Oral immunization of cattle with hemagglutinin protein of rinderpest virus expressed in transgenic peanut induces specific immune responses

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Abstract

Rinderpest is an acute, highly contagious often fatal disease of large and small ruminants, both domestic and wild. Global eradication of rinderpest needs a robust, safe and cost-effective vaccine. The causative agent, rinderpest virus (RPV) is an important member of the genus *Morbillivirus* in the *Paramyxoviridae* family. We have generated transgenic peanut (*Arachis hypogea* L.) plants expressing hemagglutinin protein of RPV and report here, the induction of immune responses in cattle following oral feeding with transgenic leaves expressing hemagglutinin protein without oral adjuvant. Hemagglutinin-specific antibody was detected in the serum as confirmed by immunohistochemical staining of virus-infected cells, and in vitro neutralization of virus infectivity. Oral delivery also resulted in cell-mediated immune responses.
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1. Introduction

Rinderpest is an acute, febrile, highly contagious disease of cattle caused by rinderpest virus (RPV), which is a member of the family *Paramyxoviridae* and genus *Morbillivirus*. In spite of availability of a highly effective live attenuated vaccine, rinderpest remains a threat to livestock in developing countries. The difficulty in maintaining the cold chain results in failure of vaccination in the hot regions where rinderpest is endemic. Attempts have been made to develop heat stable rinderpest vaccines, which include thermostable Vero cell-adapted rinderpest vaccine [1,2], Xerovac live attenuated rinderpest vaccine [3] and dry powder rinderpest vaccine [4]. RPV contains two glycoproteins, hemagglutinin (H) and fusion (F) proteins, on its host cell membrane-derived envelope. H and F proteins are known to be highly immunogenic and confer protective immunity. Efforts have been made to develop recombinant vaccinia virus expressing H and F [5–9] and recently long-term immunity in cattle has also been demonstrated [10,11]. Because of its wide host range, the use of recombinant vaccinia virus remains a matter of debate. Another pox virus (capripox virus), which has more restricted host range, has

been used to develop recombinant capripox–rinderpest virus vaccine [12,13] and vaccination with this vaccine confers long-term immunity in African cattle [14]. Further, the H and F proteins expressed by recombinant baculo virus was shown to be immunogenic [15] and a recombinant baculo virus expressed H protein could induce both humoral and cell-mediated immune response [16,17]. In addition, the recombinant H expressed as extracellular baculo virus particles has been shown to elicit cytotoxic T-cell responses and a CTL epitope on H has been mapped [18,19]. Although the above-mentioned efforts promise to provide an effective vaccine, their use becomes prohibitively expensive because of the cost of production of cell culture vaccine.

In order to effectively control and eliminate rinderpest, a vaccine is necessary which provides a handle to differentiate between animals that have been vaccinated and those, which have recovered from natural infection [20]. For pathogens, which enter and colonize in the mucosal epithelium of gastrointestinal, respiratory and genital tract, it would be better to employ a mucosal vaccine since induction of both mucosal and systemic immune responses is achieved whereas the reverse does not hold true. A recombinant subunit vaccine expressed in edible parts of transgenic plants promises to possess the desired properties. In case of foot and mouth disease and transmissible gastroenteritis, recombinant antigens expressed in transgenic plants have been reported to possess immunological properties at least

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in the mouse model [21,22]. We have recently shown that the H protein of RPV expressed in a model plant, tobacco is immunogenic and elicit specific humoral immune response [23]. Further, we have expressed the hemagglutinin protein in transgenic peanut, a crop which is also used in India for animal feeding after harvesting the nuts, and tested its antigenicity and immunogenicity. This peanut-derived H is immunologically active when delivered through parenteral or through oral route in experimental mouse model system [40]. In the present communication, we report the induction of specific immune responses in cattle upon oral feeding with transgenic leaves of peanut expressing H without mucosal adjuvant.

2. Materials and methods

2.1. Animals

Four cattle (C1–C4) were maintained at the Central Animal Facility of the Institute. C1, C2 and C4 (4–5 years age) of Holstein–Friesian cross-breed and C3 (about 2–3 years age) is a Jersey breed.

2.2. Cells and viruses

Vero cells were obtained from National Center for Cell Science, Pune, India and were maintained in MEM supplemented with 5% fetal calf serum (Gibco-BRL, USA) at 37 °C in a CO_2 incubator. A tissue culture adapted vaccine strain of RPV (RBOK) [24] was obtained from the Institute of Animal Health and Veterinary Biologicals, Bangalore, India and vaccine strain of PPRV (Nig 75/1) was provided by Dr. A. Diallo, CIRAD-EMVT, France. To prepare infected cell lysates, at 24–48 h post-infection of Vero cells when 70% CPE was seen, cells were lysed in PBS by freezing and thawing three times and stored at −20 °C till further use.

2.3. Recombinant proteins

Recombinant hemagglutinin protein expressed in insect cells secreted into the medium (SecH) was used [25]. Recombinant nucleocapsid protein of RPV expressed in *E. coli* was purified by CsCl gradient as described earlier [26]. The full length M gene of RPV (RBOK) was cloned into pBluesript KS$^+$ vector (kindly provided by Dr. M. Baron, Institute for Animal Health, Pirbright, UK) was subcloned into pRSET expression vector and expressed in *E. coli* BL21 (DE3) (Shaji and Shaila, unpublished data), as His tag protein. The protein was purified on a nickel affinity column.

2.4. Antibodies

A mouse monoclonal antibody D2F4 to RPV H protein earlier generated in the laboratory [27] was used. Polyclonal monospecific antibodies to RPV H purified from infected cell extracts were generated in rabbits [28].

2.5. Transgenic peanut plants

The hemagglutinin gene of attenuated strain (RBOK) of rinderpest virus was subcloned into binary vector pBI 121. In the recombinant binary vector pBI H, the H gene is under the control of constitutively expressed CaMV 35S promoter. pBI H was mobilized into *Agrobacterium tumefaciens* (EHA 105). Transgenic peanut plants obtained using pBI 121 served as the control and termed as vector-transformed peanut plants. Transgenic peanut plants expressing hemagglutinin protein were generated via *Agrobacterium*-mediated transformation of shoot apices [40]. Total protein from leaves was isolated employing the method of McGarvey et al. [29] used for the solubilization of rabies virus glycoprotein. The expression level of H was in the range of 0.2–1.3% of total soluble protein as estimated by double antibody sandwich ELISA using a standard curve where recombinant H was used as an antigen. Leaves from different lines expressing H were pooled and fed to the animals such that each dose contained an amount of H equivalent to 0.5% of total soluble protein.

2.6. Immunization schedule

The animals were fed with either transgenic peanut leaves expressing H (C2–C4) or with vector-transformed peanut leaves (C1) as control at weekly interval for three consecutive weeks. The first immunization was with about 7.5 g of leaves followed by 5 g of leaves at 7 and 14 days. Animals were given normal feed at all times.

2.7. Competition ELISA

The method described by Anderson and McKay [30] was used. The assay was performed in a 96-well flat bottom plate. SecH was used as the antigen (1:150 dilution in PBS) and D2F4 monoclonal antibody (1:5000) was used for competition with test serum. The reaction was developed with 50 μl of OPD (4 mg/ml) and H_2O_2 (2 μl of 30% stock) in PBS after terminating the reaction with 50 μl of 2N H_2SO_4, plate was read at 490 nm in an ELISA reader, which is attached to a computer having enzyme immunoassay (EIA) software of Biologicals Diagnostic Supplies Ltd. (BDSL, UK) and the OD values were converted to percentage inhibition (PI) values. Percentage inhibition more than 40 was considered to be significant.

2.8. Immunohistochemical staining

The method described by Naik et al. [16] was used to test the immune sera for reactivity with RPV H made in virus-infected cells.

2.9. Virus neutralization test

Serum samples collected at various time points were tested for the presence of neutralizing antibodies (both homologous and cross-neutralizing) in triplicates using flat-bottomed 96-well plates as described by Barrett et al. [6]. Attenuated strain of RPV (RBOK strain) and vaccine strain of PPRV Nig 75/1 were grown on Vero cells and titrated employing $TCID_{50}$ method [31]. Serum samples were heat inactivated at 56 °C for 30 min and then double diluted with culture medium, starting from an initial dilution of 1:20. Following incubation with 100 $TCID_{50}$ of virus at 37 °C for 1 h, 2×10^4 cells were added to each well. The wells without the sera/virus served as control. The plates were monitored for 5–7 days for cytopathic effects (CPE) for RPV and 3–5 days for PPRV. Virus neutralization titer was defined as the highest dilution of the sera, which inhibited CPE by 50%.

2.10. In vitro lymphoproliferation

Animals were bled through jugular vein puncture at specified times. The blood was diluted 1:2 in sterile PBS and was subjected to Ficoll–Hypaque (Pharmacia) density centrifugation at 3000 rpm for 30 min. The buffy coat was collected and diluted in excess PBS. The cells were recovered by centrifugation and washed and resuspended in RPMI 1640 supplemented with 10% FBS (Gibco-BRL, USA). The lymphocytes were proliferated in triplicates at a density of 2×10^5 cells per well in presence of varying concentrations of SecH, N, M proteins or PPRV infected cell lysate or uninfected cell lysate in a final volume of 200 µl per well for 5 days. The cells were pulsed with 1 µCi [^{3}H]-thymidine (specific activity 6500 mCi/mmol; Amersham) for 16 h and harvested on glass fiber filter (Nunc, USA). The incorporated radioactivity was measured in a Rackbeta scintillation spectrometer.

3. Results

3.1. Humoral immune responses

Animals were bled at regular intervals after immunization either with transgenic peanut leaves expressing H or vector-transformed peanut leaves and sera were examined for the presence of H-specific serum antibodies in ELISA using recombinant secretory form of H (SecH) in a competition ELISA. Inhibition of binding to H protein of monoclonal antibody (D2F4) generated against RPV H by immune sera (Fig. 1) demonstrates the specificity of antibody. The serum from immunized animal, which received transgenic peanut expressing H competed very well with the monoclonal antibody and no significant competition was seen in presence of serum from the cattle fed with vector-transformed peanut leaves.

The specificity of the antibody produced in response to oral delivery of recombinant H as part of food was also verified by immunostaining of the infected cells. When antibodies from the orally fed animals were used for reactivity with antigens expressed in virus-infected cells only the immunized cattle serum reacted with viral antigen expressed in infected cells and the immune serum from the control animal that was fed with vector-transformed peanut leaves did not show any reactivity (Fig. 2) suggesting the capability of induced antibodies to recognize the antigens made by infected cells.

3.2. In vitro neutralization of virus infectivity

To analyze the in vitro protective ability of the induced antibodies, virus neutralization test was performed. Results (Tables 1 and 2) clearly show that high levels of neutralizing and cross-neutralizing antibodies are present in the serum 1 week after immunization and are maintained up to the duration of the experiments in the orally immunized
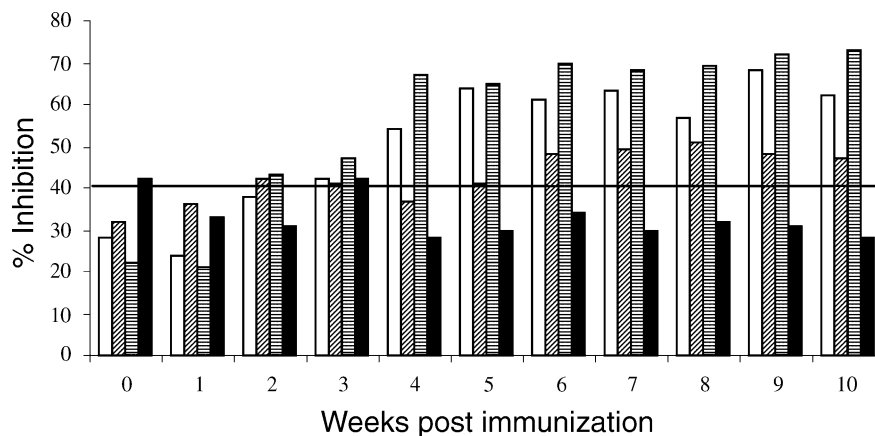

Fig. 1. Competition of immunized cattle serum with monoclonal antibody for recombinant H protein. Percent inhibition is calculated using the formula: PI = 100 − [(OD in test well/OD in 0% control well)] × 100. C1–C4 are represented as follows: C1 (■), C2 (□), C3 (▨) and C4 (▤).
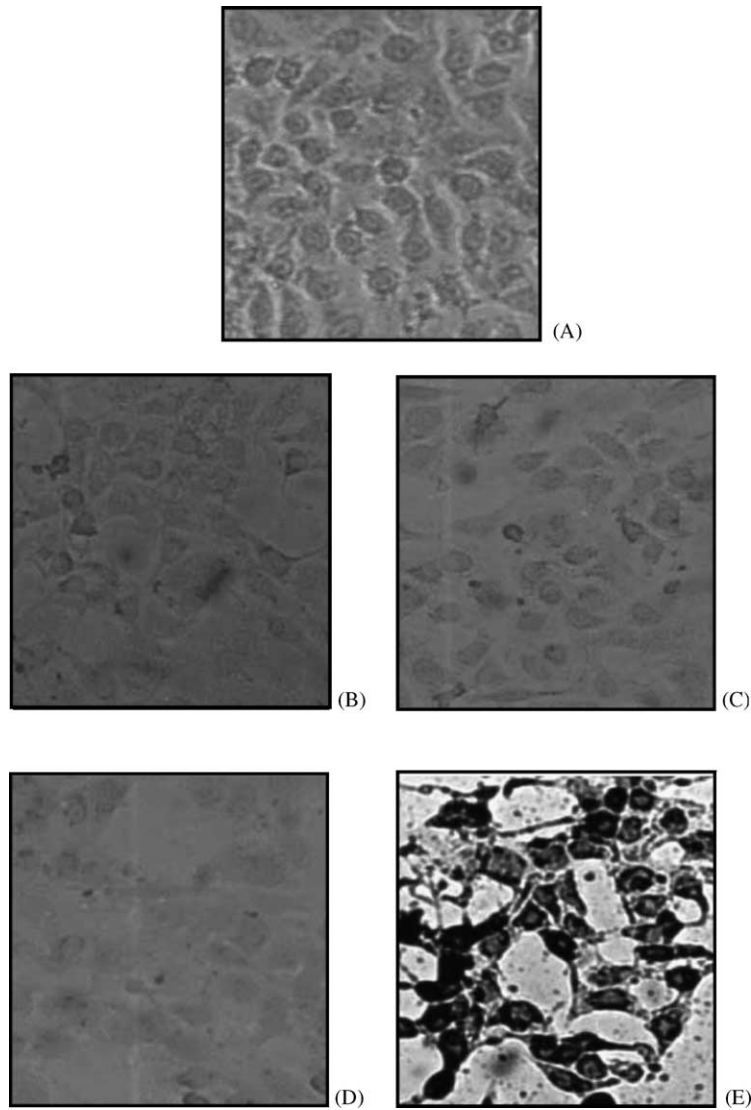
Fig. 2. Immunohistochemical staining. Panel A: uninfected Vero cells. Panels B and C: infected Vero cells immunostained with preimmune and immune serum (1:50 dilution in PBS) from animal fed with vector-transformed leaves (C1), respectively. Panels D and E: infected Vero cells stained with preimmune and immune serum (1:50 dilution in PBS) from animal fed with transgenic peanut leaves (C4), respectively. Vero cells grown on coverslips were infected with RPV (RBOK) at a multiplicity of 0.1–0.5 and incubated at 37 °C, for 48–72 h till 30–40% CPE was seen. Coverslips were washed in PBS and cells were fixed for immunohistochemical staining.

Table 1
In vitro neutralization of RPV infectivity by serum collected from cattle after oral immunization with transgenic peanut leaves or with vector-transformed peanut leaves

Animal	Virus neutralization titer[a] (days post-immunization)										
	0	7	14	21	28	35	42	49	56	63	70
C1	0	0	0	0	0	0	0	0	0	0	0
C2	0	320	320	640	80	40	40	40	40	40	20
C3	0	640	640	320	160	160	160	160	80	40	20
C4	0	160	320	320	160	160	160	40	40	40	40

[a] Virus neutralization titer is defined as the reciprocal of the highest dilution of serum exhibiting 50% protection of infected cells.

Table 2
In vitro neutralization of PPRV infectivity by serum collected from cattle after oral immunization with transgenic peanut leaves or with vector-transformed peanut leaves

Animal	Virus neutralization titer[a] (days post-immunization)										
	0	7	14	21	28	35	42	49	56	63	70
C1	0	2	2	2	2	2	4	2	2	0	0
C2	0	128	256	256	256	64	32	32	32	32	8
C3	0	0	0	0	0	64	128	128	64	64	16
C4	0	128	256	256	64	64	64	32	32	16	8

[a] Virus neutralization titer is defined as the reciprocal of the highest dilution of serum exhibiting 50% protection of infected cells.

animals. Homologous and heterologous virus neutralizing ability was absent in serum from the animal fed with vector-transformed peanut leaves. Earlier work from our group on virus neutralization titer following vaccination had shown virus neutralization titers of 20, 40, 40, 160 at 1, 2, 4 and 8 weeks post-immunization and nearly twofold lower titers of cross-neutralization against PPRV [17]. However, immunization of cattle with recombinant baculovirus expressing H (rECV-H) resulted in a twofold higher titer for both homologous and heterologous virus neutralization titers. Induction of high levels of virus neutralization titer immediately at 1 week post-immunization could be due to
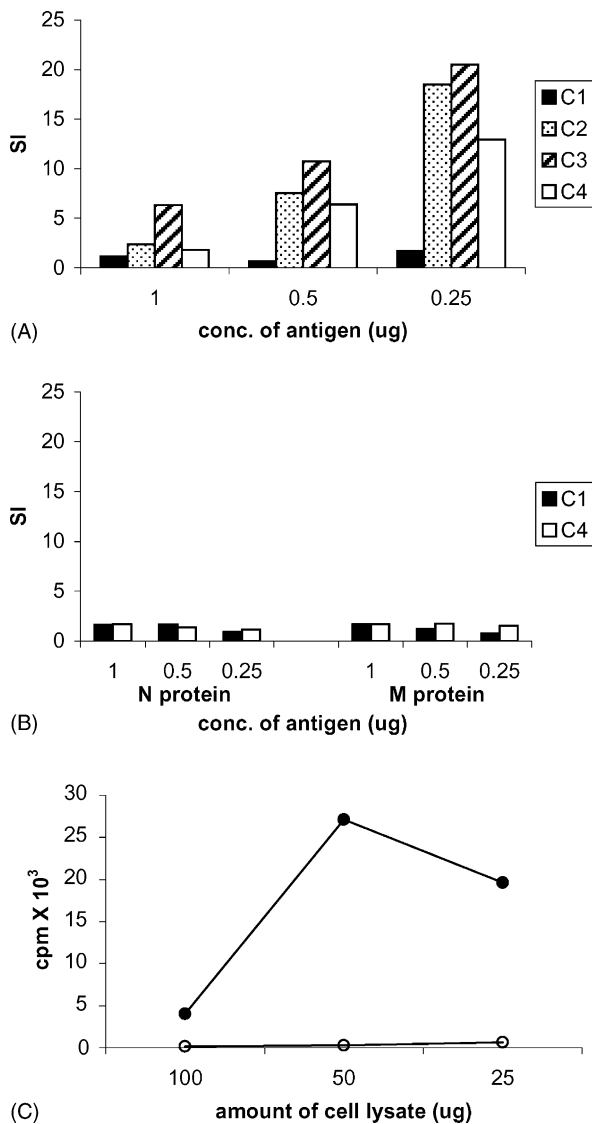

(A)

(B)

(C)

Fig. 3. (A) Lymphoproliferation of PBMC from animals fed with either vector-transformed peanut leaves (C1) or with transgenic peanut leaves (C2–C4) at 10 weeks post-immunization in presence of recombinant hemagglutinin protein. (B) Lymphoproliferation of PBMC from C1 (control animal fed with vector-transformed peanut leaves) and C4 (fed with transgenic peanut leaves expressing H) in presence of nucleocapsid (N) and matrix (M) protein of RPV. (C) In vitro proliferation of PBMC from C1 (○) and C4 (●) in presence of antigens of PPRV.

the adjuvant activity provided by some plant component. These results demonstrate that antibodies generated upon oral immunization are able to provide protection against RPV or cross-protection against PPRV infections in vitro.

3.3. Lymphoproliferative responses

At the end of 10th week post-immunization, PBMC were isolated and used for in vitro proliferation assay in presence of recombinant antigens (Fig. 3A and B). PBMC from animals fed with transgenic peanut leaves expressing H proliferated in a dose-dependent manner when SecH was used as the antigen (Fig. 3A), and the animal (C1) fed with vector-transformed peanut leaves did not show any proliferation in response to SecH. The specificity of lymphoproliferative responses was tested by stimulating the PBMC in presence of other antigens of RPV. Data shown in Fig. 3B shows that lymphocytes from orally immunized animals do not respond to other antigens of RPV (nucleocapsid (N) protein and matrix (M) protein), although the lymphocytes from vaccinated animal have been shown to proliferate in response to N protein of RPV [18]. In addition, when the cross-reactive proliferative responses to PPRV antigens were tested (Fig. 3C), lymphocytes from the animal immunized with peanut-derived H proliferated well in vitro in presence of PPRV infected cell lysate and the animal that received vector-transformed peanut leaves did not respond.

4. Discussion

As part of efforts to develop edible vaccine for rinderpest, we generated transgenic peanut (Arachis hypogea L.) plants expressing hemagglutinin protein of rinderpest virus. The antigenicity of peanut-derived H protein was established using specific antibodies and its immunogenicity was analyzed in a mouse model [40]. Oral feeding of transgenic peanut leaves induced specific mucosal (secretory IgA) and systemic immune responses (serum IgG and IgA) and also cell-mediated immune responses. In the present work, induction of immune responses in cattle was monitored upon oral delivery of hemagglutinin protein of rinderpest virus as part of food, without any mucosal adjuvant. To our knowledge, this is the first report describing elicitation of specific immune responses in the host animal by a protective antigen of a Morbillivirus expressed in transgenic plants given orally. Although small quantities of transgenic plant tissues (7.5 g for the first feeding followed by two feedings of 5 g) was given orally, the test animals developed high titer of specific antibodies. These antibodies were able to compete out monoclonal antibodies in ELISA (Fig. 1) demonstrating the specificity of the induced antibodies; in addition, these antibodies neutralized the virus infectivity in vitro. Animals were fed only thrice with plant-derived antigen at weekly intervals, which in addition to production of significant levels of specific antibody, resulted in stimulation of T cells

from immunized animals in response to specific antigens (Fig. 3A and B) indicating the induction of systemic immune response upon oral immunization. Wigdorovitz et al. [21] reported induction of protective systemic immune response in the mouse model upon oral feeding of transgenic plants expressing VP1 protein of foot and mouth disease virus. In this work, the VP1 protein expressed in alfalfa plants was not detected by Western blotting and several immunizations (three times a week for 2 months with approximately 0.3 g of leaves) were needed in order to induce a significant immune response. Similarly, Gomez et al. [22] have shown oral immunogenicity of the spike protein of swine-transmissible gastroenteritis coronavirus expressed in potato in a mouse model. This group followed almost similar immunization schedule as reported by Wigdorovitz et al. [21]. However, there was no detectable neutralization activity, which was attributed to the post-translational processing in the host plant. Compared to these two reports, in the present work, small quantities of peanut expressed H protein given orally without adjuvant induced high levels of virus neutralizing antibodies.

There are two reports where induction of specific immune response is demonstrated upon oral feeding of human volunteers with potato tubers expressing LT-B of *E. coli* [32] or Norwalk virus capsid protein-assembled as virus like particles [33]. In the first human trials, the antigen used (LT-B) is a well-known mucosal adjuvant and therefore when given through oral route, LT-B antigen induced significant systemic and mucosal immune responses. In the second trial, potato expressing Norwalk virus capsid protein was delivered orally. It has been suggested that the particulate nature of the virus like particles confer greater stability to the antigen in the stomach and resulted in specific immune response although the level of specific serum antibody was modest. Induction of specific immune response in mice upon oral delivery of measles virus hemagglutinin expressed in plant tissues has been demonstrated [34]. The induction of immune responses upon oral delivery shown in the present work might be due to "bioencapsulation" as described by Kong et al. [35]. Modelska et al. [36] have shown that expressed antigen is more immunogenic when plant material is fed orally as compared to the plant proteins present in the extract. Additionally, there is evidence to suggest that components of the plant also influence the immunogenicity of the antigen expressed [22]. The induction of serum or mucosal antibody response to orally administered antigens is often difficult and generally requires large quantities of antigen as only part of the antigen is being absorbed and is capable of eliciting an immune response. It was assumed that co-administration of a mucosal adjuvant is necessary to achieve optimum oral immunogenicity for a given antigen. Furthermore, the presentation of large amount of antigen may lead to oral tolerance and use of mucosal adjuvant will result in non-specific stimulation of mucosal immune system. de Aizpurua and Russell-Jones [37] have identified the class of proteins that provoke an immune response upon oral feeding and concluded that all the proteins that possess "lectin or lectin-like" binding activities are active in oral immunization. These molecules have the ability to bind to glycolipids or glycoproteins on the intestinal mucosae and thus transported across the epithelial barrier, to enter the circulation and elicit an immune response. More recently, plant lectins with different sugar specificities have been investigated for mucosal immunogenicity [38] and elicitation of specific systemic and mucosal antibody response was observed upon intranasal or oral administration. Since hemagglutinin protein of RPV is a cell attachment protein which binds to cell surface oligosaccharide containing protein or glycoconjugate in order for the virus to begin the infection process, it qualifies to be in the categories of antigens having "lectin or lectin-like" activities. Therefore, it is conceivable that H protein may be transported across the epithelial barrier easily through the mechanism described by Lavelle et al. [38] and leading to systemic immune responses. It remains to be seen if this protein expressed in peanut plants elicits a mucosal immune response upon oral immunization.

Rinderpest is an economically important disease of livestock and certainly remains a threat to the world because of the isolated foci of the disease. History has witnessed the outbreak of the disease after 40 years of rinderpest-free Sri Lanka [39]. Since most part of the world is declared rinderpest-free, use of time tested live attenuated vaccine is restricted. And the infection cannot be diagnosed at an early stage since there is no simple test to differentiate between animals vaccinated with currently used vaccine and infected animals. The recombinant subunit oral vaccine expressed in plants is useful not only in differentiating vaccinated and infected animals but also offers a cost-effective means of mass vaccination by production of transgenic plants expressing the vaccine antigen in developing countries. In addition, it will have the advantage provided by an oral vaccine which results in induction of both mucosal and systemic immune responses better achieved through oral administration as compared to parenteral delivery of the antigen and may help in the first line of defense at the mucosal surfaces. Although we have not carried out any challenge experiments due to lack of high disease security and containment facilities, in vitro neutralization demonstrated the protective capability of the induced antibodies and priming of T cells, which are also involved in rinderpest immunity [15] and therefore the present work clearly demonstrates the potential of edible oral vaccine against rinderpest.

Acknowledgements

References

[1] Mariner JC, van den Ende MC, House JA, Mebus CA, Salifou S, Stem C. The serological response to a thermostable Vero cell-adapted rinderpest vaccine under field conditions in Niger. Vet Microbiol 1990;22:119–27.

[2] Mariner JC, House JA, Sollod AE, Stem C, van den Ende MC, Mebus CA. Comparison of the effect of various chemical stabilizers and lyophilization cycles on the thermostability of a Vero cell-adapted rinderpest vaccine. Vet Microbiol 1990;21:195–209.

[3] Worrall EE, Litamoi JK, Seck BM, Ayelet G. Xerovac: an ultra rapid method for the dehydration and preservation of live attenuated rinderpest and peste des petits ruminants vaccines. Vaccine 2000;19:834–9.

[4] Anderson J, Fishbourne E, Corteyn A, Donaldson AI. Protection of cattle against rinderpest by intranasal immunisation with a dry powder tissue culture vaccine. Vaccine 2000;22:840–3.

[5] Yilma T, Hsu D, Jones L, Owens S, Grubman M, Mebus C, et al. Protection of cattle against rinderpest with vaccinia virus recombinants expressing the H or F gene. Science 1988;242:1058–61.

[6] Barrett T, Belsham GJ, Subbarao SM, Evans SA. Immunization with a vaccinia recombinant expressing the F protein protects rabbits from challenge with a lethal dose of rinderpest virus. Virology 1989;170:11–8.

[7] Belsham GJ, Anderson EC, Murray PK, Anderson J, Barrett T. Immune response and protection of cattle and pigs generated by a vaccinia virus recombinant expressing the F protein of rinderpest virus. Vet Rec 1993;124:655–8.

[8] Giavedoni L, Jones L, Mebus C, Yilma T. A vaccinia virus double recombinant expressing the F and H genes of rinderpest virus protects cattle against rinderpest and causes no pock lesions. Proc Natl Acad Sci USA 1991;88:8011–5.

[9] Yamanouchi K, Inui K, Sugimoto M, Asano K, Nishimaki F, Kitching RP, et al. Immunisation of cattle with a recombinant vaccinia vector expressing the haemagglutinin gene of rinderpest virus. Vet Rec 1993;132:152–6.

[10] Ohishi K, Inui K, Barrett T, Yamanouchi K. Long-term protective immunity to rinderpest in cattle following a single vaccination with a recombinant vaccinia virus expressing the virus haemagglutinin protein. J Gen Virol 2000;81(Part 6):1439–46.

[11] Verardi PH, Aziz FH, Ahmad S, Jones LA, Beyene B, Ngotho RN, et al. Long-term sterilizing immunity to rinderpest in cattle vaccinated with a recombinant vaccinia virus expressing high levels of the fusion and hemagglutinin glycoproteins. J Virol 2002;76:484–91.

[12] Romero CH, Barrett T, Evans SA, Kitching RP, Gershon PD, Bostock C, et al. Single capripox recombinant vaccine for the protection of cattle against rinderpest and lumpy skin disease. Vaccine 1993;11(7):737–42.

[13] Romero CH, Barrett T, Chamberlain RW, Kitching RP, Fleming M, Black DN. Recombinant capripox virus expressing the hemagglutinin protein of rinderpest virus: protection of cattle against rinderpest and lumpy skin disease viruses. Virology 1994;204(1):425–9.

[14] Ngichabe CK, Wamwayi HM, Ndungu EK, Mirangi PK, Bostock CJ, Black DN, et al. Long-term immunity in African cattle vaccinated with a recombinant capripox–rinderpest virus vaccine. Epidemiol Infect 2002;128:343–9.

[15] Bassiri M, Ahmad S, Giavedoni L, Jones L, Saliki JT, Mebus C, et al. Immunological responses of mice and cattle to baculovirus-expressed F and H proteins of rinderpest virus: lack of protection in the presence of neutralizing antibody. J Virol 1993;67:1255–61.

[16] Naik S, Renukaradhya GJ, Rajasekhar M, Shaila MS. Immunogenic and protective properties of haemagglutinin protein (H) of

[17] Sinnathamby G, Naik S, Renukaradhya GJ, Rajasekhar M, Nayak R, Shaila MS. Recombinant hemagglutinin protein of rinderpest virus expressed in insect cells induces humoral and cell-mediated immune responses in cattle. Vaccine 2001;19:3870–6.

[18] Sinnathamby G, Renukaradhya GJ, Rajasekhar M, Nayak R, Shaila MS. Recombinant hemagglutinin protein of rinderpest virus expressed in insect cells induces cytotoxic T-cell responses in cattle. Viral Immunol 2001;14:349–58.

[19] Sinnathamby G, Nayak R, Shaila MS. Mapping of T-helper epitopes of rinderpest virus hemagglutinin protein. Viral Immunol 2001;14:83–92.

[20] Walsh EP, Baron MD, Anderson J, Barrett T. Development of a genetically marked recombinant rinderpest vaccine expressing green fluorescent protein. J Gen Virol 2000;81:709–18.

[21] Wigdorovitz A, Carrillo C, Dus Santos MJ, Trono K, Peralta A, Gomez MC, et al. Induction of a protective antibody response to foot and mouth disease virus in mice following oral or parenteral immunization with alfalfa transgenic plants expressing the viral structural protein VP1. Virology 1999;255:347–53.

[22] Gomez N, Wigdorovitz A, Castanon S, Gil F, Ordas R, Borca MV, et al. Oral immunogenicity of the plant-derived spike protein from swine-transmissible gastroenteritis coronavirus. Arch Virol 2000;145:1725–32.

[23] Khandelwal A, Lakshmi Sita G, Shaila MS. Expression of hemagglutinin protein of rinderpest virus in transgenic tobacco and immunogenicity of plant-derived protein in a mouse model. Virology 2003; in press.

[24] Plowright W, Ferris RD. Cytopathogenicity of rinderpest virus in tissue culture. Nature 1957;179:316.

[25] Naik S, Shaila MS. Characterization of membrane-bound and membrane anchor-less forms of hemagglutinin glycoprotein of rinderpest virus expressed by baculovirus recombinants. Virus Genes 1997;14(2):95–104.

[26] Mitra-Kaushik S, Nayak R, Shaila MS. Identification of a cytotoxic T-cell epitope on the recombinant nucleocapsid proteins of rinderpest and peste des petits ruminants viruses presented as assembled nucleocapsids. Virology 2001;279:210–20.

[27] Renukaradhya GJ, Mitra-Kaushik S, Sinnathamby G, Rajasekhar M, Shaila MS. Mapping of B cell epitopes of hemagglutinin protein of rinderpest virus. Virology 2002;298(2):214–23.

[28] Devirddy LR, Raghavan R, Ramachandran S, Subbarao SM. Protection of rabbits against lapinized rinderpest virus with purified envelope glycoproteins of peste des petits ruminants and rinderpest viruses. Acta Virol 1998;42(5):299–306.

[29] McGarvey PB, Hammond J, Dienelt MM, Hooper DC, Fu ZF, Dietzschold B, et al. Expression of rabies virus glycoprotein in transgenic tomatoes. Biotechnology 1995;13:1484–7.

[30] Anderson J, McKay JA. The detection of antibodies against peste des petits ruminants virus in cattle, sheep and goats and the possible implications to rinderpest control programmes. Epidemiol Infect 1994;112:225–31.

[31] Reed LJ, Muench H. A simple method of estimating 50% endpoints. Am J Hyg 1938;27(3):493–7.

[32] Tacket CO, Mason HS, Losonsky G, Clements JD, Levine MM, Artnzen CJ. Immunogenicity in humans of a recombinant bacterial antigen delivered in a transgenic potato. Nat Med 1998;4:607–9.

[33] Tacket CO, Mason HS, Losonsky G, Estes MK, Levine MM, Arntzen CJ. Human immune responses to a novel Norwalk virus vaccine delivered in transgenic potatoes. J Infect Dis 2000;182(1):302–5.

[34] Huang Z, Dry I, Webster D, Strugnell R, Wesselingh S. Plant-derived measles virus hemagglutinin induces neutralizing antibodies in mice. Vaccine 2001;19:2163–71.

[35] Kong Q, Richter L, Yang YF, Arntzen CJ, Mason HS, Thanavala Y. Oral immunization with hepatitis B surface antigen expressed in transgenic plants. Proc Natl Acad Sci USA 2001;98(20):11539–44.

[36] Modelska A, Dietzschold B, Sleysh N, Fu ZF, Steplewski K, Hooper DC, et al. Immunization against rabies with plant-derived antigen. Proc Natl Acad Sci USA 1998;95(5):2481–5.

[37] de Aizpurua HJ, Russell-Jones GJ. Oral vaccination. Identification of classes of proteins that provoke an immune response upon oral feeding. J Exp Med 1988;167:440–51.

[38] Lavelle EC, Grant G, Pusztai A, Pfuller U, O'Hagan DT. The identification of plant lectins with mucosal adjuvant activity. Immunology 2002;102:77–86.

[39] Barrett T, Rossiter PB. Rinderpest: the disease and its impact on humans and animals. Adv Virus Res 1999;53:89–110.

[40] Khandelwal A, et al. Plant Sci 2003; in press.