% morbidity and mortality. The history of rinderpest's effects on cattle populations has been documented since 376 A.D. (1). Until recently, it has accounted for a loss of over two million cattle and buffalo per year. The etiological agent, the rinderpest virus (RPV), is a member of the family Paramyxoviridae and the genus *Morbillivirus*. Other antigenically related members of the morbillivirus group include pestes-des-petits-ruminants (PPR) virus of small ruminants (sheep and goats), measles virus of humans, canine distemper virus of dogs, and the morbillivirus of seals (2). Like other members of the paramyxovirus group, RPV has a single-stranded RNA genome with a negative polarity. We have characterized at least six proteins of the virus, including the large (L), the phosphoprotein (P), the hemagglutinin (H), the nucleoprotein (N), the fusion (F), and the membrane (M) proteins (3).

There is an effective cell culture-adapted vaccine for rinderpest (tissue culture rinderpest vaccine, TCRV) developed by the Muguga modification of the virulent Kabete O strain of RPV (1); however, its thermolability and high cost of production limit its use in the hot and arid regions of nomadic Africa and Asia (4). We have been engaged in the development of inexpensive and thermostable vaccinia virus (VV) recombinant vaccines for rinderpest. First, cDNA copies of the H and F genes of RPV were made and VV single recombinants expressing either of these genes were constructed (5–7). Cattle vaccinated with either recombinant expressing the F or the H gene were completely protected when challenge-inoculated with >1000 times the lethal dose of RPV. However, some of the cattle, particularly those vaccinated with the F recombinant, had significant anamnestic response to RPV after challenge inoculation; this indicates replication of the challenge virus (7). In contrast, no anamnestic response could be demonstrated in the groups vaccinated with a mixture of both recombinants or with TCRV. Others have confirmed our findings in a rabbit model by using VV single recombinants expressing the F (8) and H (9) genes of RPV. Since viral infection and spread are mediated by receptor binding (H) and membrane fusion (F), a vaccine preparation containing both antigens is superior to one containing either the H or the F glycoprotein alone (10, 11). The use of the mixture of recombinants in the field for vaccination against rinderpest is cumbersome and expensive, particularly taxing for developing countries. In this communication we describe the development of vRVFH, a highly attenuated VV double recombinant that expresses both the F and the H gene of RPV. Cattle vaccinated with vRVFH were 100% protected against a heavy challenge inoculation with RPV.

MATERIALS AND METHODS

Cells and Viruses. Vero (monkey kidney cell line), 143 TK$^-$ [thymidine kinase (TK$^-$) deficient human osteosarcoma cell line], and CV-1 (monkey kidney cell line) cells were propagated in Dulbecco's modified Eagle's medium supplemented with heat-inactivated fetal bovine serum. For the propagation of TK$^-$ cells, the medium was supplemented with 5-bromodeoxyuridine (BrdUrd) at 25 μg/ml. The highly pathogenic Kabete O strain of RPV was propagated in Vero cells and used in all studies. The Wyeth (New York City Board of Health) strain of VV (clone B-3-1), obtained from Flow Laboratories, was used exclusively for the generation of recombinants. All VV recombinants were propagated in Vero cells according to the guidelines of the Animal and Plant Health Inspection Service of the United States Department of Agriculture (USDA-APHIS) and the Office Internationale de Epizooties.

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Abbreviations: RPV, rinderpest virus; VV, vaccinia virus; TK, thymidine kinase; HA, hemagglutinin of VV; F, fusion; H, hemagglutinin of RPV; TCRV, tissue culture rinderpest vaccine; $TCID_{50}$, median tissue culture infective dose(s); pfu, plaque-forming units; SN, serum neutralization; USDA-APHIS, Animal and Plant Health Inspection Service of the United States Department of Agriculture.

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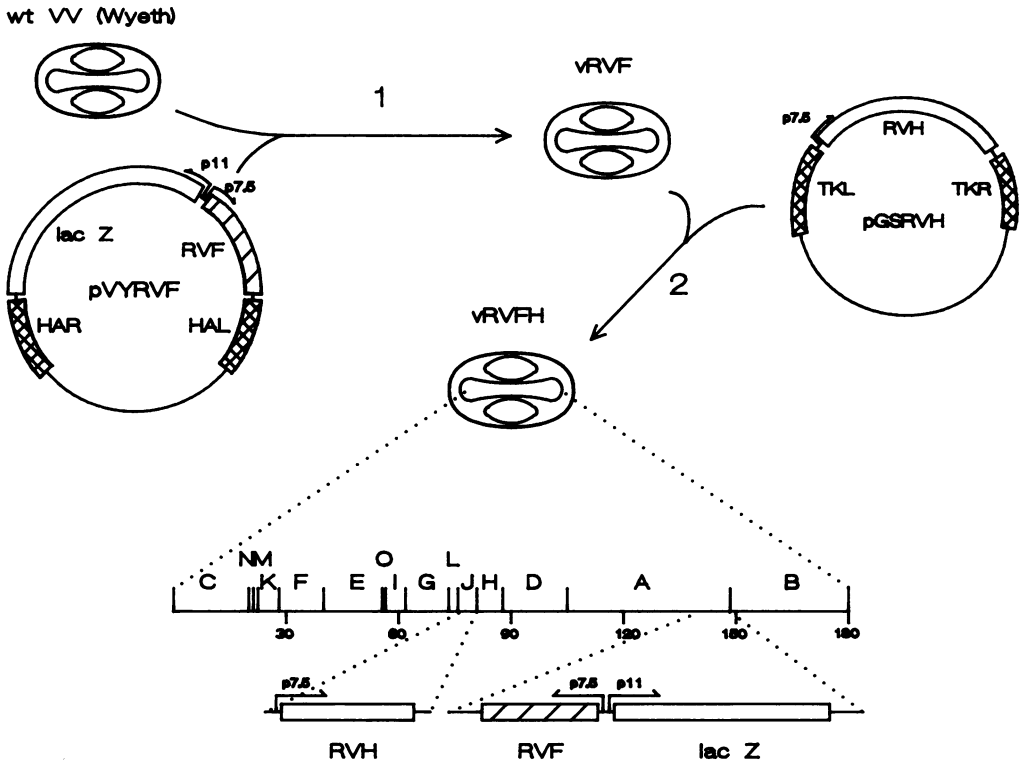

FIG. 1. Construction of a VV double recombinant (vRVFH) expressing both the F and H genes of RPV. In step 1, a VV single recombinant (vRVF) was generated by homologous recombination between plasmid vector pVYRVF and the Wyeth strain (wild-type, wt) of VV in CV-1 cells. In step 2, the VV double recombinant (vRVFH) was generated by homologous recombination between plasmid vector pGSRVH and the VV single recombinant (vRVF) in CV-1 cells. In the plasmid maps, the right and left halves of HA gene (HAR and HAL) and TK gene (TKR and TKL) are indicated, and the direction of transcription from the p7.5 and p11 promoter is shown; *lacZ* is the β-galactosidase marker gene. The linear map shows *Hin*dIII restriction fragments A–O; the scale is in kilobase pairs.

Recombinant Plasmids. The cloning and sequencing of full-length cDNAs coding for the H and F proteins of RPV have been described (5, 6). We utilized plasmid vectors pVY6 (12) and pGS53 (13) for the construction of vRVFH, the VV double recombinant expressing both the F and the H gene of RPV. Plasmid vectors pVY6 and pGS53 direct the cloning of genes in the hemagglutinin (HA) and the TK region of the VV genome, respectively.

Radioimmunoprecipitation. Proteins expressed by VV recombinants were characterized by radioimmunoprecipitation as described (3). Rabbit antiserum raised against the measles virus H protein was used for specific immunoprecipitation of the H protein of RPV. Rabbit antiserum directed against the carboxyl terminus of measles virus F protein was used for immunoprecipitation of the F protein of RPV. Except for the first amino acid, the octadecapeptide used for generation of the antiserum was completely conserved between measles virus and RPV (6, 14).

Immunization Studies in Cattle. Protective immune response studies were conducted in yearling cattle in the high-containment facility at the Plum Island Animal Disease Center (USDA-APHIS) according to proper institutional guidelines.[§] Cattle used in these experiments were shown to be seronegative to RPV and VV by serum neutralization (SN) and plaque reduction assays, respectively. One contact animal (no. 111), however, had crossreacting antibody to RPV that was not protective (see Table 1). Cattle were vaccinated with 10^8 plaque-forming units, (pfu) of VV recombinants by intradermal inoculation in the neck region. In addition, contact animals were housed with vaccinates to test for transmission of VV recombinants from vaccinated to non-vaccinated groups of animals. On the day of challenge inoculation, one additional cow was brought in as a fresh control to assure the presence of an animal susceptible to RPV, in case VV recombinants had been transmitted to contact animals. For the determination of protective immunity, all cattle were challenge inoculated with 10^3 median tissue culture infective doses ($TCID_{50}$) of RPV one month postvaccination. We demonstrated previously, in a study using 19 animals, that as little as 1 $TCID_{50}$ administered subcutaneously in the prescapular lymph node region induced clinical rinderpest with 100% mortality (7).

[§]The experiment was conducted with the approval of the USDA-APHIS (permits 15648 and 16261). The current Administrator of the USDA-APHIS is on the project's advisory panel selected by the United States Agency for International Development to ensure that no infraction of regulations occurs. The vaccine is now approved by the USDA-APHIS for field trial in Africa and Asia. When the field study is initiated, the head of the veterinary service of the host country will serve as a co-principal investigator on the project.

RESULTS

Construction of VV Recombinants. Previous reports described the generation of full-length cDNAs for the F and H genes of RPV (5, 6) and the construction of recombinants expressing either of these genes from the TK region of vaccinia virus (7).

Our strategy for the construction of vRVFH, the VV double recombinant expressing both the F and H genes of RPV, is outlined in Fig. 1. First, we constructed vRVF, the single recombinant expressing the F gene from the HA region of the VV genome. For this purpose, the F cDNA of RPV was excised from plasmid pVRVF6 (7) by digestion with *Eco*RI, filled in with Klenow DNA polymerase, and then cloned in the *Sma* I site of plasmid vector pVY6 to generate pVYRVF. The F gene was then cloned in the HA region of VV by homologous recombination between pVYRVF and the

Wyeth strain of VV in CV-1 cells. Recombinants expressing the F gene (vRVF) were selected by their blue phenotype in the presence of 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside. In preparation for the construction of vRVFH (double recombinant), we first excised the H gene of RPV from plasmid pRVH6 by digestion with *Eco*RI (7). The fragment was then filled in with Klenow polymerase and cloned in the *Sma* I site of the VV shuttle vector pGS53 to generate pGSRVH. The double recombinant (vRVFH) was generated by homologous recombination between pGSRVH and vRPVF in CV-1 cells (13). TK^- VVs were picked by BrdUrd selection, and double recombinants were distinguished from contaminating, spontaneous TK^- mutants by plaque hybridization with H cDNA of RPV.

Expression of F and H Proteins of RPV by VV Recombinants. The expression of authentic F and H proteins of RPV by vRVFH, F by vRVF, and H by vRVH was demonstrated by specific immunoprecipitation (Fig. 2). Fluorographs revealed that cells infected with vRVFH expressed both the F and the H protein of RPV of the expected molecular size. Similarly, the F protein expressed by vRVF and the H protein expressed by vRVH were of the correct size, suggesting that the extent of glycosylation was similar to that occurring in cells infected with RPV (3).

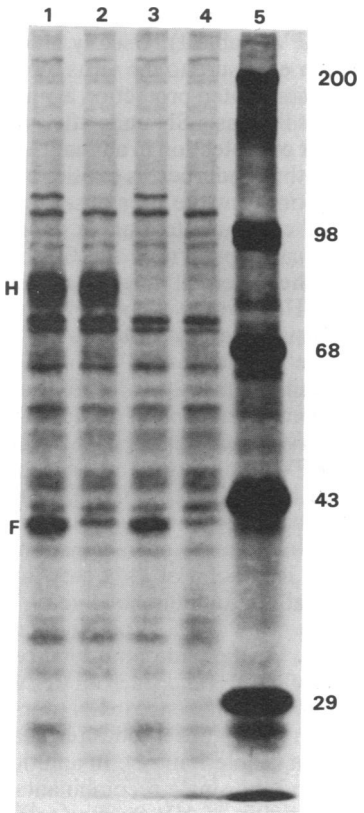

FIG. 2. Characterization of RPV polypeptides expressed by VV recombinants. Monolayers of human 143 TK^- cells were infected with VV (10 pfu per cell) in medium containing no methionine or cysteine. After 2 hr at 37°C, 100 μCi (3.7 MBq) of [^{35}S]methionine/[^{35}S]cysteine (Trans^{35}S-label, ICN) was added and the incubation was continued overnight (16 hr). A mixture of anti-measles F and anti-measles H antibodies was used to precipitate polypeptides from infected cells. Preparation of cytoplasmic extracts, immunoprecipitation, and polyacrylamide gel electrophoresis were performed as described (10). Lane 1, cells infected with VV double recombinant (vRVFH) expressing both the F and H genes of RPV; lane 2, cells infected with VV single recombinant (vRVH) expressing the H gene of RPV; lane 3, cells infected with VV single recombinant (vRVF) expressing the F gene of RPV; lane 4, cells infected with wild-type vaccinia virus (Wyeth); lane 5, molecular size markers (kDa).

Table 1. SN titers of cattle vaccinated with the double recombinant (vRVFH) expressing the F and H genes of RPV

	SN titer					
Cow	Day 0	Day 8	Day 14	Day 21	Day 28	Day 42
102	—	—	—	—	1	Dead
111	16	12	6	12	6	Dead
122	0	0	0	2	6	Dead
112	2	64	16	64	48	256
13	0	32	24	48	24	32
126	0	128	48	64	24	<100
134	0	48	64	64	96	96
135	0	12	64	32	64	64

Cow 102 was a control animal that was included in the group on the day of challenge (day 28). Animals 111 and 122 were unvaccinated, contact controls. The rest of the animals were vaccinated on day 0 with 10^8 pfu of vRVFH, and serum samples were taken weekly during the course of the experiment. SN titers were determined by the prevention of the cytopathic effects of 100 $TCID_{50}$ of RPV in Vero cells. On day 28, all animals were challenged with 1000 $TCID_{50}$ of the pathogenic Kabete O strain of RPV.

Immunization Studies in Cattle. Two groups of cattle were vaccinated with VV recombinants in separate isolation rooms. In the first group, five animals were vaccinated with vRVFH (Table 1). In the second group, four animals were vaccinated with a mixture of vRVF and vRVH (Table 2). In addition, two unvaccinated animals were included in each group to assess the transmissibility of VV recombinants from vaccinated to contact animals. Pock lesions developed as early as 4 days in cattle vaccinated with the mixture (Fig. 3*B*). The lesions were limited to the site of inoculation and were healed completely by 2 weeks postvaccination. In contrast, animals vaccinated with vRVFH developed no detectable pock lesions (Fig. 3*A*). A thorough examination failed to demonstrate pock lesions in the contact animals in both groups. Further, serum samples taken from contact animals on the days of vaccination (day 0) and challenge (day 28) were negative to VV by SN and plaque reduction assays (data not shown). All animals vaccinated with VV recombinants produced SN antibodies to RPV (Tables 1 and 2). One month after vaccination, all animals in both groups were challenge inoculated with 10^3 $TCID_{50}$ of the pathogenic Kabete O strain of RPV. Cattle vaccinated with VV recombinants (both groups) were completely protected from rinderpest, exhibiting no detectable illness and a normal temperature of 38°C. The four unvaccinated contacts (including no. 111, with the

Table 2. SN titers of cattle vaccinated with a mixture of VV single recombinants expressing the F (vRVF) and H (vRVH) genes of RPV

	SN titer					
Cow	Day 0	Day 8	Day 14	Day 21	Day 28	Day 42
121	0	0	0	0	0	Dead
133	0	3	0	1	0	Dead
101	0	24	24	96	128	256
118	0	128	64	24	12	64
124	0	24	32	64	96	64
131	0	96	384	32	128	64

Animals 121 and 133 were unvaccinated, contact controls. The rest of the animals were vaccinated on day 0 with 10^8 pfu of the vRVF/vRVH mixture, and serum samples were taken weekly during the course of the experiment. SN titers were determined by the prevention of the cytopathic effects of 100 $TCID_{50}$ of RPV in Vero cells. On day 28, all animals were challenged with 1000 $TCID_{50}$ of the pathogenic Kabete O strain of RPV.

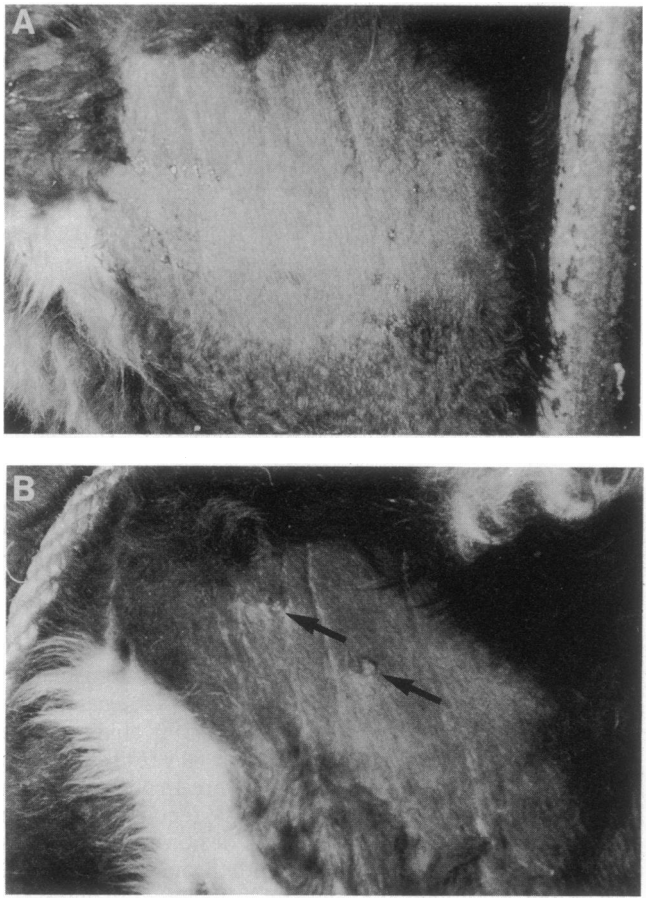

FIG. 3. Photographs of VV recombinant-induced pock lesions in the neck region of cows. Animals were vaccinated by intradermal inoculation at two sites in the shaved area of the skin. (*A*) Cow 126 was vaccinated with vRVFH (double recombinant); note the lack of detectable pock lesions at the sites of inoculation. (*B*) Cow 118 was vaccinated with an vRVF/vRVH mixture and developed pock lesions at the two sites of inoculation (see arrows).

crossreactive antibody to RPV) and the one control animal developed high fever (42°C) by day 2 and died by day 6 after challenge. They also developed lesions typical of severe rinderpest, characterized by sloughing and erosion of the epithelial lining of the gastrointestinal tract and bloody diarrhea. After daily monitoring for 2 weeks and a lack of detectable clinical disease in vaccinated animals, the experiment was terminated.

All cattle vaccinated with the recombinants produced SN antibodies to RPV as early as 8 days after vaccination. However, all contact and control animals lacked detectable SN antibodies to RPV during the course of the experiment.

DISCUSSION

Immune responses to both the H and F antigens of paramyxoviruses play an important role in the prevention and spread of the infection (10). The H protein initiates viral infection by binding to the receptor of the susceptible cell (15). In contrast, the F protein facilitates the spread of virus from infected to adjacent, normal cells by its fusogenic activities on cell membranes (16–18).

In our previous communication, we reported the construction of VV recombinants that expressed the F or the H glycoprotein of RPV (7). Further, we demonstrated that cattle vaccinated with either recombinant (F or H) or with a mixture of the two recombinants (F plus H) were completely protected against a challenge inoculation >1000 times the lethal dose of RPV. Although the single recombinants (F or H) alone provided 100% protection against rinderpest, humoral immune response of vaccinated cattle indicated the superiority of the mixture over the single recombinants. Unlike the single recombinants, the mixed (F plus H) vaccine provides protection against both the initial infection (H-mediated) and the secondary spread (F-mediated). In addition, inclusion of several immunologically relevant proteins in a single vaccine increases the number of available epitopes and thus the chances for protective immunity in an outbred population (11). Consequently, it is essential to employ a vaccine that provides both the F and the H immunogen for field use. However, the use of a mixture in the field is cumbersome and more expensive than use of a single VV recombinant. A mixture containing equivalent doses of vRVF and vRVH would be very difficult to produce by the scarification method in a calf and would require cell culture facilities for the quantification of infectious virus.

In this communication, we report the construction of a VV double recombinant (vRVFH) that expresses both the F and the H glycoprotein of RPV. Cattle vaccinated with the recombinant were completely protected against a challenge inoculation >1000 times the lethal dose of the virus. Indicating the enormity of the virus load, no contact or control animals survived beyond 6 days after the challenge inoculation, including a contact animal (no. 111) with a crossreactive antibody titer to RPV (Table 1). Usually animals can last 12 days or longer after exposure to the virus by natural contact. We previously demonstrated protection against rinderpest in animals showing as little as 4 SN units after vaccination with vRVF (7). The lack of protection in animal 111 (with 6–16 SN units) and the fact that rinderpest is exotic to North America are strong indications that the titer observed in animal 111 was the result of exposure to one or more of the other morbilliviruses of cattle present in the United States (19).

We recognize the potential safety problems with widespread use of VV. However, we have constructed the double recombinant by using the Wyeth strain of VV, which was used worldwide in the successful eradication of smallpox (20). It has been demonstrated that insertional inactivation of the TK gene further attenuates the virus (21). In the double recombinant, the TK region has been inactivated by insertion of the H gene of RPV. In addition, the insertional inactivation of the HA region by the F gene of RPV has led to further attenuation of the recombinant virus. The absence of detectable pock lesions at the site of vaccination by the double recombinant is an indication of its greater attenuation (Fig. 3*A*). This did not affect its immunogenicity, however, since it provided cattle with 100% protection against a heavy challenge of RPV. Further, in comparing results of groups vaccinated with vRVFH and the vRVF/vRVH mixture, there was no significant difference in SN titers. No significant anamnestic response was observed after challenge, indicating solid protection (Table 1 and 2). In addition, the safety of both the double and the single recombinants was confirmed by lack of transmission of VV from vaccinated to contact animals. No pock lesions or antibody to VV, as measured by SN and plaque reduction assays, could be demonstrated in contact animals. We believe that these outstanding safety features of the double recombinant make it highly suitable for field use. We also have the potential to further attenuate the recombinant virus by additional insertion of lymphokine genes such as those encoding interferon γ or interleukin 2 (22–25).

Many of the reasons for recrudescence of rinderpest in Africa are related to problems in vaccine production, preservation, and delivery. The continuous movement of nomadic herdsmen and their animals also makes it difficult to assemble groups for vaccination. We have developed an effective and safe VV double recombinant vaccine that

protects cattle against a severe challenge of RPV. Unlike TCRV, the production and use of a vaccinia vector vaccine do not require refrigeration, trained personnel, or expensive cell culture facilities. The VV recombinant vaccine can be produced with the same simple methods used during the World Health Organization smallpox eradication campaign; in that campaign, smallpox vaccine was produced by extensively scarifying the skin of a calf and seeding the wounds with VV (26).

In the large-scale vaccination program for rinderpest with TCRV, vaccination had to be repeated yearly for at least 2 years because calves under the age of 6 months could not be vaccinated. Inactivation of the vaccine virus by colostral antibody to RPV is a major problem in young calves. In contrast, calves can be vaccinated with the recombinant vaccine at any age, even in the presence of high levels of anti-RPV antibody, since anti-VV antibody is not normally found in cattle.

Rinderpest is an excellent candidate for eradication through use of the VV recombinant vaccine (27, 28). There is only one serotype of RPV, although there are different strains manifesting different degrees of pathogenicity in the field. A vaccine against one strain will immunize against all, including peste-des-petits-ruminants of sheep and goats (29). The potential to develop a polyvalent vaccine with a single VV recombinant expressing a number of heterologous genes has great significance, especially for nomadic regions of Africa and Asia (28).

The absence of pock lesions in animals vaccinated with the double recombinant, and the lack of transmission of the virus from vaccinated to contact animals, may have important application in the development of safe and efficacious recombinant vaccines for humans and animals. This becomes quite relevant with the concern of the use of VV in a population with high incidence of the human immunodeficiency virus.

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