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Development of heat-stable recombinant rinderpest vaccine

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Summary. Recombinant vaccinia virus (RVV) containing the full-length cDNA of rinderpest virus (RV)-haemagglutinin (H) gene was constructed. The H gene was inserted into the attenuated vaccine strain of vaccinia virus (VV), Lc 16 m0, with two different promoters, namely cowpox virus A-type inclusion body (ATI) promoter or VV 7.5 kilodalton ($P_{7.5}$) promoter. These RVVs produced the same sized fully glycosylated RV-H protein in RK 13 cells as that of the authentic RV-H. Their heat stability in the lyophylized state was similar to that of the parental VV. All rabbits immunized with these RVVs produced virus neutralizing (VN) antibody to RV as well as anti RV-H antibody. Four weeks after immunization, these animals were challenged with RV intravenously. None of the RVV-immunized rabbits developed any clinical signs of RV infection except one which was immunized with RVV containing the ATI promoter and developed low VN titer. These results indicate the possibility of developing a heat-stable recombinant vaccine for the eradication of rinderpest in tropical countries without cold storage systems.

Introduction

Recent epidemics of rinderpest virus (RV) infection widely spread in Africa, the Middle and Near East, and South Asia are having serious harmful effects on cattle husbandry [1]. Since the current live rinderpest vaccine is not stable enough for use in those countries with inadequate cold storage facilities, the development of a heat-stable vaccine is required for the eradication of rinderpest. Because of the high heat-stability of vaccinia virus (VV), as demonstrated by its use in the global eradication of smallpox [10], the use of VV as a vector [9] appears to be an appropriate solution to this problem.

RV is classified in the morbillivirus subgroup of the family *Paramyxoviridae* together with measles virus, canine distemper virus and peste des petits rumi-

nants virus. The haemagglutinin (H) protein of this group of viruses is considered to act as the receptor-binding protein and is a target for virus neutralization [4]. Recently, Yilma et al. [17] developed a recombinant vaccinia virus (RVV) by inserting RV-H and fusion (F) genes within the thymidine kinase site of the VV-WR strain and showed that it can confer protective immunity in cattle against challenge by virulent RV. In the present study, we developed two kinds of RVV by inserting the full-length RV-H gene [13] into the haemagglutinin site of the attenuated vaccine strain of VV; Lc16m0 and examined their heat-stability and their immunogenicity in rabbits.

Materials and methods

Cells and viruses

Vero cells were maintained as described previously [18]. RK 13 cells were maintained in Eagle's minimal essential medium with 5% foetal calf serum. The Lc 16m0 strain, which was cloned from the Lister strain [5], was used as the parental VV. The stocks of VV and RVVs were prepared in RK 13 cells. The stock viruses were lyophilized with 5% polypeptone as stabilizer. A stock of the lapinized Nakamura III (L) strain was prepared in rabbits as described previously [15], and used for the challenge test. Vero cell-adapted L strain of RV at the 13th passage [7] was used for protein analysis, and a lapinized-avianized strain of RV [11] for VN tests.

Plasmid construction, transfection, and isolation of RVV

The full-length RV-H cDNA was excised from plasmid H 1–119 by the restriction endonucleases PvuI and BamHI as described previously [13], filled in with Klenow fragment and ligated into the SmaI site of the VV insertion vector, pHA-A 2–9 containing the promoter of cowpox virus A type inclusion body (ATI) [3], pVR-1 containing the promoter of VV $P_{7.5}$ protein. These promoters were inserted into the middle of the haemagglutinin (HA) gene of VV, which is non-essential for the growth of VV [12]. RK 13 cells infected with the Lc 16m0 strain were transfected with the recombinant plasmids as described previously [14]. HA-negative recombinant viruses were screened by haemadsorption tests using chicken erythrocytes [12].

Analysis of protein expression

Vero cells were infected with the Vero cell-adapted strain of RV at an input multiplicity of 1.0 and were labelled with ^{3}H-glucosamine (30–60 Ci/mmol, New England Nuclear Corp; 100 μCi/dish) or L-[^{35}S]-methionine (> 800 Ci/mmol, New England Nuclear Corp; 50 μCi/dish) for 12–16 h from 48 to 60 h after virus inoculation. RK 13 cells were infected with RVV at an input multiplicity of 1.0. After 6 h, they were labelled with ^{3}H-glucosamine and ^{35}S-methionine for a further 16 h. Virus-infected cells were lysed with a buffer containing 1% Triton X-100, 15% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl and Tris (pH 7.4). They were reacted with the convalescent serum obtained from a rabbit infected with RV-L strain at 9 weeks post infection.

Immunogenicity of RVV

Japanese White rabbits (average body weight 2.5 kg) were immunized with 1–5 × 10^{8} PFU of RVV or parental VV intradermally on the back. The production of anti RV-H antibody after immunization in their sera was examined by VN test [15] and immunoprecipitation (IP) tests with ^{35}S-methionine-labelled RV as described previously [13].

Challenge test with RV

Animals immunized with RVV and control animals (inoculated with VV or uninoculated, rabbits no. N 1–3) were challenged intravenously with 10^3 median infectious dose (ID_{50}) of the L strain of RV 4 weeks after immunization. After challenge, rectal temperature and body weight were measured every day. Immunosuppression induced by the challenge virus [2, 15, 16] was evaluated 10 days after challenge by the blastogenic responses of peripheral blood lymphocytes (PBL) (10^6 cells/ml) to stimulation with Concanavalin A (ConA; Difco Lab., Detroit, MI) (20 μg/ml) [2] as measured by the uptake of ^{3}H-thymidine (10 μCi/ml; 10–25 Ci/mmol, New England Nuclear Corp.). Production of anti-nuclear antibody (ANA) in the sera from rabbits was examined by indirect immunofluorescent (IF) tests using HeLa cells [6] as antigens.

Results

Construction and expression of RVVs

The RV-H gene was inserted with either of two kinds of promoters into the HA gene of VV by homologous recombination, and thus two types of recom-

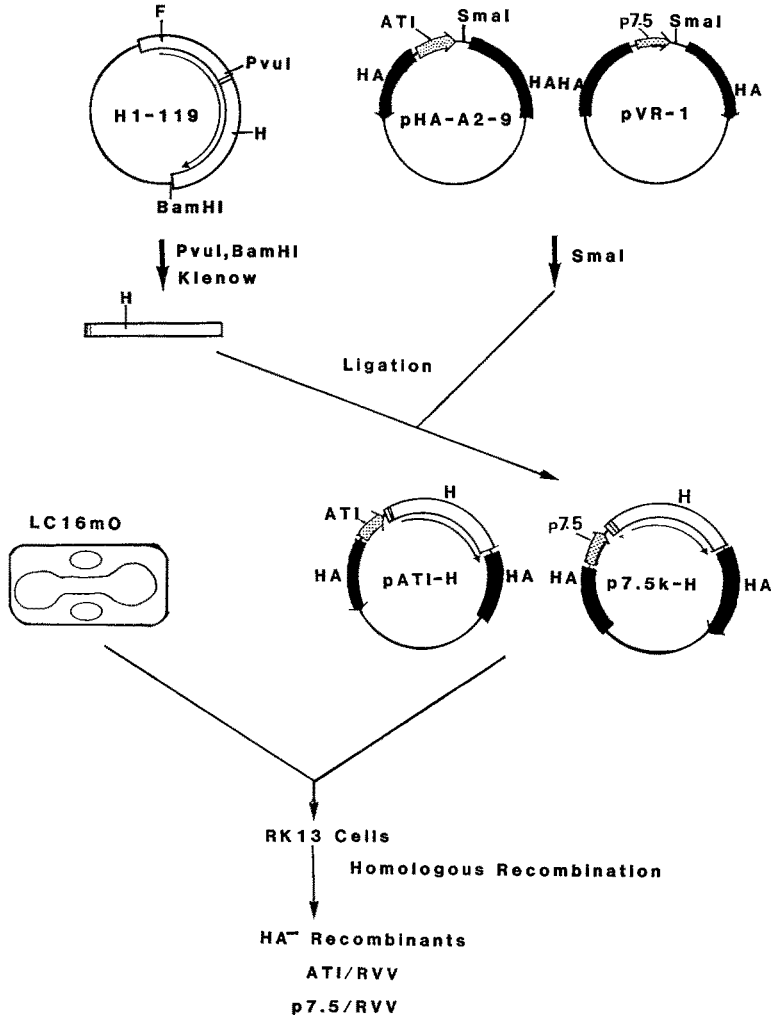

Fig. 1. Construction of the recombinant vaccinia virus

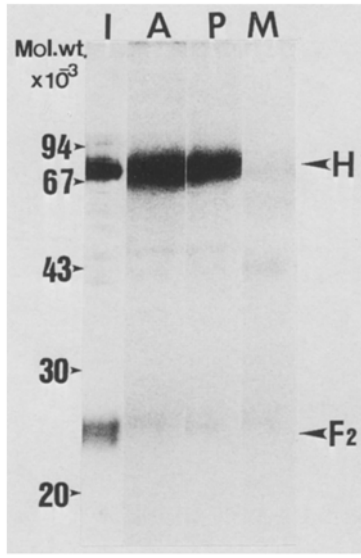

Fig. 2. RV-H protein synthesized in RK 13 cells infected with RVVs. RK 13 cells were infected with ATI/RVV (*A*), $P_{7.5}$/RVV (*P*), and VV (*M*), and labelled with ^{3}H-glucosamine for 16 h. Vero cells were infected with RV and labelled with ^{3}H-glucosamine (*I*). RV-H protein was detected by the convalescent anti-RV serum

binant virus with ATI promoter (ATI/RVV) or $P_{7.5}$ promoter ($P_{7.5}$/RVV) were obtained (Fig. 1). RK 13 cells were infected with ATI/RVV or with $P_{7.5}$/RVV, and a major protein band of about 74×10^3 molecular weight (Mol.wt.) was detected in these cells by immunoprecipitation test using anti RV serum after labelling with ^{3}H-glucosamine (Fig. 2) and ^{35}S-methionine (data not shown). The same sized glycoprotein was also detected in RV-infected cells but not in VV-infected cells (Fig. 2). The result suggests that RV-H gene was expressed under the control of ATI or $P_{7.5}$ promoter and that those products were properly glycosylated.

Heat-stability of RVV

We examined the heat-stability of RVVs and parental VV. The lyophilized stocks of ATI/RVV, $P_{7.5}$/RVV and VV had a moisture content of 1–2%. They were incubated at 37 °C or at 45 °C for one month. The titer of RVVs and VV was reduced to a similar extent (Table 1). Thus, these RVVs were considered to be heat-stable.

Immunogenicity of RVVs

Immunogenicity was examined by intradermal inoculation of each type of RVV on the back of 7 rabbits. All 14 rabbits produced anti RV-H antibodies one week after immunization (Fig. 3). The titer of VN antibody induced by ATI/RVV (rabbits no. A 1–7) was 2^{2-7} and by $P_{7.5}$/RVV was 2^{7-9} (rabbits no. P

Table 1. Heat stability of RVVs compared with parental VV

Treatment	Virus	Virus titer[a]		
		before treatment	after treatment	decrease after treatment
37 °C, 1 month	VV[b]	6.94	6.73	0.21
	ATI/RVV[c]	6.85	6.38	0.47
	$P_{7.5}$/RVV	7.23	7.08	0.15
45 °C, 1 month	VV	6.94	6.54	0.40
	ATI/RVV	6.85	6.28	0.57
	$P_{7.5}$/RVV	7.23	6.86	0.37

[a] Mean titer (log PFU) of three samples
[b] Vaccinia virus (VV)
[c] A type inclusion body (ATI)/recombinant vaccinia virus (RVV)

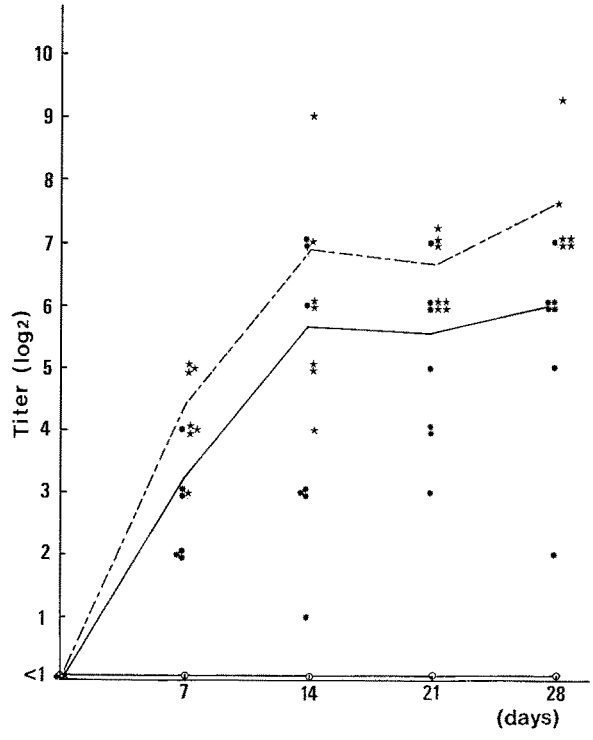

Fig. 3. The production of VN antibody to RV after immunization with RVVs. The antibody titers of ATI/RVV-immunized group (•) and $P_{7.5}$/RVV-immunized group (★) were determined every week, and geometric mean VN titers were plotted (—— ATI/RVV; – – – $P_{7.5}$/RVV). ○ VN antibody titers of the control groups

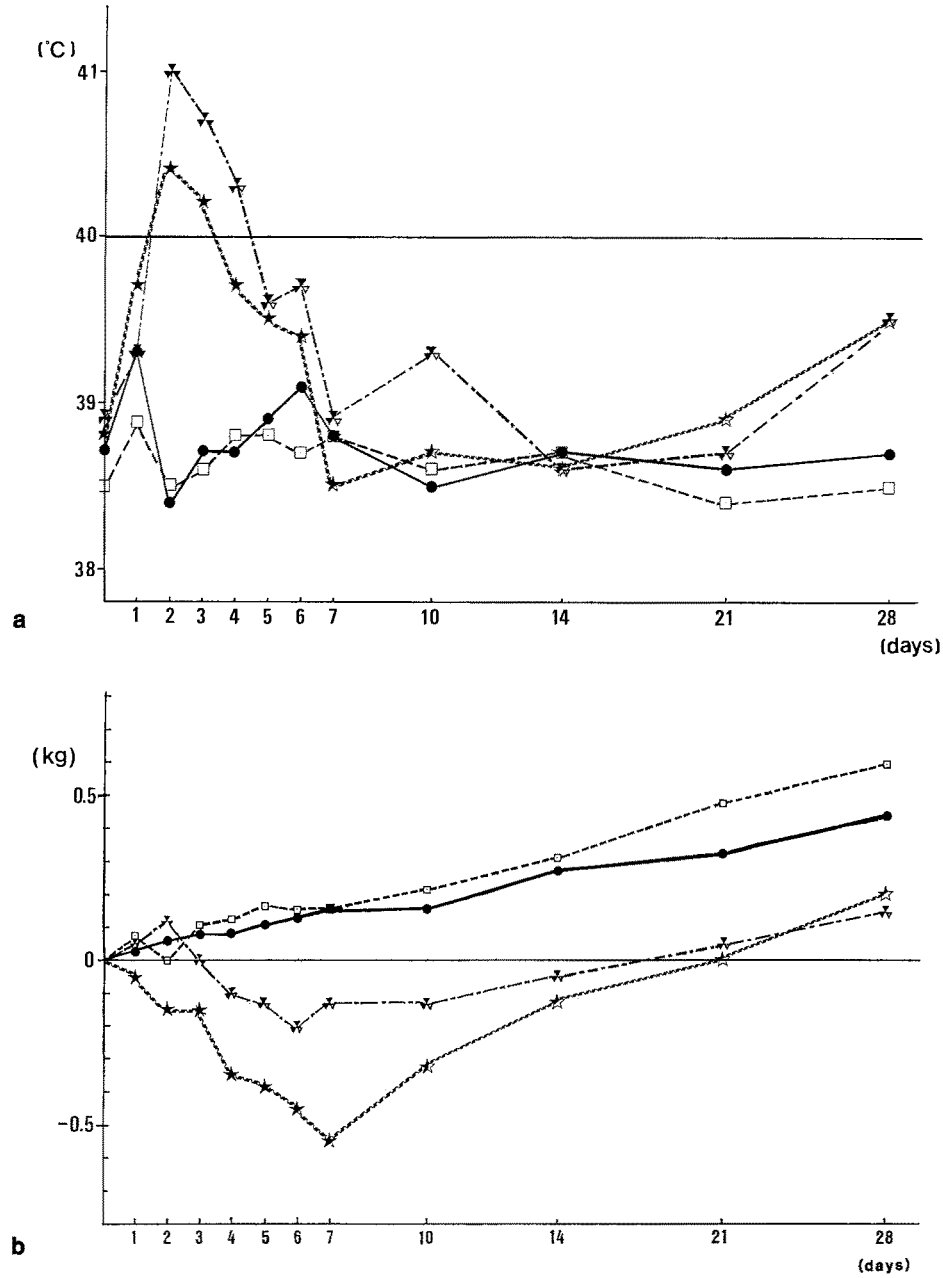

Fig. 4. a The mean rectal temperature after challenge with the L strain of RV in ATI/RVV-immunized group (●), $P_{7.5}$/RVV-immuinized group (□), VV-inoculated control group (★), and uninoculated control group (☆). **b** The mean body weight after challenge in ATI/RVV-immunized group (●), $P_{7.5}$/RVV-immunized group (□), VV-inoculated control group (★), and uninoculated control group (☆)

1–7) by 3 to 4 weeks after immunization. The immunogenicity of ATI/RVV was slightly lower than that of $P_{7.5}$/RVV. Anti RV-H and VN antibodies were not detected in VV-inoculated (rabbits no. M 1–5) or untreated (rabbits no. N 1–3) controls (Figs. 3 and 5).

Protection to challenge with RV

The protective effect of RVVs was examined by challenge with the L strain of RV, which is highly virulent in rabbits [15]. Thirteen of the 14 rabbits immunized with RVVs (A 1–7 and P 1–7) did not show any clinical signs. One rabbit in the ATI/RVV-group (A 7) which had low VN titer (2^2) before challenge showed transiently mild fever and decrease in body weight. In contrast, all rabbits in control groups (M 1–5 and N 1–3) developed typical clinical signs of RV infection, including high fever exceeding 40 °C (Fig. 4 a) by 2–4 days, inappetence and diarrhoea by 2–7 days after challenge. Their body weights were markedly decreased by 5–10 days after challenge (Fig. 4 b). Three of five rabbits inoculated with parental VV (M 4, 3, and 1) died 6, 9, and 13 days after challenge, and one out of three in the untreated group (N 2) died 4 days after challenge. At autopsy, these rabbits had coagulopathy (M 1, 3, and 4, and N 2) and showed haemorrhagic lesions in the lung (M 3 and N 2), urinary bladder (M 4), and caecum (M 1).

The L strain of RV has been shown to multiply in the lymphoid tissues of rabbits leading to marked immunosuppression and production of ANA [1, 15, 16]. Therefore, such virus-induced immune disturbance was also examined in rabbits challenged. As shown in Table 2, the blastogenic response of peripheral blood lymphocytes to ConA examined 10 days after challenge was markedly suppressed in the control groups but that of RVV-immunized rabbits remained unchanged except for rabbit A 7. ANA was detected in control animals examined

Table 2. Lymphocyte blastogenic response in rabbits after challenge with RV

Group	Rabbit no.	Before challenge		After challenge (10 days)	
		^{3}H-thymidine uptake (cpm)[a]	stimulation index	^{3}H-thymidine uptake (cpm)	stimulation index
ATI/RVV-immunized	A 4	105,913/1,355[b]	78.2	198,556/15,519	12.8
	A 5	31,172/519	60.1	157,723/5,153	30.6
	A 6	64,809/1,221	53.1	117,754/2,818	41.8
	A 7	12,327/491	25.1	38,929/27,749	1.4
$P_{7.5}$/RVV-immunized	P 4	3,820/109	35.1	18,312/1,035	74.8
	P 5	3,341/146	22.9	77,373/2,106	36.7
	P 7	13,289/282	47.1	148,120/3,494	42.4
VV-inoculated	M 2	38,666/331	116.8	906/267	3.4
	M 5	45,333/2,404	18.9	46,707/10,549	4.4
Un-inoculated	N 1	129,648/2,190	59.2	4,725/747	6.3
	N 3	121,349/2,626	46.2	1,047/584	1.8

[a] Mean values of [^{3}H]-thymidine incorporated for triplicate wells
[b] ConA +/ConA —

Table 3. Production of ANA 14 days after challenge with RV

Group	Rabbit no.	Titer of ANA
ATI/RVV-immunized	A 1	<10
	A 2	<10
	A 3	<10
	A 4	<10
	A 5	<10
	A 6	<10
	A 7	160
$P_{7.5}$/RVV-immunized	P 1	<10
	P 2	<10
	P 3	<10
	P 4	<10
	P 5	<10
	P 6	<10
	P 7	<10
VV-inoculated	M 1	200
	M 2	1,280
	M 5	1,280
Uninoculated	N 1	160
	N 3	640

14 days after challenge, but not in RVV-immunized rabbits except rabbit A 7 (Table 3). Thus, these two indicators of virus-induced immune disturbance correlated well with the clinical outcome.

Anti H antibody had increased 5 days after challenge in all RVV-immunized rabbits, and was detected 10 days after challenge in control groups (Fig. 5). Antibodies to NP, P, M, and F were produced in both RVV-immunized group and control groups but at much lower levels in the former than in the latter (Fig. 5).

Discussion

A major problem of rinderpest eradication has been the development of heat-stable vaccines since most of the areas where rinderpest is endemic lack adequate cold storage facilities. The RVVs constructed in this study were shown to be stable for at least one month at 45 °C like smallpox vaccine. Apparently, the insertion of the RV-H gene, resulting in the disruption of the VV-HA, did not affect the heat-stability of the parental VV. This degree of heat-stability is in marked contrast to that of the current rinderpest vaccine which can be stored at 45 °C for 3½ days at most [8]. Thus, this type of RVV is expected to be useful for the eradication of rinderpest.

In the present study, two types of promoters, i.e., ATI and $P_{7.5}$, were used for the construction of RVV. Although we had expected higher promoter activity

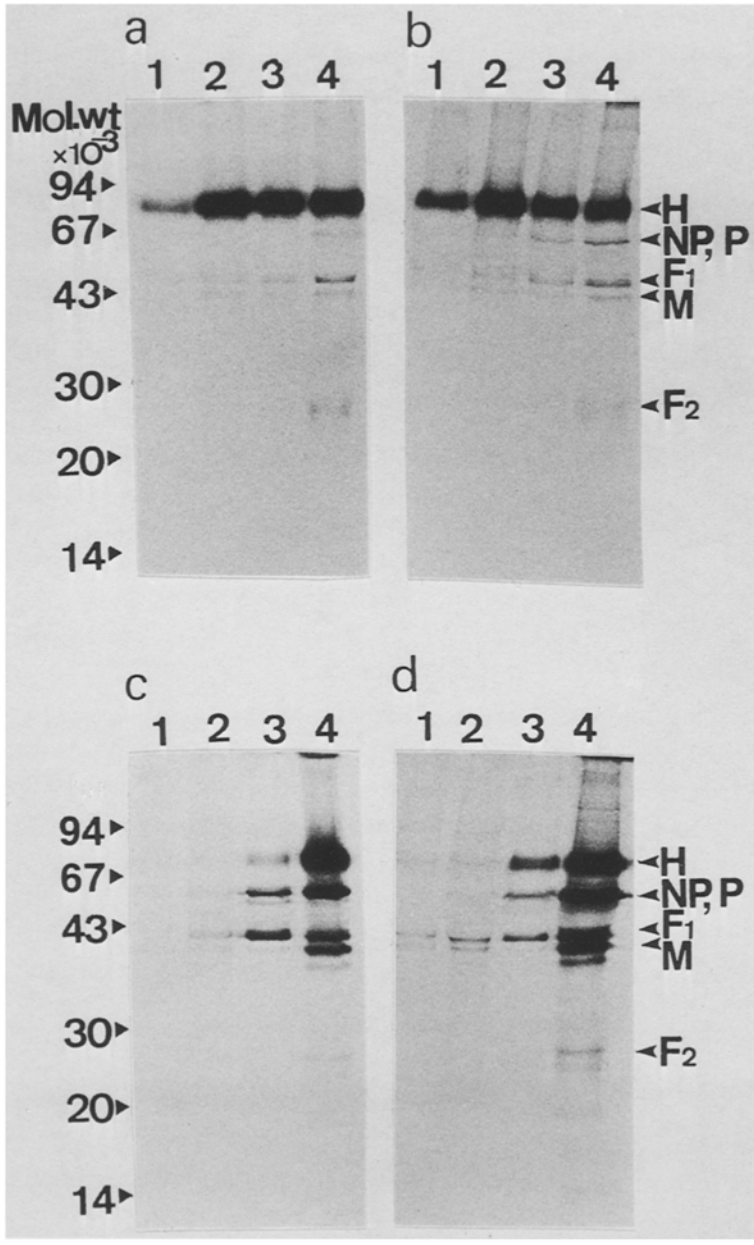

Fig. 5. The antibody responses examined by IP for **a** ATI/RVV-immunized group, **b** $P_{7.5}$RVV-immunized group, **c** VV-inoculated control group, and **d** uninoculated control group. The sera were collected before challenge (4 weeks after immunization with RVV) (*1*), 5 days (*2*), 10 days (*3*), and 4 weeks (*4*) after challenge of RV, and reacted with ^{35}S-methionine labelled RV

for ATI than for $P_{7.5}$, because of its high expression activity in vitro [3], higher immunogenicity was conferred by $P_{7.5}$/RVV than by ATI/RVV in rabbits. The reason for this discrepancy between the results obtained in vitro and in vivo is unknown.

Rabbits immunized with $P_{7.5}$/RVV were completely protected from challenge with RV as judged by clinical signs and RV-induced immune disturbance including immunosuppression and induction of autoimmunity. However, low levels of antibody to NP, P, M, and F were transiently produced in addition to an increase in antibody to H in RVV-immunized rabbits after challenge. This result may indicate that the challenge virus grew to a limited extent but did not cause clinical signs. Recently we developed another type of $P_{7.5}$/RVV by modifying the nucleotide sequence of the H gene. This RVV produced 10 times larger amounts of the RV-H protein than the original RVV in vitro, and the rabbits immunized with this type of RVV produced only anti-H antibody after challenge (unpublished data). Thus, the growth of challenge virus was apparently suppressed by the immune response to the high antigenic stimulus to this single protein. Yilma et al. [17] reported that all the cattle immunized with RVV containing RV-H gene were successfully protected, but whether there was an antibody response to structural proteins after challenge test was not mentioned.

One of the seven rabbits immunized with ATI/RVV (A 7) developed mild clinical signs, immunosuppression and ANA production. Since this was the only animal with low VN antibody titer (2^2) at challenge, this antibody level seems to be insufficient for protection from the onset of disease by challenge virus. In separate experiments (data not shown), the lowest VN antibody titer for successful protection was shown to be 2^5. Rabbit A 7 showed also low VN antibody titer to VV (2^2), probably indicating limited growth of ATI/RVV in this animal.

From these results, we selected $P_{7.5}$/RVV for further study. The minimum median protective dose has been estimated to be $10^{4.5}$ PFU in rabbits (data not shown) and we are now examining its immunogenicity in cattle. In parallel, the development of a divalent RVV by inserting both H and F genes into VV is in progress.

Acknowledgements

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