Recombinant Virus Vaccine for Bluetongue Disease in Sheep

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Bluetongue virus proteins derived from baculovirus expression vectors have been administered in different combinations to sheep, a vertebrate host susceptible to bluetongue virus, and the neutralizing antibody responses were measured. Vaccinated sheep were subsequently challenged, and the indices of clinical reaction were calculated. The results indicated that the outer capsid protein VP2 alone in doses of >50 µg per sheep elicited protection. A dose of ca. 50 µg of VP2 protected some but not all sheep. However, when used in combination with ca. 20 µg of the other outer capsid protein, VP5, 50-µg quantities of VP2 not only protected all the vaccinated sheep but also elicited a higher neutralizing-antibody response. The addition of viral core proteins VP1, VP3, VP6, and VP7, the nonstructural proteins NS1, NS2, and NS3, and the outer capsid proteins VP2 and VP5 did not enhance this neutralizing-antibody response.

Protection against a viral disease can be accomplished by using a live attenuated virus vaccine, an inactivated virus, or virus subunits either derived (extracted) from infectious material or produced by genetic engineering involving specific gene expression in a vector. Such vectors may be based on bacterial, yeast, or other cellular systems into which the gene is introduced. Certain viruses can also be used as vectors for gene expression.

Bluetongue virus (BTV) is the prototype of the genus *Orbivirus* (of the family *Reoviridae*) and is the causative agent of bluetongue disease in domestic ruminants, such as sheep and cattle. For nearly a century BTV has been associated with disease and mortality in sheep and cattle. At least 24 different serotypes (BTV-1, -2, etc.) have been identified from different parts of the world (3, 27). Modified live vaccines have been developed in South Africa and in the United States. In South Africa, sheep are presently vaccinated with three pentavalent live attenuated virus vaccines at 3-week intervals. In the United States, although five BTV serotypes have been identified (BTV-2, -10, -11, -13, and -17), a modified live vaccine is only available for BTV-10.

The 10 segments of the double-stranded RNA genome of BTV are located in the core of the virus particle. This core contains two major (VP3 and VP7) and three minor protein species (VP1, VP4, and VP6) and is surrounded by an outer capsid consisting of two major proteins, VP2 and VP5 (9, 18, 32, 33). It has been demonstrated both in vivo (using intertypic reassortment viruses) (14) and in vitro (by translation of each RNA segment) (19) that BTV RNA segment 2 codes for VP2. Using immunoprecipitation techniques, Huismans and Erasmus (10) have shown that VP2 is a major serotype-specific antigen. This has been confirmed by analyzing intertypic reassortant viruses (14). Huismans and associates (11) demonstrated that VP2 polypeptide recovered from purified BTV induced neutralizing antibodies and protected sheep against virulent viral challenge, indicating the potential of using VP2 as a subunit vaccine.

Conventional live attenuated vaccines have certain inherent deficiencies. In the case of BTV, such vaccines may cause fetal infection with resultant teratological defects.

When polyvalent vaccines are used, interference between component serotypes may occur, resulting in the development of incomplete immunity. Furthermore, since live attenuated vaccine strains are neutralized more readily by passive colostral immunity, they are less immunogenic in lambs than inactivated or subunit vaccines.

Genetic engineering techniques offer the possibility of preparing subunit vaccines without the need to grow the pathogenic organism. We recently reported the construction of a recombinant *Autographa californica* nuclear polyhedrosis virus (AcNPV) that expresses the VP2 protein of BTV-10, and we demonstrated that antisera raised against infected insect cell lysates derived from this virus contained neutralizing antibodies to BTV (13). In this paper, we present a dose-related evaluation of the protective properties of the recombinant VP2 in sheep, a natural host of BTV. Selected combinations of VP2 with other BTV-10 antigens have also been analyzed (VP1, VP3, VP5, VP6, VP7, NS1, NS2, and NS3) (4, 5, 12, 28, 30, 31; J. J. A. Marshall and P. Roy, *Virus Res.*, in press; C. P. Thomas and P. Roy, submitted for publication). The results of these studies on the development of recombinant subunit vaccines for bluetongue disease are discussed.

MATERIALS AND METHODS

Virus and cells. AcNPV and recombinant virus stocks were grown and assayed in confluent monolayers of *Spodoptera frugiperda* cells in modified Grace medium (TC 100) containing 10% fetal bovine serum by the procedures described by Brown and Faulkner (2). BTV-10 was grown and assayed in confluent monolayers of either BHK-21 or Vero cells in Eagle medium containing 10% fetal bovine serum. Purified virus particles were obtained by using the methods described by Mertens et al. (20).

Preparation of recombinant virus-infected cell lysates for sheep inoculation. *S. frugiperda* cells were propagated as suspension cultures in medium supplemented with 10% calf serum (GIBCO Laboratories) at 28°C. Each flask of cultured cells was infected with individual recombinant AcNPV at a multiplicity of 5 PFU per cell and then incubated for 2 to 3 days. The infected cells were recovered by centrifugation, washed with phosphate-buffered saline, and lysed by three freeze-thaw cycles. A portion of each sample was analyzed

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by 10% polyacrylamide gel electrophoresis (15) followed by Coomassie blue staining to estimate the amount of BTV protein present. Each sample was then divided into aliquots and stored at -20°C until the day of immunization.

Animals. Twenty-four 1-year-old Merino sheep that originated from a BTV-free region of the North Eastern Cape of South Africa were used for the vaccination trials. The lack of BTV antibody in the herd was verified by analyzing animal serum by using an enzyme-linked immunosorbent assay (12) and an immunodiffusion test for BTV group-specific antigens as well by plaque reduction neutralization tests against BTV serotype 10. Two weeks before the experiment, the sheep were transferred to an insect-proof isolation stable, where they were kept for the duration of the study.

Vaccinations. Animals were divided into six treatment groups (groups I to VI) and immunized subcutaneously with the infected-cell extracts containing the indicated BTV proteins. Three groups of animals received extracts containing only the VP2 of BTV-10 (from 50 to 200 μg [see Tables 1 and 2]). Group IV received a mixture of BTV-10 VP2 ($\sim 50 \mu\text{g}$) and BTV-10 VP5 ($\sim 20 \mu\text{g}$). Group V received a mixture of nine BTV proteins (namely, VP1, VP2, VP3, VP5, VP6, VP7, NS1, NS2, and NS3) in the indicated amounts. With the exception of recombinant VP3, which originated from BTV-17, all the proteins were derived from BTV-10 genes. Control animals received only saline. In each group, two animals were given vaccine together with incomplete Freund adjuvant; no adjuvant was used for the remaining two sheep. With the exception of three groups of sheep which received only one booster dose (groups II, III and V), all other groups of sheep received two booster injections (on days 21 and 42, respectively [see Tables 1 and 2]).

Plaque reduction neutralization test. From day 22 to day 96 after the primary inoculation, serum from each animal was collected at intervals and diluted as required with phosphate-buffered saline. Virus neutralization tests were accomplished as described elsewhere (11). Antibody titers are expressed as the reciprocal of the serum dilution causing a 50% plaque reduction.

Immunoprecipitation test. Immunoprecipitation tests were conducted essentially as described by Huismans et al. (11). In short, [^{35}S]methionine-labeled polypeptides were immunoprecipitated from cytoplasmic extracts derived from BTV-10-infected BHK-21 cells with either sheep anti-BTV-10 hyperimmune serum or serum derived from the vaccinated sheep (11). The ^{35}S -labeled polypeptides were separated by electrophoresis on 10% polyacrylamide gels and visualized by autoradiography.

BTV challenge and clinical reaction index. All the sheep were challenged by subcutaneous injection with 1 ml of infective sheep blood containing virulent BTV-10 (South African strain) at day 75, i.e., 33 days after the final immunization of sheep. The clinical reactions were monitored from 3 to 14 days postchallenge. The severity of clinical bluetongue after challenge with the virulent virus was expressed in numerical form as the clinical reaction index as described by Huismans and associates (11).

Viremia assays. After challenge with virulent virus, heparinized whole-blood samples from the sheep were collected daily for 15 days. Each sample was administered intravascularly to 10- to 12-day-old embryonated chicken eggs followed by incubation at 33°C . Embryos were monitored for 7 days. Dead embryos were harvested and suspended (10% [wt/vol]) in Eagle medium and seeded onto monolayers of BHK-21 cells. These monolayers were observed for 7 days for the appearance of cytopathic changes and recovery of

FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoretic analyses of recombinant baculoviruses (AcBTV) that express the 10 gene products of BTV-10, compared with BTV virion proteins and AcNPV-infected *S. frugiperda* cells. In addition to the BTV proteins, the AcNPV polyhedrin protein is identified. The resolved proteins were stained with Coomassie brilliant blue. Nine BTV proteins other than VP4 were used in vaccine trials (see Materials and Methods).

virus. All virus isolates were serotyped to confirm their identities.

RESULTS

Vaccination of sheep with expressed BTV antigens and induction of BTV-neutralizing antibodies. Proteins derived from nine baculovirus recombinants that express BTV genes were used for vaccinating sheep. The recombinants were made by inserting into AcNPV the appropriate DNA copies of cloned BTV genes derived from the prototype United States BTV-10 virus strain except for genome segment L3 (VP3), which came from BTV-17 (7, 16, 17, 23–26, 29, 35, 36). The BTV genes were placed under the control of the AcNPV polyhedrin promoter as described previously (4, 5, 12, 13, 28, 30, 31; Marshall and Roy, in press). The recombinants were designated as follows: AcBTV-10.1 (VP1), AcBTV-10.2 (VP2), AcBTV-17.3 (VP3), AcBTV-10.5 (VP5), AcBTV-10.9 (VP6), AcBTV-10.7 (VP7), AcBTV-10.6 (NS1), AcBTV-10.8 (NS2), and AcBTV-10.10 (NS3). The BTV-10 VP4 gene was not available for the study.

To determine the concentration of BTV protein present in each extract of recombinant virus-infected cells, polypeptides were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described in Materials and Methods. The proteins were visualized by Coomassie blue staining (Fig. 1). The amounts of the BTV proteins in the extracts were estimated by subsequent comparisons of the stained extracts to known quantities of bovine serum albumin electrophoresed in parallel in the same gel (data not shown). Aliquots of cell extract were stored at -20°C until required for immunization.

Twenty-four Merino sheep were used for the vaccination trials as follows. Three groups of four sheep each (groups I, II, and III) were injected subcutaneously with various doses of the recombinant BTV-10 VP2 protein (derived from AcBTV-10.2). VP2 is one of the two major outer capsid proteins of BTV. Booster doses were administered on day 21 (and also on day 42 for group I) (see Tables 1 and 2). The sheep in group I received approximately 50 μg of VP2

TABLE 1. Serum plaque reduction titers of sheep inoculated with recombinant BTV antigens

Group no. ^a	Antigen(s) (μg)	Sheep no.	Adjuvant	Serum neutralization titers ^b against BTV-10 on day:							
				25	42	48	50	52	60	67	74
I	VP2 (~50)	1	—	32	32	64	64	32	64	16	8
		2	—	32	32	32	32	16	8	8	4
		3	+	16	16	32	32	32	16	12	8
		4	+	<4	4	16	16	16	8	8	8
II	VP2 (~100)	5	—	>32	64	16	16	16	16	8	8
		6	—	>32	64	32	32	16	16	12	8
		7	+	32	32	16	16	16	8	6	4
		8	+	16	8	<4	<4	<4	<4	<4	<4
III	VP2 (~200)	9	—	>32	128	32	32	32	16	16	8
		10	—	>32	64	16	16	16	16	8	8
		11	+	>32	128	64	64	32	32	32	16
		12	+	>32	512	128	128	128	64	64	32
IV	VP2 (~50) and VP5 (~20)	13	—	<4	<4	16	8	8	8	4	4
		14	—	<4	4	16	8	8	8	4	4
		15	+	>32	128	512	256	128	128	128	96
		16	+	32	64	128	128	64	32	32	24
V	VP1 and VP5 (~20 each); VP2 and VP3 (~50 each); VP6 and VP7 (~100 each); NS1 and NS2 (~200 each); and NS3 (~20)	17	—	8	>4	8	8	8	16	16	16
		18	—	16	4	8	8	8	24	24	12
		19	+	>32	128	256	256	128	64	64	16
		20	+	>32	64	128	128	64	64	48	24
VI (control)	Saline	21	—	<4	<4	<4	<4	<4	<4	<4	<4
		22	—	<4	<4	<4	<4	<4	<4	<4	<4
		23	+	<4	<4	<4	<4	<4	<4	<4	<4
		24	+	<4	<4	<4	<4	<4	<4	<4	<4

^a All sheep were inoculated on days 0 and 21; those in groups I, IV, and VI were also inoculated on day 42.

^b Reciprocal of the dilution that caused a 50% plaque reduction.

antigen per inoculation, while groups II and III received approximately 100 and 200 μg, respectively, per inoculation. To investigate whether VP5 (the second outer capsid protein) plays a role in the induction of neutralizing antibodies, four sheep (group IV) were injected with a mixture of VP2 (ca. 50 μg) and VP5 (ca. 20 μg) and were similarly boosted on days 21 and 42. In addition, to determine whether other virion proteins (the core proteins VP1, VP3, VP6, and VP7) or the three nonstructural proteins (NS1, NS2, and NS3) would contribute to the protective immune response, four sheep (group V) were inoculated (and boosted once) with mixtures of recombinant virus-infected cell lysates containing VP1 (ca. 100 μg), VP2 (ca. 50 μg), VP3 (ca. 50 μg), VP5 (ca. 20 μg), VP6 (ca. 100 μg), VP7 (ca. 100 μg), NS1 (ca. 100 μg), NS2 (ca. 100 μg), and NS3 (ca. 20 μg). The amounts of BTV antigen inoculated were based on the availabilities of the viral antigens. The control group of four sheep (group VI) received only saline. To evaluate whether adjuvant enhanced the immunity and induction of neutralizing antibodies in sheep, two animals in each group received vaccine without adjuvant, while the other two were given vaccine emulsified in incomplete Freund adjuvant.

Serum samples were collected from each sheep at regular intervals between days 7 and 75 postimmunization and were tested for the presence of neutralizing antibodies against BTV-10 by the plaque reduction neutralization test. All the sheep immunized with or without adjuvant elicited BTV-10-neutralizing antibodies, albeit to various levels (Table 1). Higher antibody titers were obtained when adjuvant was included for sheep in groups III, IV, and V (Table 1). In contrast, adjuvant did not seem to have any effect when low

doses (50 to 100 μg) of VP2 antigen were used (groups I and II in Table 1). When a small amount of VP5 (ca. 20 μg) was combined with ca. 50 μg of VP2 antigen, higher titers of neutralizing antibodies were elicited (Table 1). The presence of other viral proteins (group V) did not appear to make any difference to the neutralizing antibody titers produced. In all cases, the plaque reduction titers decreased with time. No neutralizing antibodies were detected in the sera of sheep inoculated with saline alone.

Immunoprecipitation of BTV-10 proteins by immunized sheep sera. Immunoprecipitation analyses were used to analyze the specificities of the immune responses to the various combinations of expressed BTV antigens. From each group of sheep, only sera with high neutralizing-antibody titers were analyzed. The assays were performed by incubating samples of ³⁵S-labeled soluble protein fraction (S100) obtained from BTV-infected BHK-21 cell cultures with a sample of the respective serum as described in Materials and Methods. Two control sera were also included. Serum from a sheep immunized with purified BTV particles was used as a positive control, while the preinoculation serum of one of the sheep that received a low dose (group I) of VP2 served as a negative control. The 48-day serum of a sheep that received 200 μg of VP2 precipitated VP2 (Fig. 2). The 51-day serum of a sheep that received both the VP2 (50 μg) and VP5 (20 μg) precipitated both proteins, while VP2, VP3, VP5, and VP7 were detected in the immunoprecipitation analysis with the serum collected from a sheep that was immunized with a mixture of all nine BTV proteins (group V). None of the nonstructural proteins was immunoprecipitated by that serum. This was expected, since

FIG. 2. Immune precipitation of ^{35}S -labeled BTV-10 protein with sera from sheep injected with 100 μg of VP2 alone (lane b), with 50 μg of VP2 and 20 μg of VP5 (lane c), or with a mixture of nine expressed BTV proteins (lane d). Lane a shows the immunoprecipitation of ^{35}S -labeled BTV proteins with anti-BTV-10 antiserum.

the nonstructural proteins are predominantly insoluble and should not have been present in the S100 fraction of the ^{35}S -labeled infected cells. The assays failed to detect the minor core proteins VP1 and VP6.

Protection against virulent viral infection. To assess the ability of the recombinant viral antigens to induce a protective immunity, on day 75 (33 days after the second booster injection) all sheep were challenged by subcutaneous injection with infective sheep blood of a South African strain of virulent BTV-10. From day 1 postchallenge, rectal temperatures were recorded twice daily and the sheep were carefully examined for clinical manifestations of bluetongue disease. The clinical reaction index was expressed numerically (see Table 2, footnote a). Whole-blood samples were collected daily after the virus challenge for the first 15 days and were screened for viremia by passage in 10- to 12-day-old embryonated chicken eggs (Table 2). The recovered virus was identified as BTV-10. Plaque reduction titers were determined for sera taken at 21 days postchallenge (Table 2).

Apart from two sheep of group I that received a low dose (ca. 50 μg) of VP2 (Table 2, sheep 2 and 4), all of the sheep injected with recombinant BTV antigens were immune to virulent virus challenge. None of these sheep developed any clinical symptoms of bluetongue disease or demonstrable viremia, although they did show mild anamnestic antibody responses. The two immunized sheep (sheep 2 and 4) that showed mild clinical signs showed a marked antibody response indicative of virus replication. Surprisingly, however, virus was recovered from the blood of only one sheep (no. 2) and not from that of the other (no. 4). All of the control sheep, on the other hand, developed typical bluetongue disease with a relatively high clinical reaction index.

TABLE 2. Immune status of vaccinated sheep after virulent virus challenge

Group no.	Inoculum (μg)	Sheep no.	Serum neutralization titer against BTV-10 at day 21 postchallenge	Clinical reaction index ^a	Viremia ^b (days postchallenge)
I	VP2 (~50)	1	160	0.0	—
		2	640	1.4	4-6
		3	40	0.0	—
		4	320	3.1	—
II	VP2 (~100)	5	40	0.0	—
		6	<20	0.0	—
		7	<20	0.0	—
		8	80	0.0	—
III	VP2 (~200)	9	80	0.0	—
		10	40	0.0	—
		11	80	0.0	—
		12	<20	0.0	—
IV	VP2 (~50) and VP5 (~20)	13	40	0.0	—
		14	40	0.0	—
		15	120	0.0	—
		16	60	0.0	—
V	VP1 and VP5 (~20 each); VP2 and VP3 (~50 each); VP6 and VP7 (~100 each); NS1 and NS2 (~200 each); and NS3 (~20)	17	20	0.0	—
		18	20	0.0	—
		19	<20	0.0	—
		20	20	0.0	—
VI (control)	Saline	21	>640	7.4	4-9
		22	640	5.0	4-10
		23	640	4.6	4-9
		24	>640	5.1	4-10

^a Clinical reaction index = a + b + c where a = the fever score - (cumulative total of fever readings above 40°C on days 3 to 14 after challenge) (maximum score, 12), b = the lesion score (lesions of the mouth, nose, and feet were each scored on a scale of 0 to 4 and added together) (maximum score, 12), and c = the death score (4 points if death occurred within 14 days postchallenge).

^b Viremia was assayed in eggs. —, None detected; numbers refer to days that sheep blood tested positive for viremia.

In addition, the postchallenge blood of the control sheep was viremic, and their sera contained high plaque reduction titers, which is characteristic of a primary infection.

DISCUSSION

Baculovirus-expressed BTV proteins were used to induce neutralization antibodies in sheep and protection against virulent virus challenge. BTV-10 VP2 protein, in excess of 50 µg per sheep, elicited neutralizing antibodies and totally protected the animals. These results confirmed previous observations that the outer capsid protein VP2 is the main determinant of the neutralization-specific immune response (1, 10, 11, 13, 14) and that it induces protection (11). Our data indicated that 50 µg of the expressed VP2 alone was insufficient to confer total protection. Two successive injections of 100 µg of VP2 provided full protection, a finding that closely correlates with that of Huismans and associates (11). However, 50 µg of VP2 together with 20 µg of VP5 protected the sheep. Other amounts of the two antigens have yet to be assessed. Why the VP5 antigen enhances the neutralization (and protective) response is not known. No neutralizing monoclonal or monospecific antibody which reacts specifically with VP5 protein has yet been obtained. Recently, Mertens and associates (21) reported that both VP2 and VP5 are involved in the determination of BTV serotype response as analyzed by serum neutralization analyses of a reassortant virus. Our studies confirm that a combination of VP2 and VP5 antigens elicited significantly higher titers of BTV-neutralizing antibodies. It is possible that VP5 enhances the immune responses indirectly by interaction with VP2 and by affecting the conformation of VP2 and, consequently, its serological properties. In this context, though, it is noteworthy that the VP5 proteins of Kemorovo serogroup orbiviruses elicit neutralizing-antibody responses (22). It remains to be determined whether any of the BTV core proteins or nonstructural proteins play a role in protection and in the cellular immune response to BTV.

We have yet to determine the minimal amount of VP2 and VP5 antigens needed for complete protection and the duration of the immunity conferred by these antigens. It is also essential to perform similar vaccination trials in cattle, since cattle are a major reservoir host of BTV. Another important aspect of vaccine development is the role of adjuvant. Our data demonstrated that the incomplete Freund adjuvant enhanced the neutralizing-antibody responses in sheep. Whether other, more acceptable adjuvants are effective remains to be determined.

Previous studies involving cDNA-RNA hybridization experiments as well as complete sequence analysis of cDNA clones of viral RNA species have demonstrated that both outer capsid proteins VP2 and VP5 are among the most variable proteins of different BTV serotypes. Depending on the serotype, they exhibit sequence relationships to other BTV serotypes (6, 8, 26, 34). Data that indicate that the antigens of one BTV serotype elicit antibody responses that neutralize the infection by other BTV serotypes (13) have been obtained. The extent that the baculovirus-expressed antigens representing a BTV serotype or heterologous combinations of VP2 and VP5 elicit immune responses that protect sheep against heterologous virus challenges needs to be determined.

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