Veterinaria Italiana, **41** (3), 149-155

Immune response and protective efficacy in sheep immunised with hydroxylamine-inactivated bluetongue virus vaccine

M.A. Ramakrishnan, A.B. Pandey, K.P. Singh, R. Singh & M.L. Mehrotra

Summary

Vero cell adapted bluetongue virus (BTV), serotype 18 (BTV-18) was inactivated with hydroxylamine and adjuvanted with aluminium hydroxide ($Al[OH]_3$) gel, saponin, or $Al(OH)_3$ gel/saponin prior to immunisation of sheep. Group-specific non-neutralising antibodies to BTV were detected in all vaccinated sheep as soon as seven days after vaccination. However, before challenge, serotype-specific neutralising antibodies were not detected in vaccinated sheep. Upon challenge with homologous live BTV, there was a reduction in delayed type of hypersensitivity response in control sheep but not in the vaccinated animals. All vaccinated sheep had significant ($P<0.01$) reductions in their clinical reaction index (CRI) and duration of viraemia.

Keywords

Bluetongue virus - Clinical reaction index - Delayed type of hypersensitivity, Hydroxylamine - Immune response - Immunosuppression - Vaccines.

Introduction

Bluetongue (BT) is an infectious, non-contagious, insect-borne viral disease of sheep and other ruminants. The disease has major implications for international trade of livestock. Bluetongue is caused by the bluetongue virus (BTV), genus *Orbivirus*, family *Reoviridae*. The virion is a non-enveloped, triple-layered, icosahedral particle containing 10 linear dsRNA segments. The outer layer contains two major proteins, VP2 and VP5, that are involved in the generation and specificity of neutralising antibodies (1, 3). The inner bi-layered icosahedral core is composed of two major proteins, VP7 and VP3, three minor proteins, VP1, VP4 and VP6, which surround the genomic RNA. The VP7 protein is the major BTV group reactive antigen (13). In addition to these structural proteins, the BTV genome encodes four non-structural proteins, NS1, NS2, NS3 and NS3A (14).

The BTV is transmitted between its vertebrate hosts by the bites of vector species of *Culicoides* and has a global distribution that extends approximately between latitudes 35°S and 50°N (7). It has been estimated that BTV causes losses of US$3 billion each year throughout the world (16). There are at least 24 serotypes of BTV of which 21 (except 19, 22 and 24) are reported to be circulating in India based on sero-surveillance and/or virus isolation studies (12).

Clinical signs of BT are usually observed in certain breeds of sheep and certain species of deer. Cattle and goats usually experience sub-clinical infections but can serve as important reservoirs of the virus

Centre for Animal Disease Research and Diagnosis, Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh 243122 - India
drramakrishnanvir@rediffmail.com

(6). The severity of the disease in sheep is dependent on the virus serotype and strain, certain environmental factors such as solar radiation, and the breed of sheep (2). Mortality can be as high as 70% in individual flocks but generally is much lower (10-20%). However, the main consequences of BTV infection are a result of indirect losses due to abortion, loss of condition and prolonged convalescence. Clinical signs of disease in sheep include fever, oedema, congestion, lameness and depression (9).

Vaccination of susceptible species is the most efficient way of controlling the disease. Live attenuated BTV vaccines have been developed and are used in many countries. However, attenuated BTV vaccines have potential safety problems, such as abortion in pregnant sheep, vector-borne transmission, genetic recombination, reversion to virulence (8) and immunosuppression (5). It is important, therefore, to develop inactivated vaccines against BT. This study was undertaken to determine the efficacy of a hydroxylamine-inactivated BTV vaccine that incorporates three different adjuvants.

Materials and methods

Virus and cells

A strain of serotype 18 of BTV (Bhopal isolate) was isolated from sheep in 1988 during a clinical outbreak of BT disease in Khandwa village, Madhyapradesh, India. The virus was passaged three times in baby hamster kidney (BHK-21) cells and freeze dried. For the present study, the virus was reconstituted and passaged three times in African green monkey kidney (Vero) cells. The cells were grown in Glasgow minimum essential medium supplemented with 10% foetal calf serum, 2 mM L-glutamine, 100 IU/ml benzyl penicillin and 100 µg/ml streptomycin sulphate (growth medium). This medium without serum served as the maintenance medium.

Animals

All sheep (local nondescript breed) used in this study were provided by the Division of Pharmacology and Toxicology, Livestock Production and Management Section, Indian Veterinary Research Institute (IVRI), Izatnagar, India. The sheep included both male and female animals and were between the ages of two and four years. Before the commencement of the experiment, the animals were shorn, dewormed with albendazole at 7.5 mg/kg body weight and dipped in cypermethrin High Cis. They were held in insect-proof housing and blood samples were obtained two weeks before the first vaccination to ensure that they were free of BTV antibodies. Animal experimentation was performed in accordance with the guidelines of the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA), India.

Inactivation of virus

Hydroxylamine was used as hydrochloride, $NH_2OH.HCl$. A solution of 2 M hydroxylamine was freshly prepared in 5 N NaOH. The pH of the solution was adjusted to 8.8 with 12 M HCl and this solution was added to the virus suspension to achieve a final concentration of 0.25 M. The BTV was allowed to react with hydroxylamine for a total of 48 h at 37°C. Inactivation was first performed for 36 h in one vessel, and then transferred to another vessel for a further 12 h to inactivate any residual live virus. After 48 h incubation, inactivation was stopped by the addition of ice cold phosphate buffered saline (PBS) in a ratio of 1:5 (for each ml sample 4 ml of PBS) and dialysed for 24 h against PBS to remove any excess or unbound hydroxylamine. The inactivated virus suspensions were inoculated onto blood agar and Sabourad dextrose agar to confirm the absence of bacterial and mycotic contamination, respectively.

The inactivated virus suspensions were inoculated onto Vero cell monolayers to confirm complete virus inactivation.

Formulation of vaccines

$Al(OH)_3$ gel-adjuvanted BTV vaccine

Equal parts of $Al(OH)_3$ gel (kindly provided by the late S.M. Lal, IVRI, Bangalore, India) and inactivated virus suspension were stirred for 30 min on ice. The mixture was kept at 4°C for 7 days for further maturation of the gel.

Saponin-adjuvanted BTV vaccine

Saponin was prepared as a stock solution of 100 mg/ml in PBS and added to a final concentration of 1 mg/ml. The mixture was stirred for 30 min and kept at 4°C for 7 days.

$Al(OH)_3$ gel/saponin-adjuvanted BTV vaccine

In addition to equal parts of $Al(OH)_3$ gel and virus suspension, saponin was added to a final concentration of 1 mg/ml. The mixture was stirred and kept at 4°C for 7 days.

Experimental design

Twelve seronegative sheep were randomly assigned to four groups of three animals each. Sheep in groups 1, 2 and 3 each received 4 ml of $Al(OH)_3$ gel-adjuvanted vaccine, 2 ml of saponin-adjuvanted vaccine and 4 ml of $Al(OH)_3$ gel/saponin adjuvanted vaccine, respectively. The sheep in group 4 served as unvaccinated controls. An equal quantity of vaccine was inoculated intramuscularly at two sites (neck and posterior thigh) and all vaccinated animals were revaccinated on day 21 with the same dose, route and vaccine type. All animals were challenged with virulent BTV-18 six weeks after the first vaccination. A total of 4 ml (1.2×10^5 PFU/ml) of virus per animal was injected simultaneously at multiple sites by the intradermal and subcutaneous routes.

Clinical signs

Sheep were monitored daily for body temperature and other clinical signs for 14 days following challenge. Clinical signs were quantified by a clinical reaction index (CRI) as described by Huismans *et al.* (3). A maximum score of 12 was given to the cumulative total of body temperature of more than 40°C from days 3 to 14 post inoculation (dpi), a score from 0 to 4 each for mouth, nose and foot lesions, and a score of 4 for death. These scores were added to a maximum possible score of 28.

Post challenge viraemia

Heparinised blood samples were collected and centrifuged at 1 500 × g for 10 min. After removing the plasma, red blood cells (RBCs) were washed three times with PBS and lysed with chilled sterile distilled water to the original volume. Three tubes of confluent monolayers of BHK-21 cells were inoculated with 0.1 ml of lysed RBCs and kept at 37°C for 1 h for virus adsorption. After washing the cells twice with maintenance medium, 1 ml of maintenance medium per tube was added and incubated at 37°C for 7 days. The cells were observed microscopically for BTV-specific cytopathic effects (CPE). Three blind passages were performed for each sample.

Humoral immune response

The presence of group-specific, precipitating, non-neutralising antibody was measured by the agar gel immunodiffusion (AGID) test (4). Serotype specific neutralising antibody was determined by the standard beta-microtitre serum neutralisation test (10).

Delayed type hypersensitivity test

Phytohaemagglunin (PHA) was used as a non-specific mitogen to evaluate cellular immunity (11). An area of approximately 6 × 6 cm was clipped on both sides of the neck of the sheep and approximately 2 × 2 cm was delineated with an indelible marker. The initial skin-fold thickness at each site was measured using Vernier calipers. The amount of intradermal mitogen had previously

been titrated in normal sheep to determine the optimal concentration and response time. Sheep were intradermally inoculated on one side of the neck with 100 μl of PHA diluted to 1 mg/ml in distilled water. Distilled water (100 μl) was injected into the opposite side of the neck to serve as a negative control. Skin fold thickness was measured again 24 h after injection. The response to the mitogen was determined by subtraction of initial skin thickness measurement from 24 h post-injection measurements.

Statistical analysis

Statistical analysis was performed using the Student *t* test.

Results

Clinical signs

No untoward effects or clinical signs were observed in vaccinated sheep before challenge. After challenge, clinical signs were scored as CRI as shown in Table I.

The sheep in all three vaccine groups presented a significant CRI reduction ($P<0.01$). With the exception of a single animal (ID No. 6; group 2; fever on 5 dpi), none of the vaccinated sheep exhibited fever or clinical signs of BT. In contrast, the control sheep had a mean CRI of 4.43±0.23 and fever occurred in all three animals for 3 to 4 days, lameness (ID No. 11), and mouth lesions (ID No.12).

Post challenge viraemia

The duration of post challenge viraemia amongst sheep in the different groups is shown in Table I. The virus was isolated for up to 7 dpi in vaccinated and for up to 13 dpi in control sheep.

Humoral immune response

Group-specific, non-neutralising antibodies to BTV were detected in vaccinated animals as early as 7 days after first vaccination. No neutralising antibodies were detected in any of the vaccinated sheep before challenge. Neutralising antibodies

Table I
Clinical reaction index and duration of post challenge viraemia

Group	Sheep ID No.	Clinical reaction index	Duration of viraemia (days)
1. ($Al[OH]_3$) gel adjuvant	1	0	7
	2	0	5
	3	0	7
	Mean ± SE	0.00 ± 0.00*	6.33 ± 0.66*
2. Saponin adjuvant	4	0	7
	5	0	7
	6	1	5
	Mean ± SE	0.33 ± 0.33*	6.33 ± 0.66*
3. ($Al[OH]_3$) gel + saponin adjuvants	7	0	7
	8	0	7
	9	0	7
	Mean ± SE	0.00 ± 0.00*	7.00 ± 0.00*
4. Control (unvaccinated group)	10	4	11
	11	4.5	13
	12	4.8	11
	Mean ± SE	4.43 ± 0.23	11.66 ± 0.66

* $P<0.01$

were detected one to two weeks after challenge in all vaccinated and unvaccinated animals (titres 1:4 to 1:512).

Delayed type hypersensitivity response

Prior to challenge, there was no significant difference in mean skin-fold thickness following administration of PHA to either the vaccinated or unvaccinated sheep. After challenge, the control group had reduced mean skin-fold thickness compared to thickness prior to challenge; specifically, the mean skin-fold thickness of control sheep prior to challenge was 4.33±1.2 compared to 0.33±0.33, 0.67±0.17, and 1.83±0.17 at 7, 14, and 21 days post challenge, respectively. There was no such reduction in skin thickness following PHA administration to vaccinated sheep after challenge.

Discussion

The aim of this study was to develop and evaluate an inactivated BTV vaccine. Vaccination of sheep with hydroxylamine-inactivated BTV vaccine (with all three adjuvants) resulted in a significant reduction ($P<0.01$) in clinical signs and duration of viraemia. Viraemia of limited duration in vaccinated sheep would potentially reduce the spread of vector-borne BTV in field conditions, leading to effective disease control.

All vaccinated sheep developed group-specific non-neutralising antibodies as early as 7 days after first vaccination. However, before challenge, none of the vaccinated animals developed neutralising antibodies. Potential explanations for the failure of the hydroxylamine-inactivated BTV vaccine to induce neutralising antibodies in vaccinated sheep include the following:

a) hydroxylamine may have altered some antigenic properties of proteins that invoke neutralising antibodies (VP2 and/or VP5)
b) insufficient antigen was used for immunisation
c) the adjuvants used were not effective.

In our previous study, sheep vaccinated with a binary ethylenimine (BEI)-inactivated, saponin adjuvanted BTV vaccine that incorporated the same strain and concentration of BTV, route of inoculation, and challenge dose etc. developed neutralising antibodies whereas similar vaccines adjuvanted with $Al(OH)_3$ or $Al(OH)_3$/saponin did not (12). Therefore, antigen concentration and adjuvant might not be responsible for the lack of immunogenicity observed in this study. Analysis of dsRNA extracted from hydroxylamine-inactivated BTV in RNA-PAGE did not show degradation of viral RNA (M.A. Ramakrishnan, unpublished observation), therefore we assume that hydroxylamine may have altered some antigenic properties of the proteins involved in the induction of neutralising antibodies. Specifically, epitopes on outer capsid proteins VP2 and VP5 may be altered because they are involved in the generation and the specificity of neutralising antibodies (1, 3).

Vaccination with VP2 isolated from purified virus particles or expressed from recombinant baculoviruses, can protect sheep against challenge with virulent virus of the same serotype (3, 14). Co-expression of VP2 with VP5 alone, or in combination with core proteins to form double-shelled virus-like particles (VLPs) enhances the neutralising antibody response of inoculated sheep (14). However, virus neutralising antibody may not be the only immune response capable of affording protective immunity, since sheep can resist challenge with live virus in the absence of neutralising antibody (15). This implies that although neutralising antibodies may play a role in protection, other factors or mechanisms are also involved. Vaccination with recombinant capripox virus expressed VP7 protein (group reactive antigen) partially protected sheep against virulent virus of heterologous serotype (17). Stott

et al. (15) showed that an inactivated virus conferred protection through a cellular immune response in the absence of neutralising antibodies.

Phytohaemagglunin was used as a non-specific mitogen to evaluate the cellular immune response in vaccinated and unvaccinated sheep. Upon challenge with live BTV, control animals had reduced delayed type hypersensitivity (DTH) response compared to pre-challenge, as also observed by Quist *et al.* (11) and Lacetera and Ronchi (5). In a study in deer, Quist *et al.* (11) observed that both epizootic haemorrhagic disease virus- and BTV-infected deer had reduced *in vitro* and occasionally *in vivo* proliferation of lymphocytes in response to mitogen (PHA) during acute infection. Recently, Lacetera and Ronchi (5) showed that inoculation of goats with a live attenuated BTV serotype 2 vaccine caused discernible and profound depression of non-specific lymphocyte blastogenesis as measured by DNA synthesis in peripheral blood mononuclear cells stimulated with PHA, concanavalin-A, and pokeweed mitogen.

Vaccines that incorporated each of the three adjuvants elicited similar humoral and cell-mediated immune (CMI) responses. The reduced duration of viraemia and reduction in CRI that occurred following challenge in vaccinated sheep were associated with the presence of only non-neutralising antibodies prior to challenge, however CMI could also have a role in protecting the vaccinated sheep. The authors also conclude that BTV causes immunosuppression (reduced DTH response) in sheep, and that inactivated vaccines can prevent the immunosuppressive effects of virulent BTV.

Acknowledgements

This work was supported by All India Network Programme on Bluetongue Disease, Indian Council of Agricultural Research, New Delhi, India.

References

1. DeMaula C.D., Bonneau K.R. & MacLachlan N.J. 2000. Changes in the outer capsid proteins of bluetongue virus serotype ten that abrogate neutralization by monoclonal antibodies. *Virus Res*, **67**, 59-66.
2. Erasmus B.J. 1990. Bluetongue virus. *In* Virus infections of ruminants (Z. Dinter & B. Morein, eds.). Elsevier, New York, 227-237.
3. Huismans H., van der Walt N.T., Cloete M. & Erasmus B.J. 1987. Isolation of capsid protein of bluetongue virus that induces a protective immune response in sheep. *Virol*, **157**, 172-179.
4. Jochim M.M. & Chow T.L. 1969. Immunodiffusion of bluetongue virus. *Am J Vet Res*, **30**, 33-41.
5. Lacetera N. & Ronchi B. 2004. Evaluation of antibody response and nonspecific lymphocyte blastogenesis following inoculation of a live attenuated bluetongue virus vaccine in goats. *Am J Vet Res*, **65**, 1331-1334.
6. MacLachlan N.J. 1994. The pathogenesis and immunology of bluetongue virus infection of ruminants. *Comp Immunol Microbiol Infect Dis*, **17**, 197-206.
7. Mellor P.S. & Witmann E.J. 2002. Bluetongue virus in the Mediterranean Basin 1998-2001. *Vet J*, **164**, 20-37.
8. Murray P.K. & Eaton B.T. 1996. Vaccines for bluetongue. *Aust Vet J*, **73**, 207-210.
9. Office International des Épizooties (OIE) 2000. Manual of standards for diagnostic tests and vaccines. OIE, Paris, 109-118.
10. Parker J., Herniman K.A., Gibbs E.P. & Sellers R.F. 1975. An experimental inactivated vaccine against bluetongue. *Vet Rec*, **96**, 284-287.
11. Quist C.F., Howerth E.W., Bounous D.I. & Stallknecht D.E. 1997. Cell-mediated immune response and IL-2 production in white-tailed deer experimentally infected with hemorrhagic disease viruses. *Vet Immunol Immunopathol*, **56**, 283-297.
12. Ramakrishnan M.A., Pandey A.B., Singh K.P., Singh R.,

Nandi S. & Mehrotra M.L. 2005. Immune responses and protective efficacy of binary ethylenimine (BEI)-inactivated bluetongue virus vaccines in sheep. *Vet Res Comm*, **29** (in press).

13. Roy P. 1992. Bluetongue virus proteins. *J Gen Virol*, **73**, 3051-3064.
14. Roy P., Marshall J.J.A. & French T.J. 1990. Structure of bluetongue virus genome and its encoded proteins. *Curr Top Microbiol Immunol*, **162,** 43-87.
15. Stott J.L., Barber T.L. & Osburn B.I. 1985. Immunologic response of sheep to inactivated and virulent bluetongue virus. *Am J Vet Res*, **45**, 1043-1049.
16. Tabachnick W.J., Robertson M.A. & Murphy K.E. 1996. *Culicoides variipennis* and bluetongue disease. *Ann NY Acad Sci*, **791**, 219-226.
17. Wade-Evans A.M., Romero C.H., Mellor P., Takamatsu H., Anderson J., Thevasagayam J., Fleming M.J., Mertens P.P. & Black D.N.1996. Expression of the major core structural protein (VP7) of bluetongue virus, by a recombinant capripox virus, provides partial protection of sheep against a virulent heterotypic bluetongue virus challenge. *Virol*, **220**, 227-231.