PROTECTION OF RABBITS AGAINST LAPINIZED RINDERPEST VIRUS WITH PURIFIED ENVELOPE GLYCOPROTEINS OF PESTE-DES-PETITS-RUMINANTS AND RINDERPEST VIRUSES

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Summary. – Haemagglutinin (HA) and fusion (F) proteins of peste-des-petits-ruminants virus (PPRV) and rinderpest virus (RPV) were purified by immunoaffinity chromatography. The purified proteins were characterized by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE). Rabbit hyperimmune sera were raised against the purified HA and F proteins and assayed by enzyme-linked immunosorbent assay (ELISA), haemagglutination-inhibition (HAI) and virus neutralization (VN) tests. The immunized animals were challenged with a virulent lapinized (rabbit-adapted) strain of RPV. Both HA and F proteins of PPRV protected rabbits against a lethal challenge with lapinized RPV. As expected, RPV HA and F proteins also conferred a similar protection against the homologous challenge. The postchallenge antibody responses were of a true anamnestic type.

Key words: peste-des-petits-ruminants; rinderpest; vaccine; viruses; immunity

Introduction

PPRV and RPV, members of the *Paramyxoviridae* family, *Morbillivirus* genus, are highly pathogenic for small ruminants. Both viruses cause high morbidity and mortality (for review see Lefevre and Diallo, 1990). PPRV is serologically related to RPV but distinct from it as shown by cross-neutralization of sera, size of proteins and hybridization with DNA probes (Taylor, 1979; Diallo *et al.*, 1987, 1989). The disease is characterized by necrotizing and erosive stomatitis, enteritis and pneumonia (Gargadennec and Lalanne, 1942). PPRV can infect cattle which do not exhibit a clinical reaction (Diallo *et al.*, 1989). RPV can infect sheep, and the infection results in severe disease or a subclinical form that is not characterized by pneumonia (Zwart and Rowe, 1966). The envelopes of these viruses contain two main surface glycoproteins, the HA and F proteins, which are targets for the host immune defense mechanism. Evaluation of the relative roles of these components is a prerequisite for development of a subunit vaccine. The HA of morbilliviruses is devoid of neuraminidase activity unlike that of paramyxoviruses (Scheid *et al.*, 1972). In general, the HA is responsible for virus attachment to the cell receptor, the first step in infection (for review see Choppin *et al.*, 1981). The HA also mediates an important step of virus adsorption and is not a significant factor in determining the host range and tropism of the virus. The HA seems to be the major antigen involved in *in vitro* VN tests where the neutralizing antibodies interfere with virus adsorption (Orvell and Norrby, 1985). The other

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Abbreviations: BSA = bovine serum albumin; CPE = cytopathic effect; EDTA = ethylenediamine tetraacetate; ELISA = enzyme-linked immunosorbent assay; F = fusion; HA = haemagglutinin; HAI = hemagglutination-inhibition; HAU = haemagglutination unit; MoAb = monoclonal antibody; NP-40 = Nonidet P-40; p.i. = post inoculation; PMSF = phenylmethysulfonyl fluoride; PPRV = peste-des-petits-ruminants virus; RBC = red blood cell; RPV = rinderpest virus; SDS-PAGE = polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; TCRPV = tissue culture rinderpest virus; VN = virus neutralization

glycoprotein, F protein is involved in virus-induced cell fusion, a function that is related to the mechanism of virus entry into cells (Scheid and Choppin, 1974) and is primarily responsible for the haemolytic function of the virus. Thus, *in vivo*, an antibody response to the F protein appears to be essential for the prevention of spread of the infection from cell to cell within the host and for subsequent disease development (Merz *et al.*, 1980; Malvoisin and Wild, 1980).

In order to evaluate the protective capacities of HA and F protein of PPRV and RPV against homologous and heterologous challenge in laboratory experimental animals, the present study was undertaken. Although the immunization of small ruminants with a live attenuated tissue culture rinderpest vaccine (TCRPV) confers clinical protection against both RPV and PPRV, the vaccinees permit replication of PPRV in their tissues without any disease symptom (Gibbs *et al.*, 1979). However, it has also been reported that TCRPV-vaccinated goats exhibited pyrexia, abortion and clinical disease (Taylor, 1979). Besides, TCRPV needs extensive cold chain maintenance. Studies on recombinant and subunit vaccine candidates may eliminate the necessity for the development of a safe heat-stable vaccine to control PPRV and RPV in small ruminants. Additionally, such studies may help in devising better laboratory diagnostic tests to differentiate between vaccinated and naturally infected animals. In this regard, a study employing a double recombinant vaccinia virus expressing HA and F protein of RPV has been reported (Yilma *et al.*, 1992). The recombinant vaccine used in that study was shown to protect goats against a lethal challenge with PPRV. However, studies employing recombinant PPRV HA and F genes or proteins seem to be lacking. The present study was an attempt to test the possibility of utilization of purified HA and F protein of PPRV to protect rabbits against a challenge with the virulent lapinized (rabbit-adapted) RPV.

Materials and Methods

Rabbits. Four-week-old rabbits of either sex, each weighing 1.0 to 1.5 kg, were obtained from a local colony. All the animals were maintained in a well ventilated shed with proper feed. Animals were test bled from the marginal ear vein to determine the presence or absence of pre-existing specific antibodies.

Cells and viruses. The Indian strains of PPRV (TN 87/1) and RPV (86/1) (Subbarao *et al.*, 1990) were propagated in Vero cells. Confluent monolayers of cells were infected at a low multiplicity. After virus adsorption for 1 hr at 37°C, the cells were incubated in serum-free Dulbecco's Medium (Hi-media Laboratories Ltd., India) at 37°C. The cells were harvested at an advanced stage of cytopathic effect (CPE). For challenge studies involving rabbits, a velogenic lapinized RPV in the form of spleen suspension was utilized.

Purification of HA and F protein by affinity chromatography. Immunoaffinity chromatography was performed by using monoclonal antibodies (MoAbs) as described previously (Varasanyi *et al.*, 1984) with minor modifications. RPV HA-specific and measles virus F antigen-specific MoAbs were used for the purification of the respective proteins. Antigen preparations were made by lysing the PPRV- and SRPV-infected Vero cells in a lysis buffer containing 100 mmol/l Tris-HCl, pH 7.8, 150 mmol/l NaCl, 3% Triton X-100 and 3 mmol/l phenylmethylsulfonyl fluoride (PMSF). The cell lysate was then centrifuged at 222,000 x g at 4°C for 45 mins to remove the cell nuclei and viral nucleocapsids. The MoAbs were purified from mouse ascites. The immunoglobulins were cross-linked to CNBr-activated Sepharose 4B.

For HA antigen purification, the column containing an anti-RPV HA MoAb was equilibrated with 5 – 10 column volumes of a wash buffer (10 mmol/l Tris-HCl pH 7.8, 500 mmol/l NaCl, 1% Triton X-100 and 1 mmol/l PMSF) at 4°C. The clarified cell lysate was passed through the equilibrated column three times. The HA was desorbed with 200 mmol/l glycine-HCl pH 2.8, 0.2% Triton X-100 and 0.001% Neutral Red into 10 fractions. The presence of HA in these fractions was determined by SDS-PAGE, and the amount of protein was estimated according to the method of Bradford (1976). Fractions containing highest amounts of protein were pooled and neutralized with 1 mol/l Tris base.

For F antigen purification, the immunosorbent gel containing an anti-measles virus F1 MoAb was washed with the lysis buffer extensively. The gel was then mixed with the antigen and held on ice for 2 hrs with frequent agitation. The gel was then transferred to a column and washed with TEN buffer (10 mmol/l Tris-HCl pH 8.0, 1 mmol/l ethylenediamine tetraacetate (EDTA) and 0.1% Nonidet P-40 (NP-40)). The F protein was desorbed with 3 mol/l KSCN in TEN buffer into 10 fractions. The presence of F protein was determined by SDS-PAGE and its quantity was assayed similarly to HA (Bradford, 1976). The fractions with highest amounts of protein were pooled.

SDS-PAGE on 10% gels was done according to the standard procedure followed by silver staining as described by Sambrook *et al.* (1982).

Immunization of rabbits. Rabbits were divided into four groups of two each for immunization purposes. The detergent-soluble HA and F glycoproteins were used at 50 µg doses for primary and 25 µg doses for two booster immunizations. The immunizations were performed by a single subcutaneous injection of the appropriate antigen dose in 1.5 ml of saline with an equal volume of Freund's complete adjuvant (Sigma, USA). The first booster was given 21 days after the primary immunization, and the second booster 10 days after the primary booster. A similar group of unimmunized control rabbits was used at the end of the study for evaluation of protection to challenge infection. Serum samples were collected from the immunized animals at various intervals by bleeding from the marginal ear vein. The sera were heat-inactivated at 56°C for 30 mins and tested for antibody response as described below.

ELISA. Polyvinyl plates (Nunclon, Denmark) were coated with whole virus antigen (1 µg per well in 50 µl of saline) by incubation overnight at 4°C. After three washes with 0.05% Tween 20 in PBS (PBS-Tween 20), the plates were filled with 100 µl of 3% bovine serum albumin (BSA) (Sigma) in saline per well and incubated for 1 hr at 37°C to block remaining reactive sites. Following this

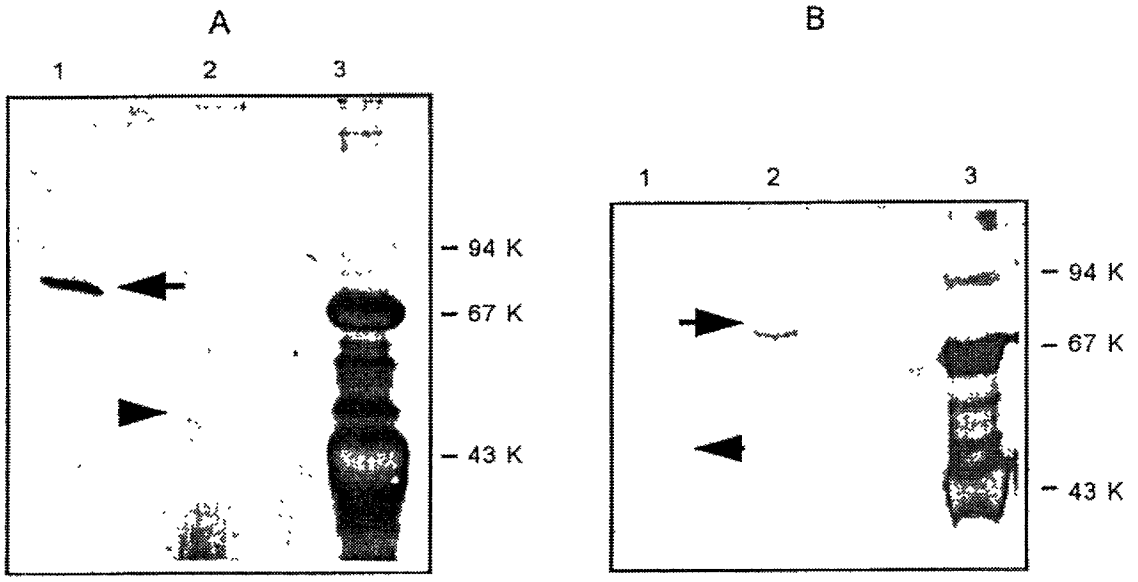

Fig. 1
SDS-PAGE of purified HA and F proteins of PPRV and RPV
A: PPRV. HA protein (lane 1), F protein (lane 2), size marker (lane 3) B. RPV. F protein (lane 1), HA protein (lane 2), size marker (lane 3).

step, 50 µl aliquots of serial two-fold dilutions of sera collected at different intervals (4, 14, 15, 16 and 20 weeks) post the second booster were added to the wells and incubated at 37°C for 1 hr. The plates were washed three times with PBS-Tween 20. This was followed by addition of an appropriate dilution of goat antibody to rabbit IgG conjugated with horseradish peroxidase to each well and incubation at 37°C for 1 hr. The plates were washed again and 50 µl of ortho-phenylenediamine (Sigma) in phosphate-citrate buffer (0.2 mol/l Na_2HPO_4 and 0.1 mol/l citric acid) pH 5.5 and 2 µl of 30% H_2O_2 was added per well. After incubation for 30 mins, the absorbance was read at 490 nm. The titre of the antibody was expressed as the reciprocal of the highest dilution of the serum giving a two-fold A_{490} of the control (sample without antibody).

HAI assay was performed in U-bottom microtitre plates (Dynatech) with sera from immunized rabbits. Serial two-fold dilutions of sera were made in PBS and applied in 25 µl aliquots to wells. Two HA units (HAU) of viral suspension (25 µl) was then added to each well and incubated at 37°C for 1 hr. Then 50 µl of 0.5% chicken red blood cells (RBCs) in saline was added per well. The plates were incubated at room temperature until the RBCs in control wells settled properly. The HAI titre was expressed as the reciprocal of the highest serum dilution showing HAI by two HAU of the antigen.

VN test. Serial two-fold dilutions of sera were mixed 1:1 with aliquots of virus suspension (100 $TCID_{50}$) in serum-free Dulbecco's Medium and incubated at 37°C for 1 hr. The mixtures were then added to Vero cell monolayers grown in microtitre plates (Nunclon). The VN titre was expressed as the reciprocal of the highest serum dilution of serum that inhibited CPE formation.

Results

Purification of HA and F proteins of PPRV and RPV

The M_r values of HA and F proteins of PPRV were 73.2 K and 46 K, while those of RPV were 74 K and 46 K, respectively (Figs. 1 A and B). Thus, one cycle of adsorption and desorption in the immunoaffinity chromatography produced highly purified preparations of HA and F proteins. Furthermore, the yields of purified HA and F proteins of PPRV from infected Vero cells were lower by about 50% than those of RPV (data not shown). This may indicate a lower affinity of the surface glycoproteins of PPRV to MoAbs employed for purification.

Antibody response of rabbits immunized with viral HA and F proteins

In this study, the rabbit was used as an animal model to evaluate the possible use of viral glycoproteins as subunit vaccines. Serum samples were collected 4, 14, 15, 16 and 20 weeks post immunization (p.i.) to evaluate the immune responses to viral envelope glycoproteins.

The specific antibody response was measured by ELISA using purified PPRV and RPV. Whereas the antibody to PPRV F protein showed a steady increase, that to PPRV HA protein increased only to week 14 and then declined (Fig. 2A). On the other hand, the antibodies to RPV HA

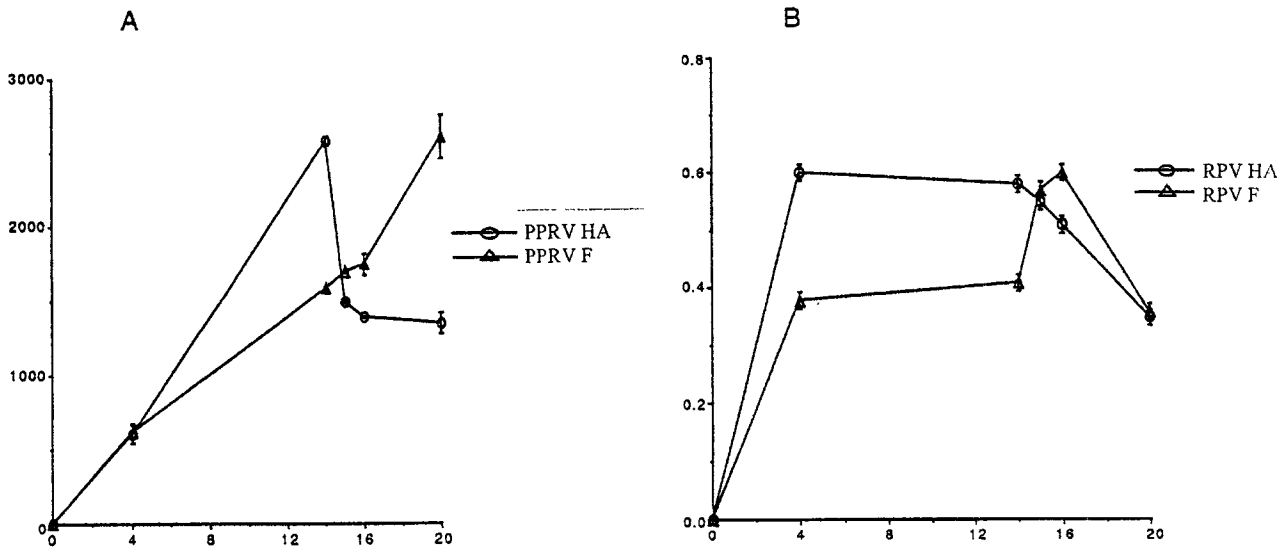

Fig. 2
Antibody responses to HA and F proteins of PPRV (A) and RPV (B)
A. Abscissa: weeks p.i. Ordinate: ELISA titre. B. Abscissa: weeks p.i. Ordinate: A_{490}.

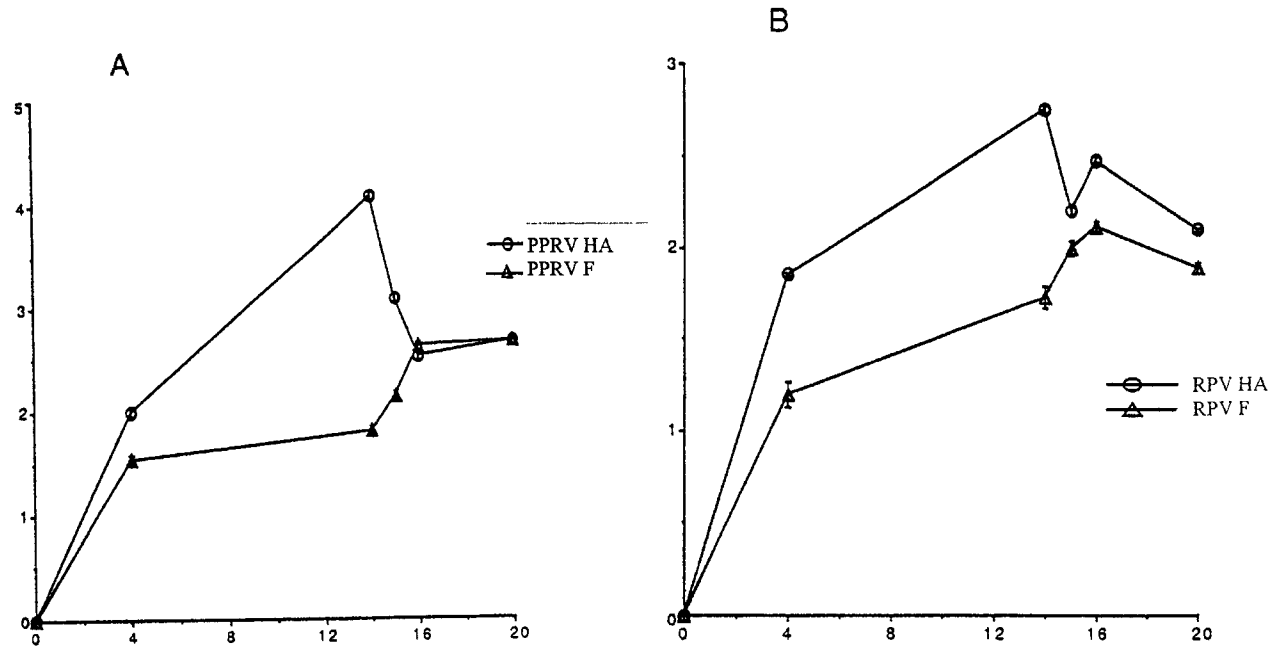

Fig. 3
VN titres of sera of immunized rabbits
A: PPRV. B: RPV. Abscissa: weeks p.i. Ordinate: VN titre.

and F proteins increased sharply within the first 4 weeks (the former antibody to a higher value than the latter) and then kept a plateau (Fig. 2B). The faster response to HA protein may indicate that this protein is immunodominant. These results show that the purified proteins have retained their immunogenicity.

The immune sera against HA and F proteins of PPRV and RPV were next analyzed by the VN test using PPRV and RPV. Whereas the antibodies to F proteins of both viruses increased steadily up to week 16, those to HA proteins of these viruses reached higher levels already at week 14 and then declined (Fig. 3). Taken together, these data suggest that antibodies to HA and F proteins neutralized their respective homologous viruses successfully.

A previous study has shown that purified PPRV can agglutinate chicken RBCs (Hegde, 1992). Therefore the immune sera against HA proteins of PPRV and RPV were subjected to the HAI test. The antibodies to HA proteins of PPRV and RPV inhibited the haemagglutination of chicken RBCs by PPRV. The HAI titres of the tested sera increased steadily up to week 14 and then declined or kept plateau (Fig. 4). Thus, these data suggest that the purified HA proteins retained their biological properties.

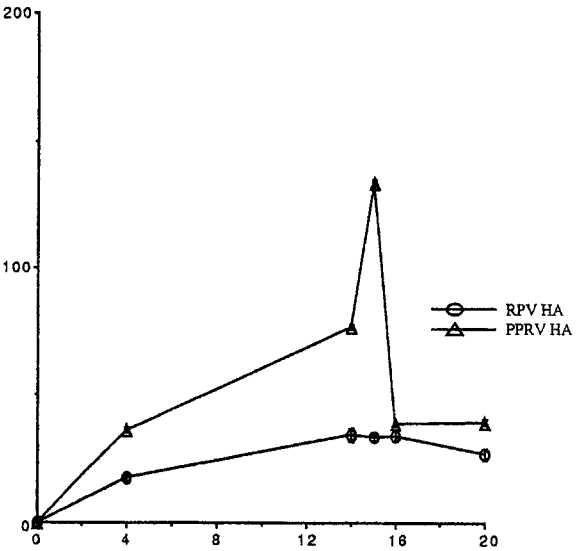

Fig. 4
HAI titers of sera of immunized rabbits
Abscissa: weeks p i. Ordinate: HAI titre.

Protective response of immunized rabbits to challenge

Immunized and control (two rabbits in each group) rabbits were monitored for body temperature and weight five days before the challenge which was performed intravenously with 1 ml of 10% spleen suspension containing virulent lapinized RPV. Then all the animals were observed for the disease symptoms, body temperature and weight (Fig. 5). The control group of animals developed symptoms of rinderpest from day 2 after infection, and hyperthermia and inappetance followed by diarrhea on day 3. One control animal was necropsied on day 6 when the fever reached 40.9°C. The main necropsy findings consisted of the enlargement of mesenteric lymph nodes and spleen (data not shown). The other control animal died on day 8 with a high fever of 40.9°C.

The PPRV HA-immunized animal remained normal. The PPRV F-immunized animal developed fever (40.1°C) on day 6 which rose to 40.5°C on the next day and returned to normal on day 9. There was a progressive loss of body weight in this animal, however, no diarrhea appeared. The RPV HA- and F- immunized animals showed a transient febrile reaction only on day 7 post challenge. Other symptoms like diarrhea or weight loss were not observed in these animals. Two conclusions can be drawn from these findings. (1) PPRV HA protein conferred a better protection than PPRV F protein against a lethal challenge suggesting that PPRV HA is immunodominant. (2) In contrast, both HA and F proteins of RPV conferred protection.

Antibody response of immunized rabbits to challenge

Antibody levels in sera of immunized and challenged rabbits were measured by VN test (Fig. 6). The titres of VN antibodies to both PPRV and RPV increased within the first 5 weeks post challenge in rabbits immunized with HA and F proteins of either virus. Then the titres declined. This may indicate an anamnestic response. The difference in titers before and after the challenge was is about 0.5 log unit. These data and those presented in Fig. 3 suggest that antibodies to HA and F proteins of PPRV and RPV not only neutralize virus *in vitro* but also protect rabbits against a lethal challenge *in vivo*.

The antibodies induced by HA and F proteins of PPRV and RPV were also assessed by ELISA and the obtained results were similar to those described in Fig. 6 (data not shown).

Discussion

The present study was undertaken to develop a subunit vaccine containing the envelope glycoproteins of PPRV and RPV. Subunit vaccines have advantages over live vaccines because they are non-infectious and their production is safe. Besides, they contain only one or two viral proteins which makes it easier to distinguish vaccinated animals from naturally infected ones by simple serological tests. Furthermore, the absence of adventitious pathogens and extraneous agents which may cause fever, allergy or immunosuppression is an added advantage.

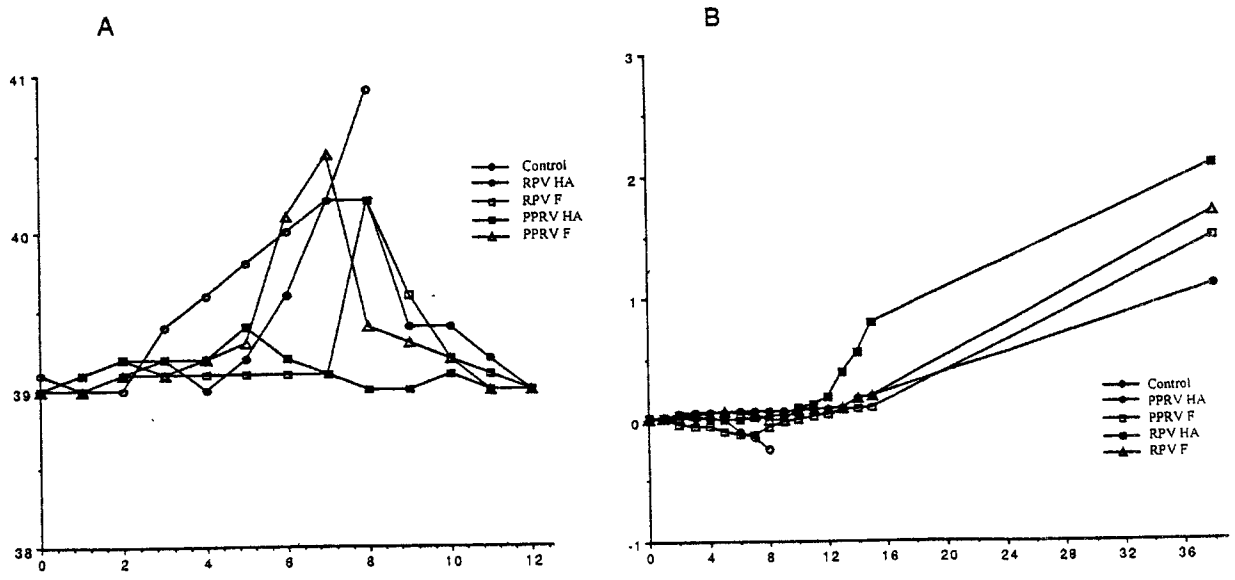

Fig. 5

Pyrogenic (A) and weight (B) responses in immunized rabbits after challenge with virulent lapinized RPV

A. Abscissa: days post challenge Ordinate: temperature in °C. B. Abscissa: days post challenge. Ordinate: weight gain or loss in kg.

The two morbilliviruses, PPRV and RPV are thought to be cross-protective. Previous reports on this subject have discussed the resistance of PPRV-inoculated cattle to challenge infection with the velogenic bovine rinderpest virus (Gargadennec and Lalanne, 1942; Mornet *et al.*, 1956) and the successful field protection of goats immunized with TC RPV against PPRV challenge infection (Taylor, 1979; Bonniwell, 1980; Nawathe, 1984; Losos, 1986). In all these studies, whole virus suspensions were used. Although a tissue culture-adapted RPV of proven immunogenic efficiency is available, a PPRV vaccine remains to be developed and standardized. Preliminary studies in French West Africa have indicated that the development of a PPRV vaccine is likely to appear in the foreseeable future (Gargadennec and Lalanne, 1942). Towards this goal, the knowledge of cross-protective properties of the surface proteins of PPRV and RPV will be of paramount value.

The choice of the surface glycoproteins HA and F in this study was based on factual knowledge that antibodies to these proteins prevent attachment of the virion to the surface receptors on permissive cells and inhibit fusion of infected cells. The HA and F proteins of PPRV and RPV are immunogenic. Recombinant RPV HA and F proteins were used to protect cattle, pigs and rabbits against homologous challenge (Barrett *et al.*, 1989; Asano *et al.*, 1991; Yamanouchi *et al.*, 1993; Yilma *et al.*, 1988). More recently, double recombinant vaccinia virus expressing RPV HA and F proteins was used to protect goats against PPRV challenge infection (Yilma *et al.*, 1992).

In the present study, we have sought to purify the envelope glycoproteins of PPRV and RPV by immunoaffinity chromatography. The immune response to these proteins was evaluated in rabbits by immunization via the intradermal route. The antibody response to HA proteins of PPRV and RPV was fast, while that to F proteins of these viruses was slow. This indicates the immunodominance of HA protein.

Although the cross-protection experiment was restricted to the use of two rabbits for each protein, the results were unequivocal. Although both HA and F proteins of PPRV protected rabbits against a lethal challenge with lapinized RPV, the HA protein conferred a better protection. As expected, PRV HA and F proteins conferred a similar protection against homologous challenge. It was intriguing to note that the PPRV HA-immunized rabbit reacted to the challenge uneventfully. On the other hand, the RPV HA-sensitized animal had a delayed thermal response, which lasted for at least six days. The thermal reaction also occurred in rabbits which were given RPV and PPRV F proteins and then were challenged. There were no other accompanying disease symptoms suggestive of active virus infection in these rabbits as seen in the controls. It is therefore justifiable to surmise that the febrile reaction to the challenge observed in the three rabbits might be an immunological sequel of pyrogenic nature. Unfortunately, no attempt was made to isolate the virus during these hyperthermic episodes. Finally, the protection conferred to a sensitive host system such as the rabbit by the two surface glycoproteins of PPRV against velogenic rabbit-adapted RPV may be regarded as a significant

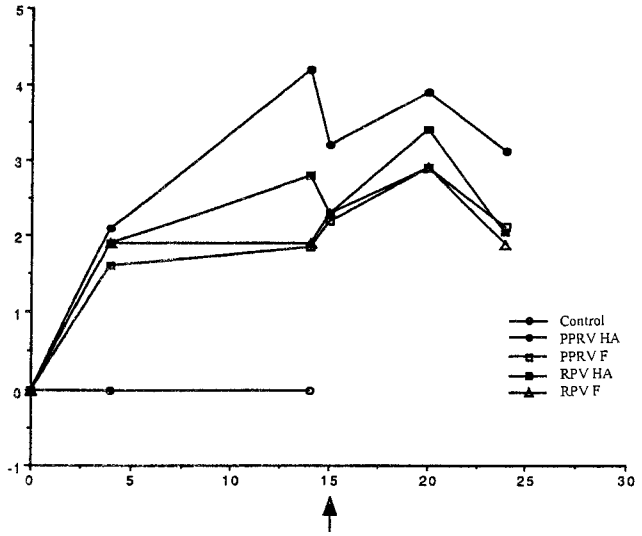

Fig. 6
VN titres of sera of immunized rabbits after challenge with virulent lapinized RPV
Abscissa: weeks before/after challenge. Ordinate: VN titres. The arrow indicates the time of challenge.

and provocative finding in the gamut of antigenic relationships in the current morbilli virus pentad.

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