Identification of Bluetongue Virus-specific Immunoglobulin E in Cattle

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SUMMARY

A modified passive cutaneous anaphylaxis test and an ELISA were used to identify IgE in calves vaccinated (sensitized) with chlorine dioxide-inactivated bluetongue virus (BTV) and in calves inoculated with infectious BTV. The levels of IgE were greatest in the vaccinated calves after challenge with infectious virus, which correlated with development of clinically apparent dermatitis and stomatitis. These findings suggest that some aspects of clinical bluetongue disease in cattle may have an immunopathological mechanism mediated by IgE (type I hypersensitivity).

Various immunological and immunopathological mechanisms may be initiated during the course of a viral infection. Although much is known about humoral and cellular immunity in many viral infections, evidence of the importance of immediate (type I) hypersensitivity reactions in viral diseases is scanty. High concentrations of IgE have been correlated with the development of allergic disease in man. Frick et al. (1979) determined that viruses could initiate immunological pathways to induce allergic sensitizations to viruses and pollens in children, probably by altering populations of T and B lymphocytes. Additionally, Ida et al. (1977) found an enhancement of IgE-mediated histamine release from human basophils by viruses, in conjunction with interferon.

Homocytotropic antibodies were demonstrated in cattle (Wells & Eyre, 1970) and bovine IgE has been characterized as a distinct Ig class analogous to human IgE (Hammer et al., 1971). Gershwin & Dygert (1983) developed an ELISA to measure relative serum concentrations of total IgE in cattle. The 72 h passive cutaneous anaphylaxis (PCA) test has been utilized for identification and/or quantification of antigen-specific bovine IgE. Ovalbumin, horse serum, rabbit serum albumin, methyl cellulose, Fasciola hepatica, and Micropolyspora faeni are antigens that have been shown to invoke IgE responsiveness in cattle (Black et al., 1975; Doyle, 1973; Gershwin, 1981; Gershwin & Olsen, 1984; Hammer et al., 1971; Wells & Eyre, 1970; Wilkie et al., 1978). In this report, we describe the identification of virus-specific IgE in calves vaccinated (sensitized) with inactivated bluetongue virus (BTV) serotype 17 and challenged with infectious virus and in calves inoculated with infectious BTV serotype 17 without prior vaccination/sensitization. Our results indicate that a type I hypersensitivity may be important in the dermatitis and stomatitis associated with bluetongue disease in cattle.

The calves used in this study were housed in insect-secure facilities and determined to be free of BTV by the agar gel immunodiffusion test and failure to isolate the virus. Twelve calves were divided into four groups as follows: vaccinated/sensitized (six calves), adjuvant control (two calves), challenge control (two calves) and animal control (two calves). The vaccinated/sensitized group was divided further into three groups: two calves received vaccine with levamisole phosphate at 3.3 mg/kg body wt, two calves received vaccine with cimetidine hydrochloride at 3.3 mg/kg body wt, and two calves received only vaccine (vaccinated controls). The vaccine consisted of chlorine dioxide-inactivated BTV emulsified in an aluminium hydroxide adjuvant (obtained from Dr T. L. Barber, USDA/ARS, Denver, Co. U.S.A.). Vaccinated and adjuvant control calves were given two inoculations of the appropriate preparation 5 weeks apart. In
addition, immunomodulators (levamisole or cimetidine) were given to four of the six vaccinated but not to the vaccinated control or adjuvant control calves.

All calves, except the animal controls, were challenged with virulent BTV serotype 17 5 weeks after the second vaccination/sensitization. Each calf was inoculated subcutaneously with blood from an experimentally infected sheep, and a total of $2.5 \times 10^6$ 50% embryo lethal doses (ELD$_{50}$) were given to each animal. All calves inoculated with virus became viraemic. Heparinized blood samples were collected by jugular venipuncture weekly, prepared as previously described (Foster & Luedke, 1968) and inoculated into embryonating chicken eggs for BTV isolation. Challenge controls were viraemic by 7 days post-challenge (p.c.) and remained viraemic for an average of 29 $\pm$ 10 days p.c. Sensitized calves were also viraemic at 7 days p.c. and remained viraemic for an average of 48 $\pm$ 14 days p.c. Clinical disease was apparent in five of the six vaccinated/sensitized calves after challenge with infectious BTV; furthermore, four of the five clinically affected animals had received immunomodulators. The clinical signs apparent on day 11 p.c. were characterized as an exudative dermatitis and an ulcerative stomatitis. Histologically, the lesions were infiltrated by mononuclear inflammatory cells, with eosinophils being prominent in the dermatitis. Challenge control calves and one calf that had been vaccinated/sensitized but not immunomodulated also exhibited inflammation within the skin and oral mucosa, but the inflammation was only apparent microscopically.

Weekly serum samples from each calf were stored at $-20 \, ^\circ C$ until tested. BTV-neutralizing antibodies were quantitatively determined by plaque reduction (Stott et al., 1978), and non-neutralizing, precipitating antibodies were detected by agar gel immunodiffusion. BTV group-specific precipitating antibodies (IgG) were detected in all sensitized calves 7 to 14 days following the second vaccination/sensitization with inactivated BTV. Minimal virus neutralization titres (titres of 1:10 to 1:40) were detected in some vaccinated/sensitized animals, and similar low level titres were present in some control calves. Following live virus challenge, virus-neutralizing antibody titres developed in all vaccinated/sensitized and challenge control calves. Vaccinated/sensitized calves tended to have lower neutralizing antibody titres than the challenge controls. Peak titres (28 to 42 days p.c.) in vaccinated/sensitized calves ranged from $2.5 \times 10^3$ to $4.0 \times 10^4$, as compared to peak titres of $1.0 \times 10^4$ to $1.6 \times 10^5$ in challenge controls. All vaccinated/sensitized animals continued to have group-specific antibodies, and the challenge controls developed these antibodies 7 to 21 days p.c.

BTV-specific IgE was determined by modified PCA tests (Gershwin, 1981; Wilkie et al., 1978) and an indirect ELISA (Gershwin & Dygert, 1982; Hübschle et al., 1981; Poli et al., 1982). BTV serotype 17 (62-45-S strain obtained from Dr T. L. Barber) was plaque-picked three times from agar-overlaid Vero cells. Virus was purified as previously described, except for centrifugation through sucrose density gradients (Huismans et al., 1979; Verwoerd, 1969). Cell culture virus was extracted three times in fluorocarbon and Sephadex G-200, and the combined water phases were extracted in a mixture of Tween 80 and ether. The virus was pelleted through 40% sucrose, resuspended in 0.002 M-Tris buffer pH 8.6 and stored at 4 $\, ^\circ C$. For use in the PCA and ELISA tests, the protein concentration of the virus was adjusted to 50 $\mu$g/ml and 5 $\mu$g/ml, respectively (Bradford, 1976).

The modified PCA tests were performed in calves as Prausnitz-Küstner tests with the addition of intravascular dye injection. Briefly, the hair was clipped on three recipient calves and intradermal injections of 0.2 ml of pre-inoculation sera, selected test sera and heat-inactivated (4 h at 56 $\, ^\circ C$) test sera, respectively, were made in the skin. After 72 h, Evans' blue dye (20 ml of a 2.5% solution in phosphate-buffered saline, PBS) was injected intravenously and 0-2 ml of partially purified BTV was injected at each serum-inoculated site. In addition, selected serum-inoculated sites were injected with 0.2 ml of a control antigen (50 $\mu$g/ml) prepared similarly to the viral antigen but from uninfected Vero cells. Histamine at 100 $\mu$g/ml, BTV antigen, Vero cells, 0.002 M-Tris buffer pH 8.6 and PBS pH 7-4 were injected (0-2 ml) into skin sites not inoculated previously with test sera. The oedema and blueing were recorded 30 min after challenge, and the reactions were evaluated 0 to 5+. Weals scored as 1+ measured at least 0-5 cm in diameter, and 5+ weals were 2-5 to 3-0 cm in diameter.

The results of PCA tests with pre-inoculation sera and sera collected at selected intervals
Table 1. *BTV*-specific PCA reactivity in calves

<table>
<thead>
<tr>
<th>Vaccinated/sensitized</th>
<th>Controls</th>
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<tr>
<td><em>+ Levamisole</em></td>
<td><em>+ Cimetidine</em></td>
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<tr>
<td>Calf</td>
<td>Calf</td>
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<tr>
<td>0*</td>
<td>1</td>
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<tr>
<td>0 ND</td>
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<tr>
<td>14</td>
<td>0</td>
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<tr>
<td>35 ND</td>
<td>0</td>
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<tr>
<td>49</td>
<td>4+</td>
</tr>
<tr>
<td>56</td>
<td>3+</td>
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<tr>
<td>Post-challenge</td>
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<tr>
<td>0§</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>4+</td>
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<td>14</td>
<td>4+</td>
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<td>21</td>
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<td>28</td>
<td>4+</td>
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<tr>
<td>35</td>
<td>3+</td>
</tr>
<tr>
<td>42</td>
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</tbody>
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* First day of vaccination/sensitization.  
† ND, Not determined.  
‡ Day of second vaccination/sensitization.  
§ Day of challenge with virulent BTV.

during the experiments are shown in Table 1. Pre-inoculation sera from all these calves failed to sensitize the skin of recipient calves, and heat inactivation (56 °C for 4 h) of the PCA-positive sera eliminated the ability to elicit a PCA reaction. Positive reactions were commonly observed at sites injected with sera from the vaccinated/sensitized group, particularly the immunomodulated calves. Three of six and four of six sera were positive 14 and 21 days after the second vaccination/sensitization, respectively. The sera from three of the four immunomodulated calves and one of the two vaccinated controls were positive at 7 days p.c. and remained positive to day 42. The greatest responses were consistently observed with sera collected 7, 14 and 21 days p.c. One calf (no. 6) receiving cimetidine failed to elicit a positive reaction after day 14 p.c., whereas one vaccinated control (no. 9) did not elicit a positive reaction until 28 days p.c. A moderate positive PCA reaction was observed with sera from one adjuvant control at day 28 p.c. (2+) and with one challenge control at days 28 and 35 p.c. (3+). Selected sera that elicited a positive reaction with the BTV antigen were also tested using the BHK control antigen in the PCA test. Of the sera tested with BHK antigen, calf 5 showed a 1+ reaction with serum collected at day 14 p.c.; this same serum elicited a 4+ reaction with the BTV antigen. Tris buffer, PBS and BTV and BHK antigen each failed to elicit a reaction when injected into unsensitized skin sites, but the response to injections of histamine varied between 3+ and 5+.

*BTV*-specific IgE was also measured by modification of the ELISA for BTV antigen described by Hübschle *et al.* (1981) and Poli *et al.* (1982) and for IgE by Gershwin & Dygert (1983). The virus was processed as indicated above, and 100 μl aliquots of the BTV preparations were fixed to the bottoms of wells of PVC microELISA plates for 12 to 16 h (4 °C) following dilution in carbonate buffer pH 9.6. Rabbit serum albumin (100 μl of 1% solution in carbonate buffer) was added and incubated at 37 °C for 2 h, to block any remaining binding sites in the well. The plates were washed six times with a solution consisting of PBS and 0.05% Tween 20. Aliquots (100 μl) of undiluted test sera, positive and negative control sera and PBS, respectively, were added in duplicate wells. A serum sample that was strongly positive with the PCA test and had a high absorbance in the ELISA (calf 1 at 21 days p.c.) was used as the positive control, and the negative controls consisted of PBS and a pool of serum collected from two healthy 6 month old calves. The plates were incubated at 37 °C for 30 min, soaked in PBS–Tween 20 for 20 min and washed six times with PBS–Tween 20. Rabbit anti-bovine IgE, produced as previously described (Gershwin & Dygert, 1983), was diluted 1:150 and added to the wells in 100 μl.
Short communication

**Fig. 1.** $A_{405}$ readings of ELISA for anti-BTV IgE in calves vaccinated/sensitized with chlorine dioxide-inactivated BTV and subsequently challenged with infectious virus. One group received levamisole (a) and one group received cimetidine (b) during sensitization/vaccination. There were control groups consisting of calves vaccinated/sensitized (vaccinated control) without immunomodulators (c), inoculated with only the alum adjuvant (adjuvant control) during sensitization/vaccination (d) and not vaccinated/sensitized but challenged with infectious virus (challenge control) (e). The absorbance for the negative control serum pool and PBS fell within the shaded background (not shown).

The development of anti-BTV IgE in the sera of the calves in the experiment is shown in Fig. 1. The sera of three of the four vaccinated/sensitized and immunomodulated calves had elevated volumes; incubation at 37°C for 30 min was followed by six washes with PBS-Tween 20. Alkaline phosphatase–goat anti-rabbit conjugate was added to each well at a 1:750 dilution in 100 μl volumes, and the plates were incubated 30 min at 37°C. Prior to the addition of substrate, plates were washed eight times with PBS-Tween 20. Finally, 200 μl of p-nitrophenylphosphate in diethanolamine buffer was added, and the $A_{405}$ were read at 5 min with an automated microplate reader. Plate-to-plate variation of the data was corrected by using the previously described method (Voller et al., 1980).

The development of anti-BTV IgE in the sera of the calves in the experiment is shown in Fig. 1. The sera of three of the four vaccinated/sensitized and immunomodulated calves had elevated
Short communication

absorbance readings, after the second vaccination/sensitization and after challenge, which peaked 21 days p.c. There was little difference in the absorbance among sera collected from one of the immunomodulated animals (no. 6), one vaccinated control calf (no. 9), the adjuvant control calves and one challenge control calf (no. 14). The other vaccinated and challenge control calves had the highest absorbance readings at 42 and 28 days, respectively.

In these experiments IgE anti-virus antibodies developed in cattle given chlorine dioxide-inactivated BTV, infectious BTV, or both. Reaginic antibodies were detected after the second vaccination/sensitization, reaching peak levels after challenge with infectious virus, and this peak correlated with the occurrence of clinical disease. The route of immunization, antigen concentrations and adjuvants are considered to be important in specific IgE production (Siraganian, 1981). Injections were subcutaneous in all our animals, using chlorine dioxide-inactivated BTV suspended in alum. Additionally, the effect of levamisole and cimetidine in these experiments is unknown. Although the specific immunomodulating mechanism of levamisole and cimetidine is incompletely understood, it is known that levamisole can potentiate the immune response in some species by modification of macrophage and lymphocyte function (Brunner & Muscoplat, 1980; Renoux, 1978), and it has been hypothesized that cimetidine may potentiate the immune response in humans by blocking the action of histamine on H-2 receptors located on suppressor T lymphocytes (Jorizzo et al., 1980; Mavligit et al., 1981). In our studies, the IgE anti-BTV response was greatest in three of the immunomodulated calves, but a challenge control calf and a vaccinated control calf also developed BTV-specific IgE. The IgE levels in the control calves were lower and they peaked 7 and 21 days later (days 28 and 42 p.c.) than the immunomodulated animals.

The 'allergic breakthrough phenomenon' proposed by Katz (1978) is a possible mechanism for a viral effect on IgE production. Chiorazzi et al. (1977) suggested that treatments with low dose irradiation, cyclophosphamide or anti-lymphocyte serum may deplete highly sensitive suppressor cell populations and therefore allow escape of helper T lymphocytes to stimulate IgE-producing B lymphocytes. Viral infections also may selectively deplete suppressor T lymphocytes and allow an 'allergic breakthrough' (Frick & Brooks, 1983).

Although specific mechanisms important in causing high levels of IgE anti-virus antibodies are poorly understood, it appears that BTV-specific IgE can be present during BTV infections and that the level of IgE may be important for development of clinical bluetongue disease. The present experiments suggest that direct interaction of BTV proteins with virus-specific IgE located on basophils or mast cells may modulate mediator release. It is well known that the triggering of these cells results in the release of histamine, leukotrienes, chemotactic and other inflammatory factors. Some of these factors, i.e. prostaglandins, thromboxane A2 and histamine, were shown to be elevated in the vaccinated/sensitized calves in our studies (Emau et al., 1984), which also could be important in the dermatitis and stomatitis observed in natural bluetongue disease. The capacity of BTV to induce specific IgE and the role of these antibodies in immunological defence and injury merit further investigation. Furthermore, the implications for vaccine development are obvious.

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Short communication


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