SHORT COMMUNICATION

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

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A recombinant capripox virus was constructed containing a cDNA copy of genome segment 7 of bluetongue virus (BTV) serotype 1 from South Africa (BTV 1SA), which expressed high levels of the major BTV core protein VP7 in infected lamb testis (LT) cells. Sheep vaccinated with this recombinant virus developed antibodies to VP7 (detected by ELISA) but no neutralizing antibodies to either the homologous or heterologous BTV serotype, prior to challenge (BTV 1 or BTV 3, respectively). Following challenge with a virulent heterotypic strain of BTV (BTV3 SA), all of the animals developed clinical signs of disease, indicating that they were infected and that the challenge virus did replicate. While all of the control animals died, six of the eight animals that were vaccinated with the recombinant capripox virus expressing VP7 recovered fully. This is the first report of a significant level of cross serotype protection against the lethal effects of a challenge with virulent BTV, produced by vaccination with a single BTV core protein, which did not generate a neutralizing antibody response. © 1996 Academic Press, Inc.

Bluetongue is an economically important noncontagious disease of sheep and cattle. The causative agent bluetongue virus (BTV), transmitted by *Culicoides* species (*1*), is the prototype member of the *orbivirus* genus, within the family Reoviridae. There are 24 known serotypes of BTV, identified by serum neutralization tests (2), which after a single vaccination do not exhibit long-term cross-protection and only generate neutralizing antibodies to the homologous serotype (*3, 4*). However, serial vaccinations with live attenuated BTV of different serotypes can generate a broad neutralizing antibody response to additional virus serotypes not previously encountered (*4, 5*). In South Africa, three pentavalent vaccines are currently used at 3-week intervals (containing a total of 15 attenuated live viruses, of different serotypes).

Genome segment reassortment can occur between different BTV strains in cell cultures, mammalian hosts, or insect vectors (*6*). Reassortant strains of BTV, African Horsesickness virus (AHSV), or Kemerovo serogroup orbiviruses have been characterized which have novel antigenic or virulence phenotypes (*7–9*). The use of multiple live viruses for vaccination is likely to increase the chances of reassortment between vaccine strains or between vaccine and wild-type viruses and could therefore generate novel progeny strains, which pose a greater disease threat or are less easy to control with the available vaccines. Attenuated strains of BTV used as vaccines can also have teratogenic effects in sheep and cows (*10, 11*). These observations provide impetus for the development of alternative, noninfectious and/or subunit BTV vaccines (*12*).

The BTV core contains two major structural proteins (VP3 and VP7) and three distinct minor proteins (VP1, VP4, and VP6/VP6a), which surround the 10 dsRNA genome segments (*13–15*). The outer capsid layer is composed of two additional major structural proteins (VP2 and VP5) and may be associated with small amounts of one of the nonstructural proteins (NS2). These outer coat proteins are involved in cell attachment and penetration, demonstrated by the large relative reduction in the infectivity of core particles for mammalian cells (*15*). VP2 and VP5 are the most variable of the BTV proteins and VP2 in particular varies in a serotype-specific manner (*16, 17*). VP2 and to a lesser extent VP5 have also been shown to be involved in the generation as well as the specificity of neutralizing antibodies, (*7, 12, 18–23*).

Vaccination with VP2 isolated from purified virus particles, or expressed using a recombinant baculovirus, can

protect sheep against challenge with virulent virus of the same serotype (*18, 23, 24*), although the titer of neutralizing antibodies produced and the level of protection observed were greater if VP2 and VP5 were used together. Although these data indicate that BTV outer capsid proteins may be useful subunit vaccine components, the protective immune response was serotype-specific and appeared to correlate with the levels of neutralizing antibodies. The outer capsid proteins of a single BTV serotype are therefore unlikely to generate significant protection against the other 23 serotypes, limiting their value for vaccination.

Jeggo *et al.* (*25*) showed that there was no correlation between the degree of protection and the level of neutralizing antibody produced after vaccination with "live" BTV, even if the vaccine and challenge viruses are of the same serotype. This implies that although neutralizing antibodies may play a role in protection, other factors or mechanisms are also involved. This has been confirmed by Stott *et al.* (*26*) who reported the protection of animals by cell-mediated immunity. Adoptive cell transfer studies in monozygotic sheep demonstrated serotype cross-reactive cell-mediated immunity, in the absence of neutralizing antibodies, although this protection is normally short-lived (2 weeks) (*3, 27*). Immunodominant serotype cross-reactive T-cell determinants have been located within the structural proteins of BTV cores (*28*) and serotype cross-reactive T-cell epitopes have been identified within VP7 (*29*). VP7 is a major BTV group reactive antigen (*30*) and was immunodominant during monoclonal antibody production using *in vitro* priming techniques (*31*). These data led us to consider VP7 as a possible candidate for use in recombinant subunit vaccines, which might induce a protective and serotype cross reactive immune response to BTV (possibly via a cell mediated mechanism).

Vaccinia virus is one of the most commonly used virus vectors for the generation of recombinant vaccines. However, it is capable of infecting many different species in addition to those hosts (cattle and sheep) which are targets for vaccination against BTV. In contrast capripoxvirus (CPV) has a restricted host range (cattle, sheep, and goats) and therefore its use as the basis of a recombinant vaccine for BTV would represent a more limited replication hazard in the field.

A plasmid transfer vector (pCR-3) was constructed containing the full-length cDNA clone of genome segment 7 (encoding VP7) of BTV serotype 1 from South Africa (BTV1 SA) (*32*), under the control of the vaccinia virus (VV) major late promoter p11. This plasmid also contains, in the opposite orientation, the *Escherichia coli* dominant selectable marker gene, xanthine-guanine phosphoribosyltransferase (Eco gpt) under the control of the VV major early/late promoter, p7.5. These two genes and their respective promoters were flanked by the entire capripox virus (CPV) thymidine kinase (TK) gene within
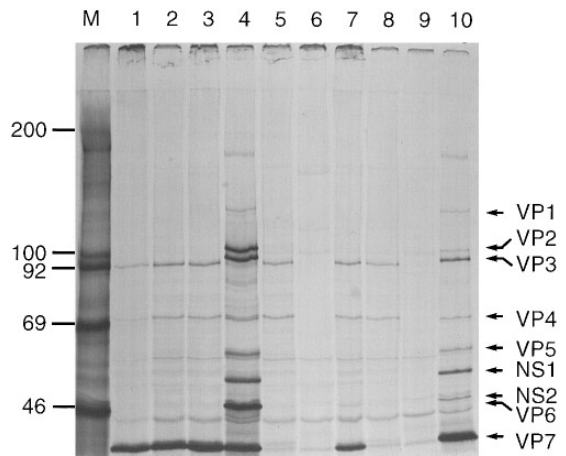

FIG. 1. Immunoprecipitation of BTV proteins synthesized in LT cells. Lysates of cells which were uninfected (lanes 6 and 9), or infected with the parental KS-1 CPV (lanes 5 and 8), rCPV.BTV1.VP7 (lanes 1, 2, 3, and 7), or BTV1SA (lanes 4 and 10), were labeled with [^{35}S]methionine. The BTV proteins were immunoprecipitated with guinea pig polyclonal antiserum raised against either BTV1SA (lanes 1, 4, 5, and 6), BTV3 (lane 2), or BTV10 (lane 3) or with the A3 monoclonal antibody (specific for VP7 (*36*)) (lanes 7, 8, 9, and 10). The precipitated proteins were analyzed by SDS–PAGE (*35*). The migration positions of the major BTV structural and nonstructural proteins are indicated.

the 2.5-kb *Hin*dIII S fragment of the KS-1-attenuated strain of CPV (*33*). A recombinant CPV (rCPV.BTV1.VP7) was generated by homologous recombination in primary lamb testis (LT) cell cultures infected with the KS-1 Pirbright vaccine strain of CPV and cotransfected with the plasmid vector pCR-3, as previously described (*34*). LT cells infected with this recombinant virus, synthesized a protein that coelectrophoresed with authentic BTV 1SA VP7 during SDS–PAGE (*35*) (Fig. 1), which was not made in cells infected with the CPV vaccine isolate (KS-1). This protein was immunoprecipitated by polyclonal antiserum to BTV 1SA, BTV 3, or BTV 10 and by the VP7-specific mAB A3 (*36*), identifying it as the BTV serotype cross-reactive antigen and major core protein, VP7 (Fig. 1). LT cells infected with rCPV.BTV1.VP7 and examined by indirect immuno-fluorescence using mAB A3 showed a high level of generalized diffuse staining as well as intensely stained cytoplasmic aggregates of VP7, which were not present in cells infected with KS-1 (Fig. 2).

Eight cross-bred Dorset horn sheep, 1–2 years old, housed in insect-proof loose boxes in the high security isolation units at the Institute for Animal Health, Pirbright, were vaccinated by two subcutaneous injections in the neck region of 1.5×10^7 plaque forming units (PFU) of rCPV.BTV1.VP7, given 21 days apart. Four control animals of similar age received 2×10^7 PFU of the parental CPV strain (KS-1). Two further control animals were not vaccinated. Two weeks after the second vaccination all 14 sheep received a normally lethal challenge by subcutaneous inoculation of 5.2 $\log_{10}$ 50% egg lethal dose (ELD$_{50}$) of a virulent strain of BTV3 SA (isolate SA535,
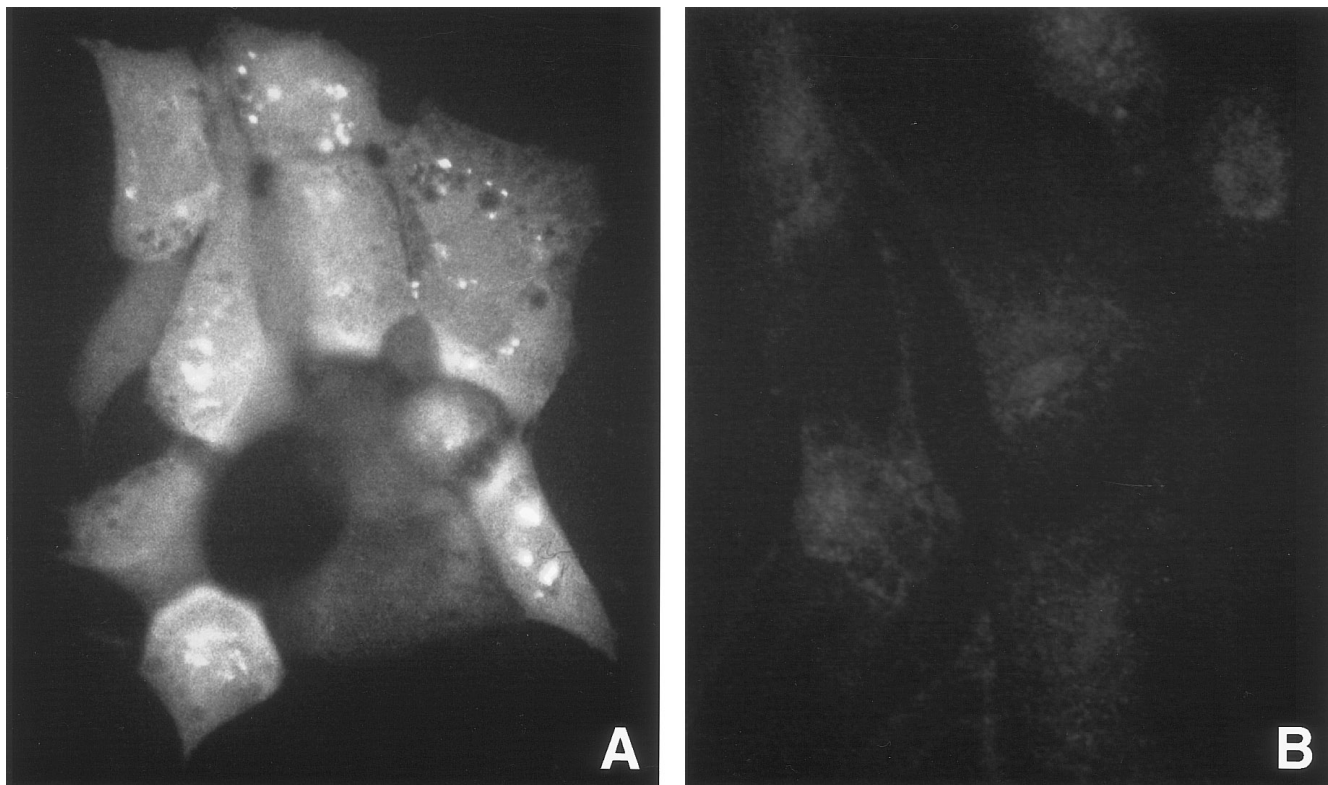
FIG. 2. Immunofluorescence in LT cells infected with either rCPV.BTV1.VP7 (A) or with the parental CPV KS-1 strain (B). Subconfluent cultures of LT cells, in Lab-Tek chamber slides, were infected with rCPV.BTV1.VP7 or KS-1 at $> 10^4$ plaque forming units (PFU) per well. On observation of cytopathic effect (CPE) the monolayers were rinsed in PBS, fixed for 2 min in acetone/methanol (1:1; v/v), and then incubated for 1 hr at 37° with an anti-VP7 monoclonal antibody, A3 (36). The cell monolayers were rinsed thoroughly with PBS + 1% Triton X-100 and then reacted for 1 hr at 37° with a 1:20 dilution of FITC-labeled rabbit anti-mouse IgG. All antibodies were diluted in PBS + 3% BSA. The slides were rinsed three times as before, mounted in glycerol/PBS (1:1; v/v), viewed using a Leitz fluorescent microscope, and photographed using identical exposure conditions.

from Dr. B. Erasmus, Onderstepoort). Rectal temperatures were measured daily and the animals monitored for clinical signs of disease. Blood samples were taken for serology at weekly intervals. Sheep inoculated with the rCPV.BTV1.VP7 produced high levels of anti-BTV VP7 antibodies, measured by competitive ELISA, reaching successive maxima 2 weeks after the first immunization and 1 week after the second (Fig. 3). After challenge, the antibody levels against VP7 increased further reaching a new maximum (Fig. 3) and remained high for the remainder of the experiment (until death or up to 4 weeks postchallenge). Although, the control sheep, both unvaccinated and those vaccinated with KS-1, did not show significant levels of antibodies to VP7 before the challenge, their VP7 antibody levels rose rapidly following challenge, prior to death (Fig. 3). After challenge all of the sheep, including those vaccinated with rCPV.BTV1.VP7, showed clinical signs of bluetongue, including fever (up to 41°), coronitis, conjunctivitis, and oedema of the lips and muzzle, indicating that in each case they were infected and the challenge virus had replicated. However, six of eight (75%) of the animals vaccinated with the recombinant capripox virus rCPV.BTV1.VP7 recovered
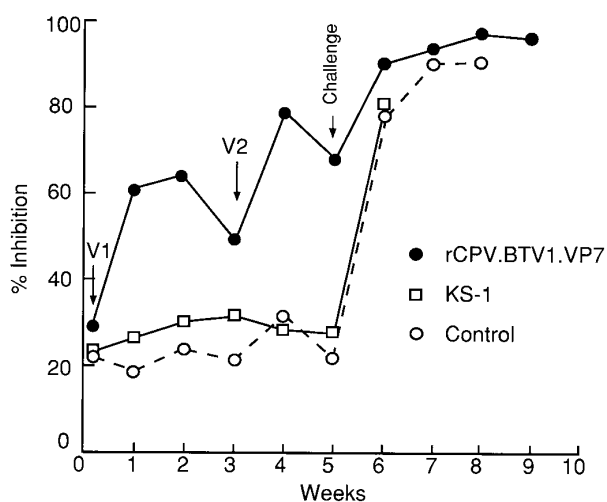

FIG. 3. A competitive ELISA (36) was used to determine the levels of anti-BTV VP7 antibodies circulating in the sheep used in the vaccination trial. Results from individual sheep sera were averaged for the surviving animals in each group (see Table 1) (vaccinated with rCPV.BTV1.VP7 (●), KS-1 (□), or unvaccinated (○)). Sheep were vaccinated twice (V1 and V2), 3 weeks apart and then challenged at 5 weeks with BTV3 SA.

TABLE 1

Neutralizing Antibody Titers to BTV-3 in Vaccinated and Unvaccinated Sheep

Sheep No.	Treatment	Weeks postvaccination						Weeks postchallenge			
		0^{V1}	1	2	3^{V2}	4	5^{CH}	1	2	3	4
39	rCP.BTV1.VP7 (1.5×10^7 PFU)	<10	<10	<10	<10	<10	<10	10	1280	1280	320
40	rCP.BTV1.VP7 (1.5×10^7 PFU)	<10	<10	<10	<10	<10	<10	<10	640	1280	320
41	rCP.BTV1.VP7 (1.5×10^7 PFU)	<10	<10	<10	<10	<10	<10	40	1280	1280	320
42	rCP.BTV1.VP7 (1.5×10^7 PFU)	<10	<10	<10	<10	<10	<10	40	1280	1280	1280
43	rCP.BTV1.VP7 (1.5×10^7 PFU)	<10	<10	<10	<10	<10	<10	20	1280	1280	640
44	rCP.BTV1.VP7 (1.5×10^7 PFU)	<10	<10	<10	<10	<10	<10	40	1280	1280	1280
45	rCP.BTV1.VP7 (1.5×10^7 PFU)	<10	<10	<10	<10	<10	<10	20	1280	1280	Dead
46	rCP.BTV1.VP7 (1.5×10^7 PFU)	<10	<10	<10	<10	<10	<10	80	Dead	—	—
47	KS-1 (2.0×10^7 PFU)	<10	<10	<10	<10	<10	<10	20	Dead	—	—
48	KS-1 (2.0×10^7 PFU)	<10	<10	<10	<10	<10	<10	<10	Dead	—	—
49	KS-1 (2.0×10^7 PFU)	<10	<10	<10	<10	<10	<10	80	Dead	—	—
50	KS-1 (2.0×10^7 PFU)	<10	<10	<10	<10	<10	<10	40	Dead	—	—
51	Nonvaccinated	<10	<10	<10	<10	<10	<10	40	Dead	—	—
52	Nonvaccinated	<10	<10	<10	<10	<10	<10	40	1280	1280	Dead

Note. Assays for neutralizing antibodies used methods similar to those described by Parker *et al.* (*41*). Serum samples were taken at weekly intervals from sheep vaccinated with either the recombinant CPV rCPV.BTV1.VP7 (expressing BTV VP7) or the parental KS-1 CPV and from the unvaccinated control animals. Twofold serial dilutions of serum (50 μl, starting at 10^{-1}) were prepared directly in 96-well microtiter tissue culture plates. An equal volume of medium (50 μl) containing 100 median tissue culture infectious doses (TCID$_{50}$) of BTV3 SA (or of BTV1 SA, data not shown) was added and the plates incubated for 1 hr at 37° then overnight at 4°. 100 μl of medium containing 2×10^5 BHK21 cells was added to each well and the plates were incubated at 37°. Plates were evaluated daily for CPE, the final reading being taken on Day 5. The neutralizing titer is defined as the reciprocal of the highest serum dilution that prevented CPE. Sheep were vaccinated twice 3 weeks apart and then challenged at 5 weeks. V1, first vaccination; V2, second vaccination; CH, challenge.

fully and all of the clinical signs had resolved by 4 weeks postchallenge. The surviving animals remained clinically normal until the end of the experiment (Week 5 postchallenge). Previous experience indicates that the animals surviving at Week 5 and no longer showing clinical signs had fully recovered and were unlikely to succumb as a result of this BTV challenge at a later date. All of the unvaccinated control animals, as well as those vaccinated with wild-type CPV, died within 4 weeks after challenge (five of six within 14 days). Sheep number 45, vaccinated with rCPV.BTV1.VP7 and unvaccinated control sheep number 52, which both died in the fourth week postchallenge, had inhaled ruminal contents as a result of smooth muscle lesions in the esophagus and died of pneumonia. Using Chi square analysis, with Yates's correction for continuity, the response (survival or death) of the two groups (sheep vaccinated with the recombinant capripox virus or the control animals) to subsequent challenge with virulent BTV3 was found to be significantly different ($\chi^2 = 5.11$, $df = 1$, and $P < 0.025$).

None of the sheep used in the trial, including those animals vaccinated with the recombinant capripox virus, developed neutralizing antibodies to BTV1 (results not shown) and no neutralizing antibodies to BTV3 were detected prior to the challenge (Table 1). Neutralizing antibody to BTV3 were detected 1–3 weeks after challenge in all of the surviving animals, which is also thought to reflect replication of the challenge virus. However, the level of neutralization produced was not affected by the prior vaccination and showed no correlation with the protection observed (Table 1). The partial protection of the sheep vaccinated with rCPV-BTV1.7 was not therefore dependent on inactivation of the challenge virus by preexisting neutralizing antibodies, which suggests that it may be mediated through a cellular immune response.

The data presented represent the first demonstration of a significant protective immune response against the lethal effects of challenge with a virulent, heterologous serotype of BTV, generated in response to the major outer core protein and serogroup cross reactive antigen VP7, by itself. This protection was not dependent on virus neutralization. These results confirm that a viral protein, other than the structural components of the BTV outer capsid layer, can play an important role in protection, possibly via a cell-mediated mechanism. These observations are consistent with those of Jeggo *et al.* (*5*), who were unable to demonstrate any evidence of antibody-dependent cell-mediated cytotoxicity (ADCC) or complement-mediated ADCC in BTV-infected sheep. Stott *et al.* (*37*) showed that an inactivated virus conferred protection via a cellular immune response, in the absence of neutralizing antibodies. BTV infection has previously been shown to induce BTV-specific cytotoxic T-cells (CTLs) in both sheep and mice (*38, 39*). These BTV-specific CTLs were serotype cross-reactive (*38*) and were able to inhibit BTV replication *in vitro* (*40*). Jeggo *et al.* (*39*) also used adoptive transfer techniques in monozygotic

sheep to show that CTLs partially protect animals from BTV challenge.

Roy *et al.* (*24*) have shown that VP2 and VP5, expressed by recombinant baculovirus in insect cell cultures, could be used as a subunit vaccine to protect sheep against BTV challenge. However, no increase in protection was observed if BTV core or nonstructural proteins were added to the vaccine. The data presented here demonstrate that VP7 by itself, if expressed by the recombinant capripox virus within the target host animal, can generate a protective immune response, although this does not appear to prevent virus replication. The expression of VP7, in conjunction with the two outer capsid proteins VP2 and VP5, within the vaccinated animal, may provide a more efficacious subunit vaccine. Recombinant capripox viruses simultaneously expressing these three proteins are currently being constructed.

ACKNOWLEDGMENTS

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