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Passive Protection of Mice and Sheep Against Bluetongue Virus by a Neutralizing Monoclonal Antibody

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A murine hybridoma antibody, 6C2A.4.2, previously characterized as an immunoglobulin G class 2a that binds in radioimmunoassay to bluetongue virus serotype 17 (BTV-17) but not the other 19 BTV serotypes, neutralizes BTV-17, inhibits hemagglutination with BTV-17, and precipitates viral polypeptides 2 and 3 from BTV-17-infected cells, was produced as an ascites in the peritoneal cavities of hybridoma-inoculated mice. This ascitic fluid, but not those containing other, non-neutralizing anti-BTV-17 antibodies of the same isotype, provided serotype-specific passive protection against BTV-17-induced death of neonatal mice. Antibody 6C2A.4.2-containing ascitic fluid was injected intravenously into sheep that were later inoculated with BTV-17. These sheep remained free of clinical signs, did not develop viremia or detectable levels of antibodies reactive in the immunodiffusion test used for routine BTV diagnosis in the United States, and developed only low levels of neutralizing antibodies. Control animals became viremic and developed immunodiffusion test reactions and high levels of neutralizing antibodies during recovery, and two of three had lesions and fevers. These results provide evidence that antibodies directed against a single epitope on BTV-17 can prevent bluetongue disease.

Bluetongue virus (BTV) is the type species of the orbivirus genus. Many of the 20 serotypes of BTV are known to infect sheep, causing hyperemia, cyanosis, edema, and ulceration of oral tissues as well as fever, laminitis, abortion, and fetal anomalies (4). Cattle, goats, deer, and other ungulates are also susceptible to BTV (23).

Immunity follows natural infection (7) and vaccination with attenuated BTV (7, 18), but the mechanisms of immunity have not been investigated extensively. Antibodies reactive in hemagglutination inhibition (8, 21), virus neutralization (1, 6), agar gel immunodiffusion (12), complement fixation (2), and immunofluorescence (19) tests are detected in recovered or vaccinated animals. Hemagglutination inhibition and serum neutralization tests done on sera from animals inoculated with a single BTV serotype are serotype specific, as is protection against challenge (15), but immunodiffusion, complement fixation, and indirect immunofluorescence tests detect cross-reactive antibodies. Thus, antibodies reactive in serum neutralization and hemagglutination inhibition have been implicated in protection against BTV. The immune status of recovered animals may be more complex, as indicated by reports that BTV and specific neutralizing antibodies coexist in the blood for weeks (14), months (16), or years (17) after infection and clinical recovery. Another orbivirus, Colorado tick fever virus, has also been shown to persist in the blood of humans and experimental animals (9).

This study was designed to clarify the participation of neutralizing antibody in immunity to BTV. A neutralizing monoclonal antibody was prepared and injected into sheep. Sheep were inoculated with homologous virus and observed for 2 months. All treated sheep remained free of clinical signs, failed to develop viremia or antibodies reactive in the standard immunodiffusion test, and developed only low levels of neutralizing antibodies.

(Results of this study were presented at the First Annual Congress for Hybridoma Research, 15–17 February 1982, Los Angeles, Calif.)

MATERIALS AND METHODS

Virus. The prototype isolate of BTV type 17 (BTV-17) was obtained by C. M. Groocock from T. L. Barber, U.S. Department of Agriculture, Denver, Colo., in a blood sample from an infected sheep. (This same virus was supplied to the American Type Culture Collection, from which it is available as VR-875.) Virus was passed once in a chicken embryo, once in

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TABLE 1. Passive protection of neonatal mice against BTV-17 by monoclonal antibodies

Pretreatment[a]	Isotype	Neutralization[b]	RIA[c]	Challenge[d]	Survivals[e]
X63-Ag8.653 MAF	Various	0	0	1.9	0/10
8B2B.3 MAF	IgG1	0	4	3.2	0/10
7D5C.1 MAF	IgG2a	0	3.7	1.9	0/10
8A3B.6 MAF	IgG2a	0	5.0	1.9	0/10
8B5B.1 MAF	IgG2a	0	4.5	1.9	0/10
6C3A.2 MAF	IgG2a	1.5	3.5	3.2	9/9
6C2A.4.2 MAF	IgG2a	3.5	3.7	3.5	10/10
6C2A.4.2 MAF	10^{-3} IgG2a	ND[f]	0.7	3.5	7/10
BTV-17 mouse antiserum	Various	ND	ND	4.4	5/6

[a] Antiserum or MAF injected intraperitoneally (30 μl per mouse).
[b] Neutralization of BTV-17 in vitro; $\log_{10}$ neutralization index.
[c] $\log_{10}$ titer at which antibody bound 50% of the maximum number of counts in radioimmunoassay (RIA).
[d] $\log_{10}$ 50% lethal doses cloned Wyoming BTV-17 injected intracranially 2 to 4 h after pretreatment (30 μl per mouse).
[e] Number of mice surviving 14 days/mice surviving 3 days after inoculation.
[f] ND, Not determined.

BHK-21 cells, cloned by three limiting dilution passages in Mengling-Vaughn porcine kidney (MVPK) cells (3), grown to a high titer in MVPK cells, and frozen in samples. This virus was generously provided by C. M. Groocock. Virus was titrated by intracerebral inoculation of newborn suckling mice. BTV-4 was obtained from the Veterinary Research Institute, Onderstepoort, South Africa, and BTV-20 was provided by the Yale Arbovirus Research Center, New Haven, Conn.

Monoclonal antibodies. Antibodies were prepared and characterized by standard methods as described elsewhere (J. A. Appleton and G. J. Letchworth, Virology, in press). Briefly, BALB/c mice (Charles River Breeding Laboratories, Inc., Wilmington, Mass.) were immunized with BTV-17-infected brain tissue from neonatal BALB/c mice. Brain tissues (10%) were emulsified in complete Freund adjuvant and injected subcutaneously. The final dose of antigen was given intravenously without adjuvant or subcutaneously with incomplete Freund adjuvant. Three days after the last immunization, splenic lymphocytes were fused with X63-Ag8.653 myeloma cells (13) in 50% polethylene glycol; hybridomas were selected in medium containing hypoxanthine, aminopterin, and thymidine. Hybridomas producing antibody that reacted with BTV-17-infected cells in radioimmunoassay were cloned once (or twice; cell line 6C2A.4.2) in soft agarose and characterized. Cell line 6C2A.4.2 produced an immunoglobulin G class 2a (IgG2a) that reacted in radioimmunoassay with live cells infected with BTV-17 but not with other BTV serotypes and not with uninfected cells, neutralized BTV-17, inhibited hemagglutination by BTV-17, and precipitated VP2 and VP3 from BTV-17-infected cell cultures (Appleton and Letchworth, in press). Hybridoma 6C3A.2 (6C2A.4.2 and 6C3A.2 were derived from the same mouse but from different fusions) produced an IgG2a that reacted with unfixed MVPK cells infected with BTV-17 and several other serotypes, but not with uninfected cells. Antibodies 6C2A.4.2 and 6C3A.2 had different patterns of reactivity on a panel of BTV-17 isolates, suggesting that they were directed against different epitopes. Antibody 6C3A.2 also neutralized BTV-17, inhibited hemagglutination by BTV-17, and precipitated VP2 and VP3 (Appleton and Letchworth, in press). Other hybridomas produced an IgG1 and three IgG2a's that bound to acetone-fixed cells infected with each of the 20 BTV serotypes; precipitated VP6, or VP9, or a nonstructural polypeptide (VP8); and failed to neutralize BTV-17 (Appleton and Letchworth, in press). Each hybridoma was grown as an ascites in pristane (2,6,10,14-tetramethylpentadecane; Aldrich Chemical Co., Milwaukee, Wis.)-primed BALB/c mice. The X63-Ag8.653 myeloma also was inoculated into mice to produce control ascites fluid. Mouse ascitic fluids (MAF) were centrifuged at 15,000 rpm for 30 min (Sorvall SS34 rotor) before use. Neutralization indices and radioimmunoassay titers of antibodies used are shown in Table 1.

Mouse passive protection tests. Neonatal mice (<24 h old) were injected intraperitoneally with 30 μl of serum or MAF. At 2 to 4 h later, they were injected intracranially with 30 μl of virus. Mice were examined daily; those surviving less than 3 days were eliminated from the test, whereas those alive after 14 days were considered survivors.

Sheep passive protection tests. Seven young (16 to 34-kg) mixed breed sheep of both sexes were randomly allocated to an experimental group (four animals) and a control group (three animals). They were housed together in a P3 isolation facility.

The experimental group was injected intravenously with MAF containing hybridoma antibody 6C2A.4.2 at a dosage of 0.03% of body weight (4.8 to 10.2 ml per animal). Control animals were injected intravenously with normal mouse serum at the same dosage. All sheep were inoculated subcutaneously 2 h later with 100 suckling mouse 50% lethal doses of BTV-17. Sheep were examined, and a blood sample was taken daily for 15 days and weekly for an additional 6 weeks. Blood samples (10 ml) were taken in heparinized tubes (Becton, Dickinson & Co., Rutherford, N.J.) and centrifuged. Plasma was stored at −20°C. The cell fraction was washed twice with serum-free minimum essential medium (GIBCO Laboratories, Grand Island, N.Y.), and packed cells were mixed with an equal amount of BLP (0.066 M sodium phosphate

TABLE 2. Clinical, virological, and immunological findings after challenge of monoclonal antibody-injected and control sheep with BTV

Animal	Serum	Days postchallenge detected[a]			Antibody first detected (days postchallenge)		
		Fever	Lesions	Virema	Neutralizing[b]	Anti-BTV[c]	Anti-mouse[c]
1697	NMS[d]	5–7	4–13	3–43	8	13	36
1699	NMS	ND[e]	ND	3–15	9	11	36
1782	NMS	7	2–13	2–29	8	9	36
1688	6C2A.4.2[f]	ND	ND	ND	8	N	36
1700	6C2A.4.2	ND	ND	ND	9	N	43
1780	6C2A.4.2	ND	ND	ND	8	N	36
1781	6C2A.4.2	ND	ND	ND	8	N	36

[a] Days postchallenge with 100 mouse 50% lethal doses of BTV-17 administered subcutaneously.
[b] Titer over 1:20.
[c] Agar gel immunodiffusion test.
[d] Normal mouse serum (NMS) given intravenously at 0.03% of body weight.
[e] ND, Not detected.
[f] MAF containing 6C2A.4.2 monoclonal antibody given intravenously at 0.03% of body weight.

buffer [pH 7.4] with 0.3 M lactose and 0.2% peptone) and frozen at −70°C.

Neutralization tests. Neutralization tests were performed by the beta procedure. Ten-fold dilutions of serum were incubated in microtiter plates with BTV-17 (200 50% tissue culture infective doses) for 60 min at 37°C and overnight at 4°C. MVPK cells (20,000 per well) were added, and plates were incubated in humidified 5% CO_2. Minimal essential medium with 10% fetal calf serum was used throughout. After 5 days, cells were stained with crystal violet and scored macroscopically for protection against the virus.

Agar gel immunodiffusion tests. Agar gel immunodiffusion tests were performed in petri dishes containing 1% agarose (Marine Colloids, Rockland, Maine) in buffer (0.1 M NaCl–0.02 M Tris-hydrochloride [pH 8.0]). Sheep sera were tested for activity against normal mouse serum and standardized BTV diagnostic antigen (Veterinary Services Laboratory, Ames, Iowa). In bluetongue tests, standard positive sera (Veterinary Services Laboratory) were alternated with test sera in well patterns; test sera causing precipitin lines of identity or causing a curving of adjacent positive control precipitin lines were considered positive.

Virus isolations. Virus isolations were performed by adding gentamicin (Schering Corp., Bloomfield, N.J.) to blood cell samples to a concentration of 25 μg/ml, sonicating for 15 s, and injecting 0.1 ml intravenously into each of six 11-day-old embryonating chicken eggs. Embryos dying before 3 days were discarded, and those dying later were pooled, homogenized in a tissue grinder, sonicated, diluted in 10-fold steps, and inoculated onto MVPK cell monolayers. Cultures were overlaid with medium containing 0.65% gum tragacanth, incubated for 5 days in humidified 5% CO_2, stained with crystal violet, and examined for viral cytopathic effects. Virus isolated from each animal was tested for neutralization by mouse antiserum specific for BTV-17.

RESULTS

Mouse passive protection tests. Mice pretreated with MAF containing X63-Ag8.653 myeloma or non-neutralizing monoclonal antibodies failed to survive challenge with BTV-17 (Table 1). Mice pretreated with neutralizing monoclonal antibodies 6C3A.2 or 6C2A.4.2 or with mouse antiserum against BTV-17 survived challenge with BTV-17. Moreover, 7 of 10 mice pretreated with a 1:1,000 dilution of 6C2A.4.2 MAF survived challenge with 3,000 50% lethal doses of BTV-17. The specificity of antibody 6C2A.4.2 was demonstrated by its inability to protect mice against BTV-4 and BTV-20 (data not shown).

Sheep passive protection tests. Sheep pretreated with normal mouse serum and inoculated with BTV-17 became infected (Table 2). All three developed viremia for 12 to 40 days, neutralizing antibody titers greater than 1:2,000 by 10 days after inoculation, and antibody reactive with BTV antigen in the agar gel immunodiffusion test. Virus isolated from these animals was identified as BTV-17 by neutralization testing. Two of the sheep had mild fevers (40.7°C) for 1 and 3 days, respectively, and both of these animals had several 2- to 8-mm pustular lesions on their lips. The third animal failed to develop observable lesions and maintained a normal (40°C) temperature. All three animals maintained their appetites during the experiment.

The four sheep pretreated with hybridoma antibody 6C2A.4.2 did not develop fever, lesions, or antibody reactive with BTV antigen in agar gel immunodiffusion tests. Virus was not detected in the blood cells of any of these animals on any day. All four sheep had a neutralizing titer of 1:20 after the injection of murine antibody, and all had a rise in neutralization titer to 1:200 8 to 9 days after challenge. Two sheep failed to maintain this titer; their titers dropped to 1:20 by 16 and 30 days post-inoculation, respectively.

All sheep developed antibody reactive in agar gel immunodiffusion testing with mouse serum by 36 to 43 days postchallenge.

DISCUSSION

Pretreatment of mice and sheep with a neutralizing monoclonal antibody clearly prevented bluetongue disease. This result is in striking contrast to the inability of neutralizing titers 100-fold higher to clear virus from the blood of control sheep, a phenomenon previously reported (14, 16, 17). Thus, this study presents direct evidence that neutralizing antibody is a pivotal component in protection against bluetongue disease but may not provide complete protection against infection and may make little or no contribution to recovery.

Protection of mice and sheep against BTV with a type-specific neutralizing monoclonal antibody directed against a single antigenic determinant clarifies previous studies on BTV. Viral polypeptide 2 has been suggested to be the vehicle of serotype specificity by experiments involving RNA cross-hybridization (11), oligonucleotide fingerprinting (20), and cross-immunoprecipitation (10). The exposed position of this polypeptide on the outer capsid of the virion (22) adds credence to this suggestion. Reports that protection against BTV is serotype specific (7, 15) imply that VP2 is involved in the generation of protective immunity. The results of this study provide direct evidence for such a relationship. The participation of VP3 in an important antigenic determinant of BTV has not been reported previously and requires further investigation. It is possible that VP2 and VP3 are immunoprecipitated together because they are aggregated during preparation of viral antigens or because VP3, or a protein that comigrates in polyacrylamide gels with VP3, is a large fragment of VP2 and therefore shares antigenic determinants with VP2. The latter explanation is favored by the observation that polyclonal antiserum immunoprecipitates proteins that comigrate with both VP2 and VP3 from in vitro translation reactions containing BTV-17 gene segment 2 (J. A. Appleton, M. J. Grubman, and G. J. Letchworth, unpublished data).

The fact that monoclonal antibody-treated sheep failed to develop clinical signs of BTV and failed to have circulating virus detectable by intravenous inoculation of blood into chicken embryos, a test that is as sensitive for virus detection as the injection of blood into susceptible sheep (5), suggests that vaccines containing a single antigenic determinant might generate a similar neutralizing antibody and may both prevent bluetongue disease and perhaps interrupt natural virus transmission.

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