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Vaccine 00 (2001) 000–000

Vaccine

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Recombinant hemagglutinin protein of rinderpest virus expressed in insect cells induces humoral and cell mediated immune responses in cattle

G. Sinnathamby [a,1], Sangeeta Naik [a,2], G.J. Renukaradhya [b], M. Rajasekhar [b], R. Nayak [a], M.S. Shaila [a,*]

[a] *Department of Microbiology and Cell Biology, Indian Institute of Science, Bangalore 560 012, India*
[b] *Animal Disease Monitoring and Surveillance Unit, Institute of Animal Health and Veterinary Biologicals Campus, Hebbal, Bangalore 560 024, India*

Received 28 November 2000; received in revised form 13 March 2001; accepted 26 March 2001

Abstract

Rinderpest virus causes a highly contagious and often fatal disease in domestic and wild ruminants. The surface glycoproteins, hemagglutinin (H) and fusion (F) proteins of this enveloped virus are known to confer protective immunity in cattle. We have reported the generation of a recombinant baculovirus expressing H protein and studied its protective properties in cattle. In this report, we demonstrate that the recombinant baculovirus encoded H protein expressed in insect cells gets incorporated into extracellular baculovirus. Single administration of low doses of purified recombinant extracellular virus with or without adjuvant induces virus neutralizing antibody responses and bovine leukocyte antigen (BoLA) class II restricted helper T cell responses in cattle.

Keywords: Hemagglutinin; Immune responses; Rinderpest

1. Introduction

Rinderpest, also known as 'cattle plague', is a highly contagious and often fatal disease of wild and domestic bovids with serious economic consequences. The causative agent rinderpest virus (RPV) belongs to the genus *Morbillivirus* within the family *Paramyxoviridae* and is antigenically closely related to measles virus. Rinderpest is prevalent in parts of Africa and Asia [1] and the mortality rate of the disease is close to 90%. This disease has been eradicated from the rest of the world with the time-tested, attenuated, tissue culture adapted rinderpest vaccine, developed by Plowright and Ferris [2]. Though single dose vaccination confers life-long immunity in animals, the vaccine is heat labile and is considered to be one of the reasons for its failure in developing countries. For the global eradication of the disease, there is still a requirement for a heat-stable, effective vaccine. Attempts made towards this goal include the development of a thermostable Vero cell-adapted rinderpest vaccine [3] and recombinant vaccines using vaccinia [4–10] and capripox [11,12] vectors expressing the surface glycoproteins hemagglutinin (H) and/or fusion (F) proteins of RPV, which are known to confer protective immunity in animals. Recombinant baculoviruses expressing these surface glycoproteins have also been tested in cattle for their protective abilities [13,14]. Involvement of cell mediated immune responses in conferring protection against this disease has been suggested earlier [5,13]. Recently, RPV-specific lymphoproliferative responses were demonstrated, following challenge with virulent RPV, in cattle immunized with a recombinant vaccinia virus expressing the H protein [15].

* Address for correspondence. Tel: +91-80-3092702/3600411, ext. 2702; fax: +91-80-3602697.
E-mail address: shaila@mcbl.iisc.ernet.in (M.S. Shaila).
[1] Present address: 233, South 10th Street, Room 726, Bluemle Life Sciences Building, Kimmel Cancer Institute, Thomas Jefferson University, Philadelphia, PA 19107, USA.
[2] Present address: Cadila Pharmaceuticals Ltd, Biotechnology Division, R & D, 244, Ghodasar, Maningar, Ahemadabad 380 050, India.

0264-410X/01/$ - see front matter
PII: S0264-410X(01)00127-X

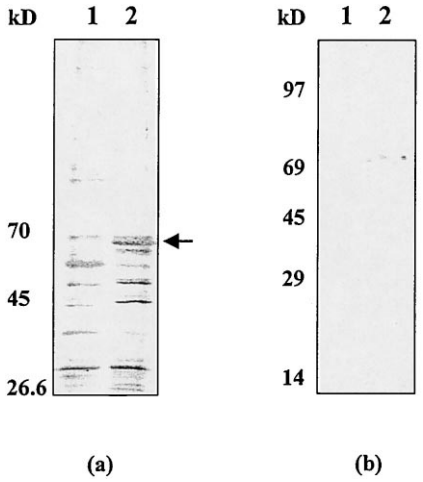


Fig. 1. SDS-PAGE and immunoblot analysis of rECV. (1)WT-AcNPV and (2) rECV (AcSNH3.41) were purified from infected S121 culture supematants by ultracentrifligation on a 25–56% linear sucrose density gradient (l00 000 × *g* for 1 h at 37°C) according to the method of Summers and Smith (17). 20μg of purified virus preparations were subjected to 10% SDS-PAGE and coomassie stained (a) or immunoblotted (b) using a rabbit polyclonal serum against RPV-H (18).

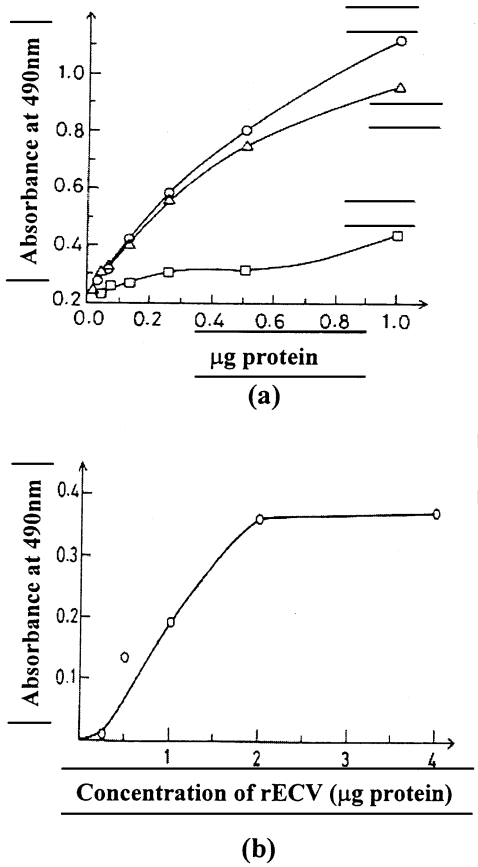


Fig. 2. ELISA reactivity of purified rECV. Indirect ELISA was performed using purified rECV (1 μg/ml, △) and its reactivity compared with RPV (○), to (a) rabbit hyperimmune serum and (b) cattle RPV-convalescent serum. WT-AcNPV (□) was used as the non-specific antigen.

We have earlier reported the expression and characterization of recombinant H protein of RPV in a membrane bound form as well as a secreted soluble form using baculovirus expression system [16] and studied the immunogenic and protective properties of the recombinant baculovirus expressing the membrane bound H protein in cattle [14]. In this report, we demonstrate that the membrane bound form of H protein expressed in insect cells gets incorporated into recombinant extracellular baculovirus (rECV), which can be purified from infected insect cell culture supernatant. Single administration of low doses of purified rECV induces strong virus neutralizing antibody response and bovine leukocyte antigen (BoLA) class II restricted helper T cell response in cattle, with or without adjuvant.

2. Materials and methods

2.1. Cell lines and viruses

Recombinant baculoviruses coding for a membrane bound form of RPV-H protein (AcSNH3.41 or rECV) and a secreted form of soluble H (SecH) have been described earlier [16]. These viruses were propagated in *Spodoptera frugiperda* (Sf) 21 insect cells grown in insect cell culture medium TC-100 supplemented with 10% fetal bovine serum (FBS) [Gibco-BRL, USA]. Sf21 cells were infected at multiplicity of infection (MOI) 1 for 72 h and rECV released into the culture medium was purified by sucrose density gradient ultracentrifugation according to the method of Summers and Smith [17]. Briefly, supernatants of Sf21 cells infected with either wild type AcNPV or recombinant AcSNH3.41 were subjected to ultracentrifugation on a 25–56% linear sucrose density gradient (100 000 × *g* for 1 h at 4°C), the virus bands were collected, diluted in sterile ice-cold phosphate buffered saline (PBS) and pelleted down by ultracentrifugation. The pellets were soaked overnight at 4°C in sterile PBS and used as purified virus stock. For production of SecH protein, Sf21 cells were infected with the recombinant baculovirus coding for the secreted form of H at MOI 20. The supernatant was collected after 72 h and subjected to immunoaffinity purification using rabbit polyclonal antibodies to H [18] coupled to CNBr activated Sepharose 4B (Pharmacia, Sweden).

Rinderpest virus (RBOK, vaccine) was obtained from the Institute of Animal Health and Veterinary Biologicals, Bangalore, India and was grown in Vero cells in Minimum Essential Medium (Gibco-BRL, USA) supplemented with 5% FBS. Titration of these viruses by TCID50 determination was performed according to standard methods. The virus was titrated using $TCID_{50}$ method of Reed and Muench [19].

Table 1
Humoral immune responses of rECV immunized and TCRV vaccinated cattle[a]

Group	Cattle #	Antibody Titre ($\times 10^3$) Weeks post-immunization					
		1	2	3	4	6	16
I	RS1	160	25.6	40	80	54.2	51.2
	RS2	80	25.6	ND	20	25.6	51.2
	RS3	40	ND	40	40	ND	ND
Group	Cattle #	Antibody Titre ($\times 10^3$) Weeks post-immunization/vaccination					
		1	2	3	8	26	
II	GS1	12	24	12	6	3	
	GS2	12	12	24	12	6	
	GS3	12	12	24	12	6	

[a] ELISA titres: Cattle sera collected at different weeks p.i. were subjected to indirect BLISA using SecH as the antigen (1:20 diluted supematant from 5121 cell culture, infected with the recombinant baculovirus expressing soluble secreted form of H [16]). Animals of group I (RS 1, RS2 and RS3) were immunized with rECV in FCA, while in group II, GS 1 and (352 were immunized with purified rECV without adjuvant and (353 is TCRV-vaccinated control. The highest dilution of the antiserum which showed twice the OD as that of the background was taken as the titre. (ND-Not Determined).

2.2. SDS-PAGE analysis and immunoblotting

Purified wild type AcNPV and recombinant Ac-SNH3.41 were subjected to 10% SDS-PAGE and the gel was coomassie stained. A similar gel was electro-transferred onto nitrocellulose membrane (Amersham, USA) using LKB semidry transfer apparatus. The blot was blocked with 2% gelatin and probed with rabbit polyclonal antibodies against RPV-H [18] and goat-α-rabbit IgG coupled to horse radish peroxidase (HRP) [Gibco-BRL, USA]. H_2O_2 and Diaminobenzidine (Sigma Chemicals, St Louis) were used to develop the blot.

2.3. Vaccination/immunization of cattle

Two groups of outbred calves of approximately 1 year of age were employed in this study. In Group I, three animals (RS1, RS2 and RS3, Jersey breed) were immunized intramuscularly with 2 mg of purified Ac-SNH3.41 emulsified in Freund's Complete Adjuvant (FCA) and one animal (RS5, Jersey breed) was vaccinated with one dose of RPV (RBOK) vaccine (obtained from Institute for Animal Health, Pirbright, UK). In Group II, two animals (GS1 and GS2, Holstein Friesian cross breed) were immunized with 500 μg of purified rECV in PBS without adjuvant while the third animal (GS3, Jersey breed) served as vaccinated control. One animal (GS4, Holstein Friesian cross breed) was used as unimmunized control animal.

2.4. Antibody assays

2.4.1. ELISA

Purified recombinant AcSNH3.41 or SecH was used as ELISA antigen. RPV(TCRV) or wild type AcNPV served as positive and negative control antigens respectively and 2% gelatin as the blocking agent. Goat-α-rabbit IgG (Gibco-BRL, USA), goat-α-cow IgG (Dacopatts, Denmark) coupled to HRP, H_2O_2 and *o*-Phenylenediamine (Sigma Chemicals, St Louis) were used in ELISA. Competition ELISA employing monoclonal antibodies to H (C1 and D2F4) was performed according to Anderson and McKay [20]. C1 Mab was kindly provided by Dr T. Barrett, Institute for Animal Health, Pirbright, U.K. D2F9 Mab which recognises a linear epitope on H was generated employing sec H as the immunising antigen and characterised in the laboratory (Renukaradhya et al., unpublished).

Table 2
Humoral immune responses of rECV immunized and TCRV vaccinated cattle[a]

	% Inhibition of binding of C1 — Weeks post-immunization		
	0	2	4
Cattle # (Group I)			
RS1	22.5	45.6	58.8
RS2	15.8	34.8	53.6
RS3	22.7	38.3	60.9
Cattle # (Group II)			
GS1	20.2	72.4	85.5
GS2	25.4	85.1	95.3
GS3	24.7	82.3	90.8

[a] Competition ELISA: Sera collected from cattle at different time points were subjected to competition ELISA employing monoclonal antibodies to H (Cl or D2F4). The assay was performed and % inhibition calculated as described earlier [19]. 1:200 diluted Ac-SNH3.41 infected 5121 cell extract (group I) and SecH antigen (as described above, for group II) were used as antigens. 1:5 (group I) and 1:10 (group II) diluted cattle sera were tested for their ability to inhibit binding of Cl (group I) or D2F4 (group II).

Table 3
Humoral immune responses of rECV immunized and TCRV vaccinated cattle[a]

Cattle # (Group II)	Preimmune Serum	Virus NeutralizationTitres — Weeks post-immunization/vaccination				
		1	2	3	8	26
GS1	–	40	160	160	80	ND
GS2	–	40	80	80	20	20
GS3	–	20	40	160	80	40
GS4	–	–	ND	–	–	ND

[a] Virus Neutralization Titres: Virus Neutralization Test (VNT) was performed according to the method of Barrett et al. [5]. Sera collected from cattle of group II (GSl, GS2-rECV immunized, GS3-TCRV-vaccinated and GS4-unimmunized control) at different time intervals afier immunization/vaccination were subjected to VNT to test for their ability to neutralize RPV in vitro, at various dilutions. The reciprocal of the highest dilution of serum that showed a 50% or less CPE compared to the virus infected control well was taken as the VN titre. (ND-Not Determined).

2.4.2. Virus neutralization test (VNT)

VNT was performed according to Barrett et al. [5]. Briefly, 100 $TCID_{50}$ units of RPV was incubated with dilutions of test sera at 37°C for 1 h and then added to Vero cells in a flat bottomed 96-well tissue culture plate (Costar, USA). The plate was microscopically examined for cytopathic effect (CPE) after 3–4 days. The reciprocal of the highest dilution of the test serum at which the CPE was 50% or less than that of the virus infected control well was taken as the VN titre.

2.5. Lymphoproliferation assay

Peripheral blood mononuclear cells (PBMC) were isolated from the experimental animals according to standard procedure using Ficoll-Hypaque (Sigma Chemicals, St Louis) density gradient centrifugation at different time points after immunization. PBMC were plated [1×10^5 cells in 100 μl of complete RPMI 1640 medium supplemented with 10% FBS (Gibco-BRL, USA), 50 μM β Mercaptoethanol, 1 mM Sodium pyruvate, 2 mM L-glutamine (Sigma Chemicals, St Louis) in a 96-well tissue culture plate (Costar, USA)] and in vitro stimulated with varying concentrations of extracts of Sf21 cells infected with wild type AcNPV or recombinant AcSNH3.41 or purified SecH or purified bacterially expressed nucleocapsid (N) protein of RPV (Shaji and Shaila, unpublished) in 100 μl of complete RPMI 1640 medium at 37°C with 5% CO_2 in a humidified chamber. After 5 days, the cells were pulsed with 1 μCi [^{3}H]-thymidine (Specific activity 6500 mCi/mmol, from Bhabha Atomic Research Centre, Mumbai, India) for 16 h. Cells were then harvested onto GF/A glassfibre filters (Gelman, USA) and trichloroacetic acid insoluble radioactivity was measured in a Rackbeta liquid scintillation spectrometer. The results were expressed as counts per minute (cpm) or stimulation index (SI) calculated as follows:

SI = [Radioactivity (cpm)
incorporated in the experimental well]
÷ [Radioactivity (cpm)
incorporated in the medium control well]

Lymphoproliferation assays were performed in triplicate wells and at least two independent experiments were carried out. In inhibition assays, various dilutions of hybridoma culture supernatants containing mouse monoclonal antibodies against BoLA class II molecules (kind gift of Dr C Howard, Institute of Animal Health, Compton) were used.

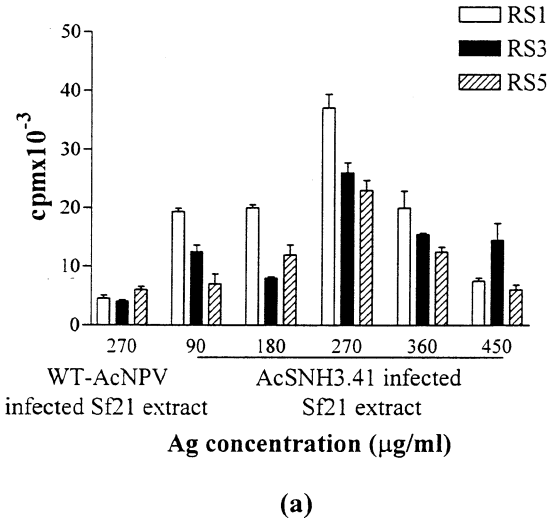
3. Results and discussion

3.1. Recombinant H gets incorporated into ECV particles and is antigenically authentic

When proteins present in purified wild type AcNPV and recombinant AcSNH3.41 were subjected to SDS-PAGE analysis, a protein band of molecular weight ~ 70 kD was detected as an additional band in the lane corresponding to recombinant AcSNH3.41, which is absent in wild type AcNPV (Fig. 1a). This protein, on immunoblotting, reacted specifically with polyclonal antibody against RPV-H protein (Fig. 1b). This confirmed that the recombinant H protein expressed in Sf21 insect cells gets incorporated into ECV. The authenticity of this rECV was further confirmed in ELISA using hyperimmune serum raised in rabbit against RPV-H (Fig. 2a), RPV-convalescent sera from cattle (Fig. 2b) and a conformation specific monoclonal antibody C1 (data not shown). rECV reacted very well with all these antibodies to levels comparable to that of RPV suggesting that the H protein expressed in insect cells and subsequently incorporated into ECV particles is antigenically authentic.

3.2. rECV immunized cattle generate H-specific humoral immune responses

Sera collected from rECV immunized animals showed RPV-H specific reactivity in indirect ELISA when SecH was used as the antigen (Table 1). All the animals developed high titred antibodies against RPV-H as early as 1 week after immunization and the antibody levels could be detected at least upto 16 weeks in group I and 26 weeks in group II after immunization. Antibody titres were higher in cattle of group I. The specificity of the antibodies was further tested using a specific competition ELISA and these sera showed inhibition of binding of monoclonal antibodies C1 and D2F4 to RPV-H (Table 2). However, when sera from rECV immunized animals were subjected to competition ELISA employing a monoclonal antibody specific for RPV-N [21], there was no inhibition of binding of this monoclonal antibody when RPV-infected Vero cell extract was used as the antigen. As expected, sera from vaccinated cattle showed high inhibition in this assay (data not shown).



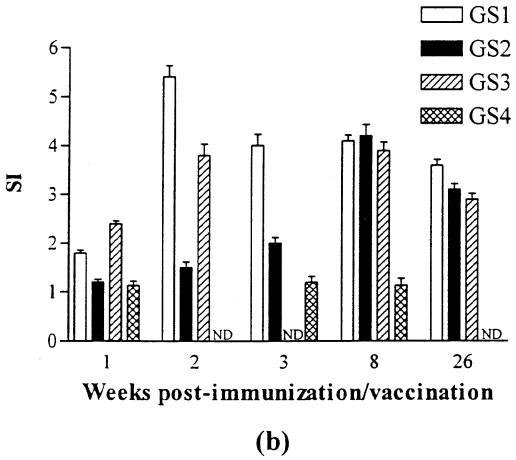


Fig. 3. Lymphoproliferative responses of cattle PBMC to recombinant H protein. (a) PBMC from cattle of group II [RS1, R53 (rECV in FCA immunized) and RSS (TCRV-vaccinated)] were in vitro stimulated with the recombinant baculovirus coding for the membrane bound H (AcSNH3.41) infected Sf21 cell extract at the indicated total protein concentrations (H corresponds to approximately 15% of the total protein in the infected cells). PBMC were stimulated with WT-AcNPV infected Sf21 cell extract as the control antigen. Lymphoproliferative responses to WT-AcNPV infected Sf21 cell extract at concentration 270 μg/ml which resulted in the highest stimulation are represented in the figure. This assay was performed 2 years afier immunization/vaccination of cattle. (b) PBMC isolated from rECV immunized cattle GS1 and GS2, TCRV-vaccinated cattle GS3 and unimmunized control GS4 of group I were in vitro stimulated with immunoaffinity (18) purified SecH at a concentration of 8 μg/ml at different time points afier immunization/vaccination. Stimulation index (SI) was calculated using the following formula: SI = Radioactivity (cpm) incorporated in the experimental well/Radioactivity (cpm) incorporated in the control well. ND-Not Determined.

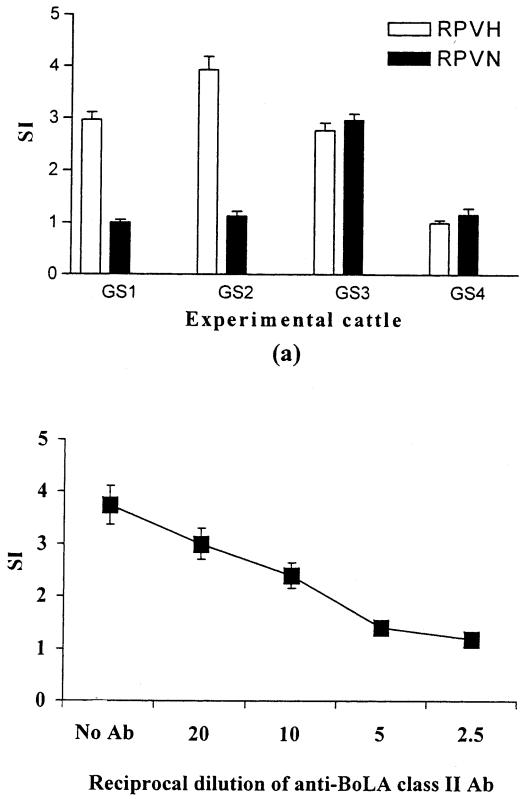


Fig. 4. Specificity of lymphoproliferative responses. (a) PBMC isolated from cattle of experimental group I [GS1, GS2 (rECV immunized), GS3 (TCRV vaccinated) and GS4 (unimmunized control)] were in vitro stimulated with varying concentrations of immunoaffinity purified SecH or purified bacterially expressed N protein. Results at protein concentrations of 8 μg/ml for SecH or 4 μg/ml for N protein at which the proliferative responses were the highest are represented in the figure. (b) PBMC from cattle of experimental group I were in vitro stimulated with SecH (8 μg/ml) in presence of varying dilutions of anti-BoLA class II antibodies. Results of a representative experiment with PBMC from GSl are presented. Stimulation index (SI) was calculated using the following formula: SI = Radioactivity (cpm) incorporated in the experimental well/Radioactivity (cpm) incorporated in the control well.

The immune sera were tested for their capacity to neutralize virus infectivity in vitro by VNT. Sera from animals of group II were subjected to this test and high levels of virus neutralization (VN) antibodies were detected (Table 3). The levels were comparable to that generated in vaccinated animal and VN antibodies could be detected at least upto 26 weeks after immunization.

3.3. H-specific and BoLA class II restricted lymphoproliferative responses in rECV immunized cattle

When the ability of PBMC from animals of group I to proliferate in vitro in response to varying concentrations of wild type AcNPV or recombinant AcSNH3.41 infected Sf21 cell extracts was tested, PBMC responded very well to recombinant AcSNH3.41 infected Sf21 cell extract though there were low levels of proliferation in response to wild type AcNPV infected Sf21 cell extract. PBMC from rECV immunized animals, RS1–RS3, responded better than vaccinated cattle, RS5, in this assay. PBMC from all the animals exhibited the highest proliferative response at 270 μg/ml protein concentration (Fig. 3a). This assay was performed 2 years after the animals were immunized or vaccinated. When PBMC from animals of group II were subjected to proliferative assays in presence of purified SecH, the responses could be detected in all the animals at least for 26 weeks after immunization/vaccination. Responses were at their peak between 2–8 weeks post-immunization/vaccination (Fig. 3b). This response was found to be H-specific as PBMC from rECV immunized animals failed to respond N protein of RPV. However, as expected, PBMC from vaccinated animal proliferated in response to N protein (Fig. 4a). Further, the lymphoproliferative responses were predominantly BoLA class II restricted as antibodies against BoLA class II molecules specifically inhibited antigen driven proliferation in a dose dependent manner (Fig. 4b).

These results demonstrate that recombinant H protein expressed in insect cells gets incorporated into ECV particles and is antigenically authentic. Antibodies generated in the rECV immunized animals were H-specific and could neutralize RPV infectivity in vitro. These data suggest that H protein present on ECV particles is 'seen' by the immune system as the native RPV antigen. Further, there were long lasting H specific lymphoproliferative responses in all the immunized animals. The cellular immune responses could be detected in rECV immunized animals of group I even 2 years after immunization while in group II, the cellular responses declined and became undetectable after 7 months although antibody responses persisted at least for a year after immunization. This could be because the animals of group I were immunized with higher antigen dose in FCA. To our knowledge this is the first demonstration of cell mediated immune responses in cattle following immunization with one of the protective antigens of RPV. It has been demonstrated that an inactivated recombinant vaccinia virus expressing the rabies virus glycoprotein gene generates high levels of rabies virus neutralizing antibodies and protects mice and rabbits from severe challenge with rabies virus [22]. Thus, non-replicating and innocuous virus particles have the potential to confer protection against lethal challenges with disease causing agents. Further, rECV particles are stable at 4°C at least upto 1 year without apparent decrease in the titres (Naik and Shaila, unpublished observation). Moreover, as non-infectious vaccines are generally preferred over their infectious counterparts owing to the safety considerations, rECV particles are a potential vaccine candidate and future challenge experiments with virulent RPV should prove useful in evaluating the protective efficacy of the humoral and cellular immune responses generated in immunized animals.

Acknowledgements

This work was supported by the Department of Biotechnology, India under the Molecular Virology Programme. We thank Drs T. Barrett and C. Howard, Institute of Animal Health, U.K. for antibody reagents used in this study. G.S. is a recipient of University Grants Commission Research Fellowship.

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