THE IMMUNE RESPONSE TO BLUETONGUE VIRUS INFECTION

By


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I am also indebted to many members of the Institute staff for helpful discussions and criticisms and in particular I would like to thank Miss Amanda Corteyn and Miss Pam Hildred for invaluable laboratory assistance, Mr Ken Herniman and Mr Ian Gumm for constant on-hand advice and Penny Scott, the librarian for her help and forbearence. I wish to thank Dr. J. Brownlie of the ARC institute at Compton for surgical assistance during ovine cell transfer experiments and Dr. G. Letchworth for the supply of monoclonal antibodies.

I also wish to thank Dr. J. Mann and Mr Alwyn Breame for their advice and use of computer equipment in the preparation of this manuscript.

Finally I would like to acknowledge the encouragement, support and patience given to me by my wife during the three years of this work.
The outcome following the single, serial and simultaneous inoculation of different bluetongue virus (BTV) types into sheep and cattle was examined in terms of temperature, viraemia and neutralising antibody responses.

Animals inoculated with one BTV type and challenged with the same type were shown to be protected from this challenge and to have produced only homotypic neutralising antibodies. Following the inoculation of one type and challenge with a different BTV type no protection could be demonstrated. When rechallenged these animals were found to be resistant to a third BTV type. Animals serially inoculated with two BTV types were shown to produce a transient heterotypic neutralising antibody response to a number of BTV types and although the level of this heterotypic response diminished with time the inoculation of a third BTV type gave rise to a further heterotypic response. The simultaneous inoculation of three BTV types however, resulted in replication of only two of the three inoculated viruses and a neutralising antibody response to only those two types.

The ability of BTV to induce a cell-mediated immune response, in terms of the production of cytotoxic T lymphocytes (CTL's) was examined in both mice and sheep. After
inoculation with live BTV, mice produced CTL's which showed virus and $H_2$ restriction. On secondary \textit{in vitro} stimulation, specifically stimulated sensitised memory cells also produced high numbers of CTL's. Inactivation of virus preparations, either by BPL or glutaraldehyde induced only a low level response and the use of Freunds adjuvants and double immunisation procedures failed to improve that response. However, mice immunised with a single BTV type were shown to produce CTL's which cross-reacted with a number of BTV types. These cross-reactive CTL's could be induced by both primary \textit{in vivo} and secondary \textit{in vitro} stimulation and a varying degree of cross-reactivity occurred with the six BTV types examined. Following these observations in mice, the ability of BTV to induce CTL's in ovines was investigated. Presumptive ovine CTL's were shown to occur, their activity to be maximal around day 14 post inoculation (pi) of virus and their activity to be genetically restricted.

BTV immune sera appeared incapable of participating in antibody dependent cell-mediated cytotoxicity (ADCC) reactions and although BTV induced high levels of interferon it was itself not susceptible to the effects of this interferon.

Following the demonstration that the serial inoculation of two BTV types protected against challenge with a third type and that both the humoral and cellular components of the immune response were capable of heterotypic activity, work was carried out to evaluate the relative importance of antibody
and CTL's in recovery and protection from reinfection with BTV.

Using antibody transfer techniques it was possible to show that immune sera has a role to play in protection from challenge with homologous virus type although that protection did not appear to correlate with neutralising antibody levels and no protection could be demonstrated against heterologous virus challenge.

Cellular transfer experiments in monozygotic sheep showed that BTV-induced thoracic duct lymphocytes were able to afford some degree of protection from homologous virus challenge and that this activity was probably T cell-mediated. It was also shown that this T cell-mediated response gave solid protection against challenge with a BTV type different from that used to induce these cells.

The work in this thesis is discussed with relevance to other virus diseases, BTV immunology and the present BTV vaccine policies.
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History

Bluetongue (BT) was first described by Hutcheon in South Africa in 1881 (Hutcheon, 1902) as an epizootic catarrhal infection affecting recently imported Merino sheep. Spreull (1905) named the disease bluetongue (BT) and suggested that the causative agent was an intra-corpusscular parasite. Theiler (1906) showed by passing the infective fluid through a Berkfield filter, that the agent was filterable and probably a virus. It became increasingly evident during the next ten years from observations on the location and seasonality of outbreaks that an insect vector played a part in the transmission of the disease. Neischulz, Bedford and DuToit (1934) isolated an agent from Aedes mosquitoes which was thought to be responsible for the disease in sheep although conclusive evidence of vector involvement by demonstrating that infected midges (Culicoides sp.) could transmit the disease to susceptible sheep was not obtained until the 1940's (DuToit 1944). Evidence of biological transmission by Culicoides sp. was not confirmed until the work of Foster,
Jones and McCory (1963).

Control of BT by means of vaccination was soon available following the recognition of the viral nature of the disease. Theiler (1908) reported on the apparent attenuation of a strain of bluetongue virus (BTV) after limited serial passage in sheep. This strain was used as a vaccine for nearly 40 years with over 50 million doses being issued. However, the rapid growth of the sheep breeding industry in the 1940's brought to light the limited protective qualities of the Theiler vaccine.

Neitz in 1948 published results of extensive studies involving cross protection experiments in sheep with different BTV isolates and it became apparent that a plurality of antigenically different strains of BTV existed in nature. His attempts to classify BTV based on the results of these cross protection experiments however were disregarded in favour of the *in vitro* virus neutralisation system developed by Howell (1960). This work followed on the studies of Fernandes (1959a, b and c) demonstrating the growth of BTV in primary lamb kidney monolayers and neutralisation by its specific anti-serum. Howell (1970) classified isolates from South Africa into sixteen serotypes based on plaque neutralisation tests and the present twenty two serotypes are characterised by similar means.

Bluetongue as a clinical entity was not recognised outside South Africa until the 1940's at which time the disease was first noted in 1943 in Cyprus (Gambles, 1949) and then in 1948 in Texas (Price and Hardy, 1954). Since that time BTV has been recognised in Israel in 1951 (Komarov and Goldsmit, 1951) and in 1956/59 in the Iberian peninsula (Campano, 1957;
Manso-Ribeiro, 1958) and West Pakistan (Howell, 1963). Since then surveys have indicated both through virus isolation and serological studies the widespread distribution of the virus (Herniman et al., 1980).

The Virus

Definition: BTV is the type species of the orbivirus group in the family Reoviridae. This group is separated from the rest of the reoviridae by virtue of its lability at pH 3.0. The virus consists of ten segments of double stranded ribonucleic acid (RNA) coding for seven structural and three non-structural viral proteins (Fenner, 1976).

Morphology: The name orbivirus was derived from the doughnut shaped rings appearing on negatively stained electron microscope preparations of the nucleocapsid. This ring or orbis appears following the loss of two surface proteins (Borden et al., 1971). In original descriptions of the infective agent of BT disease (Studdert et al., 1966; Owen and Minz, 1966; Bowne and Jones, 1966) the inclusion of the pseudo-envelopes gave rise to size discrepancies. However removal of these structures with Tween 80 showed the virus to have a diffuse outer protein layer containing two polypeptides surrounding the structural nucleocapsid (Verwoerd et al., 1972). In other reoviruses the outer layer consists of structural units arranged in a regular way and an inner capsid that has an ill-defined structure. BTV however has a structured
nucleocapsid containing 32 capsomeres clustered as pentamer-hexamer morphological units with a diameter of 63nm. This is surrounded by a diffuse outer coat containing two polypeptides giving a particle size of 69nm (Martin and Zweerink, 1972).

**Physio-chemical Properties:** BTV is thermo-stable as indicated by the storage and recovery of the virus at room temperature after 25 years (Nietz, 1948). Rapid loss of infectivity though occurs at -20°C. The virus is relatively stable to lipid solvents and sodium deoxycholate whilst labile at pH 3.0 (Borden et al., 1971). Analysis of the genomes by polyacrylamide gel electrophoresis shows the ten segments of double stranded RNA (Knudson et al., 1982) which code for the ten viral proteins. Five of the proteins are contained in the nucleocapsid and designated P1, P3, P4, P7 and P9. Two are the surface proteins P2 and P5 and three are non-structural P6, P8 and P10 (Huismans, 1979). Recent work involving *in vitro* RNA translation and using a different protein numbering system has indicated similar coding assignments (Sangar and Mertens, 1983).

**Classification:** Placed in the family Reoviridae, BTV is taken as the type member of the orbivirus group. The orbiviruses are listed in Table 1a. Morphological, physiochemical and serological characters clearly define the orbiviruses (Borden et al., 1971; Murphy et al., 1971; Fenner, 1976; Della-Porta et al., 1979). The orbiviruses have been subdivided into groups by means of the complement fixation, agar-gel diffusion and fluorescent antibody tests (Boulanger and Frank, 1975; Jochim and Chow, 1969) and further subdivision into
Table 1a: Serological subgroups, members and antigenic relationships of the Orbivirus group

<table>
<thead>
<tr>
<th>Serological group</th>
<th>Members</th>
<th>Antigenic relationships</th>
</tr>
</thead>
<tbody>
<tr>
<td>African Horse Sickness</td>
<td>AHS (9 serotypes)(^a)</td>
<td></td>
</tr>
<tr>
<td>Bluetongue</td>
<td>BTV (22 serotypes)(^a) related to EHD and Eubanangee groups</td>
<td></td>
</tr>
<tr>
<td>Changuinola</td>
<td>Changuinola</td>
<td></td>
</tr>
<tr>
<td>Corriparta</td>
<td>Acado</td>
<td></td>
</tr>
<tr>
<td>Epizootic Haemorrhagic Disease</td>
<td>EHD (2 serotypes)(^a) related to EHD</td>
<td></td>
</tr>
<tr>
<td>of deer</td>
<td>EHDV-Alberta(^b)</td>
<td></td>
</tr>
<tr>
<td>Eubanangee</td>
<td>Eubanangee</td>
<td></td>
</tr>
<tr>
<td>Palyam</td>
<td>Pala</td>
<td></td>
</tr>
<tr>
<td>Wallal</td>
<td>Mitchell river</td>
<td></td>
</tr>
<tr>
<td>Warrego</td>
<td>Ibaraki(^a)</td>
<td></td>
</tr>
<tr>
<td>Ibaraki</td>
<td>EHDV-Alberta(^a)</td>
<td></td>
</tr>
<tr>
<td>Colorado Tick Fever</td>
<td>Colorado Tick Fever(^b)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Eyach</td>
<td></td>
</tr>
<tr>
<td>Kemerovo</td>
<td>Baku, Bauline, Cape Wrath, Chenuda, Great Island, Huacho, Kemerovo(^b), Lipovnik, Mono Lake, Nugget, Okhotskiy, Seletar, Sixgun City, Tribec, Sixgun City, Tribec, Wad Medani, Yaquina Head.</td>
<td></td>
</tr>
<tr>
<td>Unalged</td>
<td>Japanaut, Lebombo, Orungo, Umatilla</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Causes disease in animals. \(^b\) Causes disease in humans.
serotypes is based on virus neutralisation tests (Howell, 1970; Barber and Jochim, 1973; Stott et al., 1978).

In BTV a core protein P7 has been shown to be responsible for group specificity whilst the main determinant for serotype specificity is the outer capsid layer protein P2 (Huismans and Erasmus, 1981) with P5 playing a minor role (De Villiers, 1974). However, both inter and intra group serological classification of BTV has given rise to conflict. Cross-reactions between BTV and other orbiviruses such as Epizootic Haemorrhagic Disease virus have been demonstrated (Campbell et al., 1975; Gorman et al., 1981; Della-Porta et al., 1983) and within the BTV group, serotype classification of recent isolates by the in vitro serum neutralisation test has also given rise to debate (Della-Porta et al., 1981; Della-Porta et al., 1983).

Studies using oligonucleotide finger printing (Sugiyama et al., 1982) and cross-hybridisation (Huismans and Bremer, 1981) show a high degree of reassortment and heterologous hybridisation within the BTV group, but indicate that the gene coding for type specificity is conserved. Recently work with monoclonal antibodies directed against BTV type 17 have shown at least 3 type specific epitopes as measured by virus neutralisation (Letchworth and Appleton, 1983) and thus even virus isolates belonging to the same BTV type may show differing activity against a neutralising serum.

Within the orbivirus genus the recognised serologically distinct groups are considered species of virus. The viruses of the bluetongue, Eubanangee and Wallall subgroups are designated as distinct species and available evidence indicates that genetic reassortment only occurs within defined
species of viruses. The shared antigens between the viruses of the bluetongue and Eubanangee serogroups probably reflect common ancestry of two distinct virus species (Gorman et al., 1979; Gorman et al., 1982.)

The Disease

Definition:- BT is an infectious, non-contagious viral disease of ruminants, transmitted by insects and characterised by congestion, facial oedema and coronitis, especially in sheep.

Epizootiology:- a) Host range. Sheep and cattle are believed to be the primary hosts of BTV (Bowne et al., 1968; Bowne, 1971) and the outcome following infection is highly variable with large differences between the breeds. (Neitz, 1948; Lawman, 1979; Gorman and Taylor, 1982). Goats are susceptible to BTV but infection usually results in a mild or inapparent reaction (Haig, 1959; Luedke and Anakwense, 1972; Barzilai and Tadmor, 1971).

A number of species of wild ruminants have been shown, either by virus isolation, or serological evidence, to harbour BTV (Table 1b). The only report of the virus occurring in wild animals other than ruminants was by Du Toit and Goosens (1955, quoted by Howell, 1963) who isolated BTV from rodents. BTV has been shown to replicate in a limited number of laboratory animals in particular newborn mice, suckling hamsters and chicken embryos (Alexander et al., 1947; Goldsmit and Barzilai, 1968). BTV is reported not to replicate in adult mice (Oellerman et al., 1976).
<table>
<thead>
<tr>
<th>Animal</th>
<th>virus isolation</th>
<th>serological evidence</th>
<th>disease location</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>White Tailed Deer</td>
<td>yes</td>
<td>yes</td>
<td>acute to America</td>
<td>1, 2, 3</td>
</tr>
<tr>
<td>(Odocoileus virginianus)</td>
<td></td>
<td></td>
<td>inapparent</td>
<td></td>
</tr>
<tr>
<td>Pronghorn Antelope</td>
<td>yes</td>
<td>yes</td>
<td>clinical N. America</td>
<td>4, 5</td>
</tr>
<tr>
<td>(Antilocapra americana)</td>
<td></td>
<td></td>
<td>mild</td>
<td></td>
</tr>
<tr>
<td>North American Elk</td>
<td>yes</td>
<td></td>
<td>inapparent N. America</td>
<td>6, 11</td>
</tr>
<tr>
<td>(Alces americana)</td>
<td></td>
<td></td>
<td>or mild</td>
<td></td>
</tr>
<tr>
<td>Kudu</td>
<td>yes</td>
<td>-</td>
<td>acute N. America</td>
<td>7</td>
</tr>
<tr>
<td>(Strepsiceros imberbis)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reeves Muntjac</td>
<td>yes</td>
<td>-</td>
<td>inapparent Africa</td>
<td>8</td>
</tr>
<tr>
<td>(Muntiacus)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bles Buck</td>
<td>yes</td>
<td>-</td>
<td>inapparent New Mexico</td>
<td>12</td>
</tr>
<tr>
<td>(Damaliscus albofrons)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Topi</td>
<td>yes</td>
<td>no</td>
<td>clinical Uganda</td>
<td>9</td>
</tr>
<tr>
<td>(Damaliscus korrigum)</td>
<td></td>
<td></td>
<td>severe</td>
<td></td>
</tr>
<tr>
<td>Mountain Gazelle</td>
<td>yes</td>
<td>-</td>
<td>inapparent Israel</td>
<td>10</td>
</tr>
<tr>
<td>Mule Deer</td>
<td>-</td>
<td>yes</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>(Odocoileus hemionus hemionus)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

References:—
1-Robinson, Stair and Jones, 1967
2-Stair, Robinson and Jones, 1968
3-Thomas and Trainer, 1970
4-Hoff and Trainer, 1974
5-Hoff and Trainer, 1972
6-Murray and Trainer, 1972
7-Hoff, Griner and Trainer, 1973
8-Neitz, 1933
9-Wells, 1962
10-Barzilai and Tadmor, 1971
11-Trainer and Jochim, 1969
b) Transmission. The horizontal transmission of BTV from host to host has been shown to be primarily through the bites of infected Culicoides sp. and the species so far incriminated are shown in Table 1c.

BTV has been isolated in areas in which Culicoides sp. do not exist, but as yet no other vector has been implicated (Parsonson, 1979) and although BTV replicates in other insects these are not thought important in the field (Jennings and Boorman, 1980; Luedke et al., 1965).

Of significance in considering the horizontal transmission of BTV is the work of Luedke, Jones and Walton (1977). They were able to demonstrate that cattle became persistently infected with BTV after infection in utero, and that although virus could not be normally recovered from the blood of these animals, following the bites of uninfected Culicoides sp. a massive viraemia rapidly ensued giving the potential for vector transmission. They suggest that this is an important mechanism of viral survival during periods of low insect activity. These observations have yet to be substantiated by other workers.

Recent observations that BTV can be recovered from the semen of experimentally infected bulls (Parsonson et al., 1981) raises the possibility of horizontal transmission at insemination. However further work using mouse and cattle has indicated in such situations foetuses do not become infected and survive till full term (Bowen et al., 1982). However infected semen can result in infection of the female at insemination (Bowen et al., 1983).

The vertical transmission of the virus is not thought to be the primary method of spread of BTV from host to host.
Table 1c: *Culicoides* species implicated in the transmission of BTV

<table>
<thead>
<tr>
<th><em>Culicoides</em> sp.</th>
<th>location</th>
<th>degree of reference importance</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. imicola</em></td>
<td>Africa</td>
<td>+++</td>
<td>Du Toit, 1944</td>
</tr>
<tr>
<td><em>C. obsoletus</em></td>
<td>Cyprus</td>
<td>++</td>
<td>Mellor and Pitzolis, 1979</td>
</tr>
<tr>
<td><em>C. variipennis</em></td>
<td>U.S.A.</td>
<td>+++</td>
<td>Foster and Jones, 1973</td>
</tr>
<tr>
<td><em>C. milnei</em></td>
<td>Kenya</td>
<td>+</td>
<td>Walker and Davies, 1971</td>
</tr>
<tr>
<td><em>C. austeni</em></td>
<td>Sudan</td>
<td>+</td>
<td>Boorman and Mellor, 1982</td>
</tr>
<tr>
<td><em>C. moreli</em></td>
<td>Sudan</td>
<td>+</td>
<td>Walker and Davies, 1971</td>
</tr>
<tr>
<td><em>C. grahamii</em></td>
<td>Kenya</td>
<td>+</td>
<td>Walker and Davies, 1971</td>
</tr>
<tr>
<td><em>C. kingi</em></td>
<td>Sudan</td>
<td>(?)</td>
<td>Boorman and Mellor, 1982</td>
</tr>
<tr>
<td><em>C. avarititia</em></td>
<td>Australia</td>
<td>+++</td>
<td>St. George, Cybinski, Standfast, 1982</td>
</tr>
<tr>
<td><em>C. brevitarsis</em></td>
<td>Australia</td>
<td>+++</td>
<td>Snowden, 1979</td>
</tr>
<tr>
<td><em>C. shultzei</em></td>
<td>Australia</td>
<td>++</td>
<td>Dyce and Standfast, 1979</td>
</tr>
</tbody>
</table>
(Bowne, 1971). In periods of decreased vector activity however mechanisms are needed to explain the persistence of the virus in a host population. Several methods of overwintering have been postulated (Luedke et al., 1977; Reeves, 1974). Of these transplacental spread through BTV infected dams to their offspring has received attention (Gibbs et al., 1979; Luedke et al., 1970; Luedke et al., 1977a and b). For this to be successful a live foetus must be born, capable of harbouring the virus for long enough and at high enough levels for vector transmission. Work by Gibbs, Lawman and Herniman (1979) was able to show that BTV infected ewes produced lambs from which virus could be isolated for up to two months. However whether viraemias were at high enough levels for transmission to occur was not ascertained. Similar results were obtained in experiments with goats (Gibbs et al., 1979) and calves (Luedke et al., 1977a and b).

c) Geographical Distribution. The widespread distribution of the virus (Table 1d) correlates to areas in which the vector is found. In the case of Culicoides sp. this involves a large area of the globe (40°N to 30°S). However the clinical disease has a much more limited incidence and its presence will depend on the interplay between infected vector movement and the availability of susceptible animals. Indeed the disease was first recognised in South Africa following the importation of susceptible breeds of sheep (Hutcheon, 1902) and it was not recognised outside South Africa until the 1940's.

The discovery of BTV in Australia in 1977 (St. George et al., 1978) resulted in retrospective serum surveys being carried out. Sentinel herds contained serological evidence of virus activity as far back as 1953 (St. George et al., 1982).
Table 1d: Geographical distribution of BTV as detected by virus isolation or the presence of antibody

<table>
<thead>
<tr>
<th>Country</th>
<th>Virus Types</th>
<th>Antibody Types</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AFRICA</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>South Africa</td>
<td>1-15, 18, 19</td>
<td>1-15, 18, 19</td>
<td>Hutchc. 1881, Howell, 1970</td>
</tr>
<tr>
<td>Sudan</td>
<td>agar gel.</td>
<td>1, 2, 5, 7, 11, 12</td>
<td>Eisa, Osman, Karrar, Abdel Rahim, 1980</td>
</tr>
<tr>
<td>Kenya</td>
<td>K1, K11, K111</td>
<td>1-16, K1, K11, K111</td>
<td>Davies, 1978</td>
</tr>
<tr>
<td>Egypt</td>
<td>1, 4, 10, 12, 16</td>
<td>4, 13, 15, 16</td>
<td>Soliman, Hafez and Ozawa, 1977</td>
</tr>
<tr>
<td>Nigeria</td>
<td>6, 7, 10, 16, 15</td>
<td>1-4, 5, 6, 7, 11</td>
<td>Lee, Causey and Moore, 1974; Herniman, Boorman and Taylor, 1983</td>
</tr>
<tr>
<td>Niger</td>
<td>1, 2, 5, 7, 11, 12</td>
<td></td>
<td>Herniman, Owen, 1979</td>
</tr>
<tr>
<td>Ghana</td>
<td>3, 12</td>
<td></td>
<td>Taylor and Sellers, 1980</td>
</tr>
<tr>
<td>Cameroon</td>
<td>1, 4, 5, 12, 13</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>EUROPE</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyprus</td>
<td>1, 4</td>
<td>3, 6, 10, 12</td>
<td>Gamble, 1949; Sellers, 1975</td>
</tr>
<tr>
<td>Turkey</td>
<td>4</td>
<td>4</td>
<td>Yonguc, Taylor, Csontos, Worrall, 1982</td>
</tr>
<tr>
<td>Portugal</td>
<td>10</td>
<td></td>
<td>Howell, 1961</td>
</tr>
<tr>
<td>Spain</td>
<td>10</td>
<td></td>
<td>Manso-Ribeiro, 1958</td>
</tr>
<tr>
<td><strong>MIDDLE EAST</strong></td>
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<td></td>
</tr>
<tr>
<td>Israel</td>
<td>2, 4, 6, 10, 16</td>
<td></td>
<td>Goldsmit and Barzilai, 1979</td>
</tr>
<tr>
<td>Iraq</td>
<td>agar gel</td>
<td></td>
<td>Hafez, Pollis and Mustafa, 1978</td>
</tr>
</tbody>
</table>
Table 1d (cont.): Geographical distribution of BTV as detected by virus isolation or the presence of antibody

<table>
<thead>
<tr>
<th>Country</th>
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*Personal communications, Taylor, W.P., Gumm, I. and Herniman, K.A.J.*
However no disease has been recorded in Australia (Snowden and Gee, 1978) and even in fully susceptible non-indigenous sheep only a mild form occurs (Flanagan et al., 1982). At present four BTV types have been isolated from Australia.

In the United States of America the virus and the disease have been shown to be widespread in the south western states both in domestic and to a lesser extent in the wild ruminant populations (Trainer and Jochim, 1969). The virus has been isolated from all the states except North Dakota and the Northern New England States (Parsonson, 1979). There are now five serotypes of BTV in the United States 2, 10, 11, 13, and 17 of which 11 and 17 appear to be the most common (Barber, 1979; Gibbs, 1983; Carlson, 1983).

Serological surveys in the Sudan, Turkey, Saudi Arabia, Yemen and Iran show the presence of group reacting antibodies in sera which do not neutralise any of the known twenty two BTV types (Taylor, Gumm and Herniman, personal communications) and there exists the possibility of cross-reactions occurring with other orbiviruses or the presence of a new BTV serotype in these areas.

In Europe the disease periodically occurs due to abnormal infected vector movement into areas containing susceptible sheep populations (Sellers, 1975 and 1980; Sellers et al., 1978). In these areas movement restrictions, vaccination programmes and the lack of a continual vector presence prevents the virus becoming permanently established (Yonguc et al., 1982; Sellers, 1975 and 1980).

**Pathogenesis. a) Routes of inoculation:** The normal route of infection is through intradermal inoculation from the bite of
infected *Culicoides* sp. and although reports vary, approximately 100 tissue culture infective doses (TCID$_{50}$) of virus are required to cause infection (Lawman, 1979). Other possible routes of virus entry have been examined and repeated oral administration of BTV infected blood did result in a BTV infection as did the subcutaneous inoculation of BTV infected urine (Jochim et al., 1965). The intravenous inoculation of BTV results in a more rapid onset of viraemia although the duration does not appear to be shortened (Taylor, personal communications).

b) Clinical findings:- The outcome following the inoculation of BTV into animals depends on several factors; the type and strain of virus used (Neitz, 1948; Flanagan et al., 1982; Grocock et al., 1982) environmental conditions pertaining around the time of virus inoculation (Neitz and Riemerschmid, 1944; Eisa et al., 1980), the species and breed of animal inoculated (Bowne, 1971; Gorman and Taylor, 1982) age of animal (Moulton, 1961) and the previous history of exposure to BTV (Bowne, 1971). How these interact with each other is not known.

The incubation period following the bite of an infected vector can be from 2 to 15 days (Moulton, 1961) and is followed by a pyrexia which may last from 1 to 7 days and peaks around day seven following virus inoculation. As the pyrexia develops the buccal, nasal and ocular membranes become reddened and oedema of the lips is often seen. A muco-serous discharge occurs from around the eyes and nose and the nasal discharge may become blood tinged. Animals frequently salivate and smack their lips. Ocular and nasal discharges lessen and the nares become encrusted with mucous. Petechial haemorrhages
appear on ocular and buccal membranes at around 7 days post-inoculation (pi), the time of peak pyrexia. Initially the animal will retain its appetite but by day four or five following the first clinical signs it becomes anorexic. The animal can now appear to be making a recovery but can then become recumbent and refuse to rise. Examination of the coronary band will reveal pathognomic lesions of coronitis with often intense local pain. Discomfort from these lesions and a painful myositis results in the animal remaining recumbent. Death may ensue at this stage through aspiration pneumonia (Luedke et al., 1964; Leudke and Jochim, 1968). Recovery is frequently prolonged with wool break and severe loss of condition. In cattle clinical disease is far less common but when it does occur lesions will be similar to those seen in sheep (Hourrigan and Klingsporn, 1975).

Goats and wild ruminants are not as susceptible to BTV infection as sheep and exhibit only a mild clinical or inapparent infection. (Luedke and Anakwensa, 1972). However in the white tailed deer of North America acute disease resembling that in sheep may be seen. (Thomas and Trainer, 1970).

c) Necropsy findings:- Post mortems of BTV infected animals are characterised by their lack of abnormal findings (Bowne, 1971; Bowne et al., 1968). However in nearly all animals affected ecchymotic haemorrhages can be seen at the base of the heart around the left atrium and on the pulmonary artery close to the heart. Frequently petechial haemorrhages occur on the peri- and endo-cardium. Haemorrhages will be seen on the surfaces of skeletal musculature and intestinal mucosa (Bowne, 1971).
Histologically, hyperaemic lymph nodes show separation of cellular elements, deposition of haemosiderin and infiltration by neutrophils and monocytes. Similar changes occur in the spleen with perifollicular neutrophil invasion. These lesions result in the haematological findings of a pan-leucopeania (Lawman, 1979).

d) Virological findings:- Although the clinical outcome following the subcutaneous inoculation of BTV into sheep and cattle is extremely varied the level and duration of the viraemia is surprisingly constant. Virus can first be detected in the blood of sheep two to three days after subcutaneous inoculation. Peak levels are reached four days later with the viraemia persisting from twenty-five to thirty days (Jochim et al., 1965; Lawman, 1979; Osburn et al., 1981; Flanagan et al., 1982). In cattle the development of the viraemia is similar but the duration is much longer, often up to four or five months. Recently it has been shown that sheep and cattle naturally infected can harbour more than one sero-type in the blood at any one time (Stott et al., 1982).

BTV can be recovered from apparently normal offspring at birth and these animals can remain viraemic, in sheep for at least two months (Gibbs et al., 1979) and in cattle possibly indefinitely (Luedke et al., 1977 a and b; Stott et al., 1982).

Studies using both immunofluorescence (Stair, 1968) and virus isolation techniques (Lawman, 1979) have shown that on entry into the host via the subcutaneous route, replication in the draining lymph node is followed by a primary dissemination and secondary replication cycle in the blood vascular system. The resulting viraemia is initially white cell associated but
within one to two days virus becomes red blood cell associated (Lawman, 1979; Alstad et al., 1977). It is not clear whether this red cell association is because virus is on the surface or whether it is present inside the red blood cell.

e) Foeto-pathogenesis: Observations that virus was capable of producing foetal death were first made following the use of live attenuated vaccines (Shultz and Delay, 1965). Since then, congenital abnormalities have also been reported after natural infection and include hydraencephaly, porencephaly, cerebrael mineralisation, encephalitides, arthrogryposis and dwarfism (Enright and Osburn, 1974; Barnard and Pienaar, 1976; Schmidt and Panciera, 1973). The outcome for the foetus following exposure to BTV will depend on the stage of gestation at which infection took place (Osburn, 1968; Osburn et al., 1971). Exposure early in the gestation period causes congenital abnormalities of the central nervous system resulting in cell destruction and cavitating deformities (Narayan and Johnson, 1972). Infection of ewes between sixty one and seventy four days of gestation does not produce obvious abnormalities although the virus crosses the placenta and results in viraemic lambs (Gibbs et al., 1979).

Diagnosis.

A provisional diagnosis of BT can be made on clinical findings but laboratory confirmation is usually sought by either virus isolation or serological evidence of the presence of BTV in the infected animal.

A number of methods can be used for the isolation of
BTV from either the host or vector and the choice will depend on factors such as cost, speed, availability of reagents and laboratory animals and sensitivity (Neitz, 1948; Howell, 1960; Stott et al., 1978; French, 1972).

The inoculation of susceptible sheep was the earliest system used for virus isolation (Alexander, 1947; Owen et al., 1965) and the use of a blood autograph technique has been reported to improve the sensitivity of this method (Metcalf, 1977; Pini et al., 1966; Luedke et al., 1970). However, it is costly, slow and requires the availability of susceptible sheep. It was shown early in the studies of BTV that virus replication would occur in embryonated chicken eggs (Alexander, 1947) and that the intravenous inoculation and incubation at 33°C of these embryos provided a sensitive isolation system (Goldsmit and Brazilai, 1965; Gleisser et al., 1969). It has also been shown that virus growth will occur in a number of continuous cell lines (Fernandes, 1959a and c; Pini et al., 1966; Sawyer and Osburn, 1977; Bando, 1976). Of these the African green monkey kidney line (Vero) is highly sensitive although baby hamster kidney-21 (BHK) cells are the most widely used for virus isolation and propagation (Barber and Jochim, 1973; McPhee et al., 1982) and controversy exists as to which of all the above methods is the most sensitive for virus isolation (Foster et al., 1972; Goldsmit et al., 1975; Thomas et al., 1976; McPhee et al., 1983). The intra-cerebral inoculation of newborn suckling mice does offer a fourth method for virus isolation but it has tended to be used more as a method for virus propagation and characterisation (Verwoerd et al., 1979).

Following viral isolation presumptive confirmation of the
presence of BTV can be obtained by electron microscopy or the
demonstration of the group antigen using group specific tests
like immunofluorescence (Stair, 1968; Jennings and Boorman,
1980) and immunodiffusion (Jochim and Chow, 1969). The virus
can then be typed by a number of methods based on its
neutralisation by specific antisera (Howell et al., 1970;
Stott et al., 1978; Thomas and Somagh, 1978; Jochim and Jones,

The tests used for the serological diagnosis of BTV by
identification of its specific antibody can be divided into
those recognising either group or type antibody (Boulanger and
Frank, 1975).

The most widely used group test at present is the agar-gel
precipitation test (Jochim and Chow, 1969; Boulanger and
Frank, 1975; Snowdon, 1979) which has the advantage of speed,
simplicity and ease of reagent production (Gumm and Newman,
1982). However it does have problems of cross-reactions with
other closely related orbiviruses (Della-Porta et al., 1983).
The fluorescent antibody test (Pini et al., 1968; Carlson,
1981), modified complement fixation test (Boulanger and Frank,
1975), passive haemagglutination (Blue et al., 1974) and ELISA
(Hubshle et al., 1981) are also suitable for identification of
group specific antibodies.

The recognition of type specific antibodies to BTV
relies on virus neutralisation by antisera measured by a
reduction of CPE (Parker et al., 1977; Herniman et al., 1983),
or plaque formation reduction/inhibition (Jochim and Chow,
1976; Della-Porta et al., 1981; Porterfield, 1960).
The control measures adopted by a particular country will be decided by the presence or absence of the virus. Three types of areas can be identified. Firstly areas in which there is no serological or clinical evidence for the disease ever having occurred, non-enzootic areas. Secondly areas in which the disease has periodically occurred due primarily to abnormal vector movement and has not become established, the partially enzootic areas. Thirdly areas in which the disease occurs annually or continually, the enzootic areas (Sellers, 1980; Anon, 1976).

Non-enzootic areas:— Control in these areas rests on the prevention of entry of the virus by restrictions on importation of animals or animal products. Thus animals should only be imported from other clean areas and semen and embryos for implantation will only be imported following clear evidence of freedom from BTV (Bowen et al., 1982; Parsonson et al., 1981).

Partial enzootic areas:— In these areas the use of monovalent vaccines directed against the BTV type causing the periodic outbreaks along with suitable movement restrictions will provide some measure of control following an outbreak (Manso-Ribeiro, 1958; Sellers, 1975). Work by Sellers (Sellers, 1980; Sellers et al., 1978) has suggested that it may be possible to predict outbreaks caused by abnormal vector movement and in these cases prophylactic vaccination ahead of an expected outbreak may be beneficial. Following the entry of virus into partially enzootic and non-enzootic areas a number of measures can be used to limit its spread. In particular, restrictions
on animal movements, slaughter and burning of infected stock and measures to reduce virus transmission by insects (FAO handbook, 1981) are of use.

Enzootic Areas: In those parts of the world in which BTV is endemic vaccination is in large part the only satisfactory method of control (Howell, 1979). Attempts have been made to curtail vector activity at breeding sites (Holbook, 1980) and by attracting the vector away from the more susceptible sheep by the use of cattle herds (DuToit, 1944). At present vaccination is carried out using live attenuated virus vaccines. In the United States the only licensed vaccine currently available contains a modified BTV 10 and is approved for use in sheep in California (Stott et al., 1979). In South Africa vaccines have been used for many years (Theiler, 1908) and at present the vaccination regime consists of the use of live attenuated pentavalent vaccines given three times at three week intervals. Thus fully vaccinated sheep receive fifteen BTV types over a six week period (Howell, 1979). The degree of immunity conferred by this vaccination protocol is highly unpredictable and at best evokes antibodies to only ten or eleven types (Erasmus, personal communication). These problems and others associated with the use of live virus vaccines (Stott et al., 1979) prompted a number of workers to investigate the use of inactivated virus preparations (Stott et al., 1979; Parker et al., 1975). These inactivated preparations elicit either a very low neutralising antibody response (Parker et al., 1975,) or none at all and in the latter case a cell mediated immune response was demonstrated along with a degree of protection (Stott et al., 1979). How long this immunity lasts and whether it produces heterotypic
immunity is not known.

The Immune Response.

In the last fifteen years great strides have been made in the study of the immune system and the way it responds to infection. It is now clear that both antibody production and the mediation of immune responses by cells are end products of cellular collaboration which is associated with unique subsets of both thymus derived (T) and bone marrow derived (B) lymphocytes, and that the regulation of this response is under genetic control (Oldstone, 1979; Katz, 1977). The end product is measured as a specific immune response initiated by a specific antigen and associated with unique interactions among macrophages, T cell subsets and B cells. The immune product results from a series of minute regulations from a network of unique cells (Jerne, 1974) involving events such as cell to cell interactions and release of mediators that can enhance or suppress the immune response.

Viruses can replicate in those very cells which form the constituent parts of the immune system (Notkins et al., 1970; Wheelock and Toy, 1973) and can also alter the normal route of travel and localisation of immuno-competent cells (Woodruff and Woodruff, 1975). In addition viral antigens per-se actively compete with non-viral antigens in stimulating immune responses (Oldstone et al., 1973). Therefore the effects of viruses on the already complex immune system presents an additional dimension of subtlety.

The immune response to viruses can best be considered as consisting of both non-specific and specific, humoral and
cellular components although they function synergistically
in vivo (Burns et al., 1975). It is often stated that humoral
immunity is probably responsible for preventing reinfection
(Blanden, 1971 and 1974) whereas cellular immunity mediated by
T lymphocytes or by the interaction of antibody and Fc
receptor bearing cells is more important in recovery from
viral infections (Bloom and Rager-Zisman, 1975; Rouse and
Babiuk, 1978). In specific viral infections detailed work on
host/virus interactions needs to be done before such
generalisations can be applied (Rouse and Babiuk, 1978). In
the case of BTV as the review of the literature below will
indicate little work in this direction has been carried out.

**Immune Response To BTV.**

**Non-specific Humoral :-** The role of interferon as a non­
specific mediator of immunity was shown by Isaacs and Hitchcock
(1960) working with influenza virus in mice. Several studies
have indicated that BTV is an exceptionally potent interferon
inducer both in vitro (Eksteen and Huismans, 1972; Fulton and
Pearson, 1981) and in vivo in a mouse model (Jameson et al.,
1978). The ability of BTV to induce interferon in sheep and
cattle and the susceptibility of BTV to interferon has not,
however, been examined.

**Specific Humoral :-** Antibody Type. The inoculation of BTV into
an animal results in the generation of a number of serum
reactive antibodies. Precipitating (Jochim and Chow, 1969),
complement fixing (Boulanger et al., 1967), immunofluorescing
(Ruckenbauer et al., 1967), haemagglutination inhibiting
Hubschle, 1980), ELISA reacting (Hubschle et al., 1981), and neutralising (Haig et al., 1956) antibodies have been demonstrated.

The inoculation of BTV inactivated by beta-propiolactone (BPL) induces neutralising antibodies (Parker et al., 1975) whereas the use of a binary ethylenime (BEI) inactivated preparation results in precipitating and complement fixing but no detectable neutralising antibody (Stott et al., 1979).

Antibody Class. The class of antibody produced following the inoculation of BTV has been examined only in mice (Oellerman et al., 1976). In this system using a haemolytic plaque assay they could show no real difference between the onset of the IgM and IgG responses although the IgM response was of shorter duration.

Antibody Duration:—Neutralising antibodies to BTV are first detected in the serum of infected animals between ten and fifteen days following virus inoculation (Groocock et al., 1982; Bowne, 1971; Neitz, 1948; Luedke and Jochim, 1968). Complement fixing antibodies often appear later, and may not be detected until forty days post-inoculation (Parsonson, 1979). Precipitating and neutralising antibodies endure for at least five years in British sheep (Herniman, personal communication) whereas complement fixing antibodies may decline by one year (Pearson et al., 1973).

Antibody Specificity:—Antibodies responsible for in vitro BTV sero-type specificity (Howell, 1960) have been shown to be directed against viral polypeptide two, by experiments involving cross-hybridisation (Huismans and Howell, 1973) oligonucleotide finger-printing (Sugiyama
et al., 1981) and cross-immuno precipitation (Huismans and Erasmus, 1981). Viral polypeptide seven has been identified as that responsible for generating BTV group reactive antibodies (Huimans and Erasmus, 1981). Of the antibody populations only the neutralising and haemagglutination inhibiting antibodies have been shown to be BTV type specific (Luedke and Jochim, 1968, Hubschle, 1980).

Recent work with an Australian isolate, BTV 20 has shown that the inoculation of this type generates not only antibodies to BTV 20 but also antibodies to BTV 17. When these BTV 20 inoculated sheep were challenged with BTV 17 they showed an increase in neutralising antibodies to both types (Groocock et al., 1982). In contrast, recent experiments in cattle infected simultaneously with two BTV types failed to develop even a precipitating antibody response (Stott et al., 1982). Thus it would appear that an ill-defined range of antibody responses can occur following the inoculation of one or more BTV types. Serological surveys have also uncovered serum samples containing neutralising antibodies to several BTV types (Davies, 1978; Hafez and Ozawa, 1981) but it is not known whether this demonstrates exposure to each virus serotypes or to one serotype which gives a heterotypic antibody response.

Non-specific cellular:- The fundamental importance of the macrophage in immunity and pathogenesis in other virus infections has been clearly shown (Mims, 1977; Glasgow, 1970; Gresser and Lang, 1969). BTV can replicate in cells of the mononuclear phagocytic system (Lawman, 1979) and in vitro macrophages may become persistently infected. The relevance of
this observation and the role of other antigen processing
cells in the immune response to BTV infection have yet to be
studied.

**Specific cellular** :- Only one study has been carried out on
the specific cellular immune response to BTV and this involved
the use of an inactivated BTV preparation (Stott et al.,
1979). In these experiments lymphocyte stimulation by BTV
antigen was employed as an in vitro correlate of a cellular
immune response. Following vaccination a clear blastogenic
response was recorded and on challenge a strong secondary
response occurred.

**Mechanisms in Protection and Recovery** :- Little work has been
carried out to investigate mechanisms for protection and
recovery in BTV infections but field and experimental
observations give some indications of the role of various
components of the immune response (Neitz, 1948).

The series of cross-protection experiments of Neitz (1948)
demonstrated a clear protection against subsequent homologous
but not heterologous type challenge. He suggested that the
inoculation of a BTV resulted in a degree of 'basal immunity'.
This would reduce the clinical response to subsequent
heterologous BTV challenge but this would not increase
following subsequent challenge either by the same or a
different BTV type. Howell (1960) was able to type these virus
isolates by means of an in vitro virus neutralisation test.
Since this work the criteria used to select suitable vaccines
indicates that a clear assumption has been made that the
presence of neutralising antibody correlates with protection (Howell, 1979). However the demonstration that virus can persist in the blood of animals showing high levels of specific neutralising antibody (Luedke et al., 1970; Luedke et al., 1983) and that protection against challenge can occur in the absence of a demonstrable neutralising antibody response but a clear cellular immune response (Stott et al., 1979) throws doubt on this assumption.

Recently Letchworth and Appleton (1983b) have demonstrated protection against challenge with BTV 17 using a monoclonal antibody raised against the viral polypeptide 2. They suggest neutralising antibody is a pivotal component in protection against BT disease but may not provide complete protection against infection and make little or no contribution to recovery.

Foeto immuno-pathology and immuno-tolerance :- The immunological and virological outcome of intra-uterine infection with BTV has been studied by a number of workers (Gibbs et al., 1979; Luedke et al., 1977 a and b; Stott et al., 1982). Their results show that 1) the infection of pregnant dams can result in offspring being viraemic at birth and remaining so for different periods of time. 2) The viraemias as such may disappear although they may remain persistently infected 3) at birth these infected animals may appear to be immuno-tolerant; or show fluctuating antibody responses. Although it has been suggested that the variable response may be due to differences in virus strains, time of onset of foetal infection and sensitivity of assay system used, the underlying immunological mechanisms are not
understood.
MATERIALS AND METHODS

1) Experimental Animals.

a) Sheep and Cattle.

Male cross bred Dorset Horn sheep and Jersey calves were used at six to eight months of age. Monozygotic sheep were supplied by the Animal Breeding Institute, Babraham at approximately one year of age. All animals were held in insect proof and BTV secure accommodation during the experiments.

b) Mice.

C\textsubscript{3}H\textsubscript{2}K mice were supplied by the Laboratory Animal Centre, Carshalton; the Balb C\textsubscript{H\textsubscript{2}}D mice were reared at the Animal Virus Research Institute (AVRI), Pirbright.

2) Inoculation Procedures and Sampling.

a) Sheep and cattle.

Animals were infected by intradermal inoculation in the left
ear of $10^6$ median tissue culture infective doses (TCID$_{50}$) of BTV. After exposure to BTV any subsequent inoculation was begun only when the previously established viraemia was no longer detectable. A clinical examination of animals was carried out daily, for up to three weeks after inoculation. Rectal temperatures were also recorded at the same time each day. Daily blood samples for serum and heparinised blood (5 iu heparin per ml final concentration) for virus isolation were collected.

b) Mice.

Six to eight week old mice were immunised by intraperitoneal inoculation of 0.5ml of live or inactivated virus preparations with or without adjuvant. For primary CTL assays mice were killed by cervical dislocation seven days later. In double immunisations procedures, mice were immunised fourteen days apart and then killed seven days after the second inoculation. For secondary in vitro studies mice were killed at least fourteen days after immunisation.

3) Cells.

a) Established cell lines.

Baby Hamster Kidney-21 clone 13 (BHK) and IB-R S-2 cells were supplied by the AVRI and Georgia Bovine Kidney (GBK) by the
Central Veterinary Laboratory (CVL) Weybridge. L929(H2K) and 3T3B(H2D) cells were obtained from Flow laboratories. Monolayers of these cells were removed using a solution of versene/trypsin (0.5% trypsin) for 5 minutes at 37°C. Cells were re-established in a growth medium of Glasgow modified Eagle's medium supplemented with 15% tryptose phosphate broth, penicillin (110 iu/ml) and streptomycin (0.2 mg/ml) (Eagle's supp.) together with 10% newborn calf serum. When re-established as confluent monolayers, cells were maintained on Eagle's supp. plus 5% newborn calf serum.

b) Primary ovine cell lines.

Primary lamb foetal kidney cells (LFK), lamb testis cells (LT) and lamb skin cells were obtained from newborn lambs or experimental animals at the AVRI. Tissue was removed from the animal, finely minced and trypsinised for two hours at 37°C. The resulting suspension was pelleted by centrifugation (200xg, 5 minutes at 4°C) and the cells resuspended in Eagle's supp. with 10% newborn calf serum.

c) Mouse spleen cells.

Suspensions of these cells were prepared by forcing cells through a fine wire mesh using the technique described by Lawman et al., (1980). They were subsequently resuspended in RPMI 1640 medium containing 10% foetal calf serum, 25mM Hepes buffer, 2mM glutamine, penicillin (100iu/ml) and streptomycin
Cell viability was assessed by trypan blue dye exclusion and was usually greater than 95%. T cell enriched populations were obtained from mouse spleen cell suspensions by the nylon wool adherence technique of Julius, Simpson and Herzenberg (1973) or by treating suspensions with rabbit anti-mouse IgG serum plus normal rabbit serum to provide a source of complement. T cell depleted populations were obtained by treating cells with anti-thymocyte sera (Miles Laboratories) and rabbit complement.

d) **Peripheral blood lymphocytes (PBL's).**

Heparinised blood from animals known to be free of BTV was centrifuged (220xg, 20 minutes at 4°C) and the buffy coat removed. The buffy coat was then diluted with 5 ml of Glasgow modified Eagle's medium and layered onto 3 ml of "Ficoll-Paque" (Pharmacia Fine Chemicals) (1.0774g/cu^3 at 10°C) and centrifuged (350xg, 30 minutes at 10°C). The interface cells were removed and contaminating erythrocytes lysed by hypertonic flash lysis with sterile distilled water. The resulting lymphocyte suspension was washed three times with Eagle's medium and finally suspended in RPMI-HEPES.

e) **Bovine and ovine udder cells.**

These were obtained as described previously (Wardley et al., 1976, Lawman, 1979). In sheep 2ml and in cattle 5ml, of a solution containing 5mg/ml of lipopolysaccharide (serotype no. 33)
0127:B8. Sigma London Chemicals Co.) was inoculated via the teat duct into the mammary gland. After the appropriate interval, 18 hours for neutrophil collection and 96 hours for macrophages, 10ml of prewarmed saline was infused into the gland, via the teat duct, the gland gently massaged and the saline expressed into a sterile container. The cells were centrifuged (200xg, 10 minutes at 4°C), contaminating erythrocytes flash lysed and the cells washed twice in Eagle's medium and finally resuspended in RPMI 1640.

4) Viruses

a) BT viruses.

BTV types except BTV4 and BTV17 were obtained from the Veterinary Research Institute, Onderstepoort as low passage isolates. They were passaged once or twice in egg embryos (E1 and E2) before adaption by passage to BHK cells (BHK1 etc.). BTV4(E1BHK1) was obtained as an isolate from the 1969 Cyprus BT outbreak. Each BTV type was plaque purified three times in BHK cells using the agar gel double sandwich suspension technique (Cooper 1961). Following plaque purification virus type specificity was verified in a virus neutralisation test using type specific hyperimmune guinea pig anti-sera. BTV 4 mouse brain virus stocks were obtained by a further three passages in brains of suckling mice. BTV17 (Wyoming strain) was kindly supplied by G. Letchworth of Plum Island Animal Disease Centre, U.S.A. It had been passaged once in
embryonated eggs, once in BHK cells and three times in Mengling Vaughan porcine kidney cells.

The following BTV types were used: BTV1(E3BHK7), BTV2(E1BHK6), BTV3(E1BHK5), BTV4(E1BHK4), BTV5(E2BHK4), BTV6(E3BHK4), BTV10(E1BHK7), BTV16(E2BHK6), BTV17 (Wyoming strain).

b) Other viruses.

Pseudorabies virus was supplied by the Central Veterinary Laboratories (CVL), Weybridge. Ibaraki virus (BHK3) was obtained from the National Institute of Animal Health, Tokyo, Japan. Epizootic Haemorrhagic Disease (EHD) virus, New Jersey strain (BHK5) was supplied by K. Herniman (AVRI). Corriparta (BHK6) by Miss J. Taylor (Queensland Institute for Medical Research, Australia) and Vesicular Stomatitis virus, Indiana strain (BHK5) and aphtho virus (type O) by Dr. Hedger (AVRI). Infectious bovine rhinotracheitis virus (IBR) was supplied by P. Roeder (CVL).

c) Growth and storage.

All BTV types, other orbiviruses and VSV were grown on BHK cells. Pseudorabies virus was grown in renal swine cells (IB-RS-2) and IBR virus in GBK cells. Virus was added to a confluent monolayer of the appropriate cell line at a multiplicity of infection (MOI) of 1. After absorption for 1
hour at 37°C monolayers were refed with maintenance media. When approximately 70% of the cells showed evidence of cytopathology the media was removed, centrifuged (400xg, 10mins. at 4°C) and the resulting supernatant stored in 1ml amounts at -70°C. Titrations of virus were carried out in roller tubes in the appropriate cell line.

d) **Virus Inactivation.**

Beta-propriolactone (BPL) inactivation of virus was carried out using 0.3% BPL buffered with 0.1 M TRIS (Parker *et al.*, 1975). The mixture was incubated at 37°C for 30 minutes and then overnight at 4°C before use.

Virus infected fixed cell vaccines were prepared according to the method of Powell (1975). Briefly BTV infected L929 cells expressing maximal surface membrane viral antigen (24 hours post virus inoculation) were reacted with 0.15% glutaraldehyde for 30 minutes. These fixed cells were washed three times in PBS and finally emulsified with equal volumes of Freund's complete or incomplete adjuvant before intraperitoneal inoculation into mice.

Heat inactivation of virus was carried out by incubating virus at 56°C for 60 minutes.

After all these inactivation procedures preparations were innocuity tested by titration in BHK cells.
e) Virus Isolation.

1) From blood. Heparinised whole blood was centrifuged and the packed cells washed three times in an approximately equal volume of isotonic saline solution. These washed cells were then sonicated at 110 watts for 15 seconds (M.S.E. Soniprep 150). This solution was then diluted in 10 fold steps in phosphate buffered saline (PBS) containing 0.2% bovine serum albumin (BSA) before titration for the presence of BTV in roller tubes. Each of five roller tubes containing a 24 hour old monolayer of BHK cells was inoculated with 0.2 ml of the appropriate blood dilution and after adsorption of virus for 30 minutes at 37°C, monolayers were refed with Eagle's supp. The tubes were then rolled at 37°C. The following day all cultures were washed twice with PBS and refed. The monolayers were examined daily, for eight days, for BTV cytopathology and the media changed every second day. Viraemia levels were expressed as \( \log_{10} \text{TCID}_{50}/\text{ml} \) of original packed cells. In each experiment virus isolates were retyped using the virus neutralisation test.

Because of the relative insensitivity of BHK cells in detecting low levels of BTV in tissues (McPhee et al., 1982) the more sensitive 11 day old chick embryo inoculation system was used on occasions for blood found negative for the presence of BTV by titration in BHK cells. Eggs were inoculated intravenously and incubated at 33°C. Embryos that died between three and seven days post inoculation were then ground up and inoculated onto BHK cells to confirm the presence of BTV.
2) From infected mice. The heart and a small section of the spleen was removed immediately after killing the mice and stored at -70°C. Before titration the heart and spleen were ground up with sterile sand and the mixture suspended in PBS containing 0.2% BSA to give a 10% (w/v) suspension. After sonication the samples were centrifuged (200xg for 5 minutes) and the resulting supernatant diluted in 10 fold dilution steps in PBS + 0.2% BSA. Dilutions were then titrated in monolayer roller tubes cultures of BHK cells using 5 tubes per dilution.

5) Serological Procedures.

a) Precipitating antibody detection.

Agar gel tests for BTV group-specific precipitating antibodies (Jochim and Chow, 1969) were conducted on microscope slides using antigen derived by ammonium sulphate precipitation of infected cell cultures (Eisa et al., 1982). Two ml of a 1% solution of litex agarose in borate buffer (9.0gm boric acid + 2.0gm sodium hydroxide in 1 litre of distilled water, pH 9.0) was added to each microscope slide (size 76x26mm). A cutter was used to give a six-well rosette around a central well (Fig 2a). Each well had a diameter of 5mm and there was a distance of 8mm between the middle of the central well and the middle of peripheral wells. The reagents and test sera were placed in the wells as shown in Fig 1. The slides were incubated at room temperature and examined for
Fig. 2a: Distribution of reagents and test serum in wells, used in the agar gel precipitation test for the diagnosis of BTV antibodies.
precipitin lines daily for 3 days.
Positive control sera was placed next to test sera to enable lines of identity to be recognised.

b) Neutralising antibody detection.

Tests for neutralising antibodies to BTV types were carried out using a micro-neutralisation system with an initial serum dilution of 1/10, and 100 TCID of virus per well. Equal amounts (100μl/well) of serum and virus were incubated in duplicate for 1 hour at 37°C and then overnight at 4°C before the addition of cells (50μl/well, 5x10^5 cells/ml). It was necessary to double the number of cells added to test wells when examining sera from animals which had received three different BTV types owing to the cytotoxic effect of these sera against BHK cells (Herniman et al., 1983). Plates were incubated at 37°C and examined 3 and 5 days later for evidence of virus induced cytopathology. Results were expressed as that dilution of serum neutralising 50% of virus infected wells (VN_{50}). Virus and positive and negative serum controls were included with each batch of sera tested.

c) ELISA fixing antibodies.

Sera from mice infected with BTV were titrated for the presence of BTV antibodies by the group specific ELISA test (Hubschle et al., 1981). Wells of 96 well microtitre plates
(Flow Lab. Ltd.) were labelled with freon extracted BTV group antigen (Hubschle and Yang, 1983). Test mouse serum diluted in PBS containing 1% BSA plus 0.05% tween 20 (PBS:BSA) was added to the wells and the plates placed in an orbital shaker for 1 hour at 37°C. Following this horse radish peroxidase conjugated rabbit anti-mouse serum (DAKO Immunoglobulins, Denmark) diluted 1/2000 in PBS:BSA was added and the mixture again shaken at 37°C for 1 hour. Between each stage plates were washed 4 times in PBS:BSA. Finally the substrate (orthophenylene diamine, OPD) was added and the reaction stopped 10 minutes later with H$_2$SO$_4$. The optical density was read using an automated ELISA scanner (Flow Lab. Ltd.)

6) **BTV3 Immune Sera Production.**

Six weeks after inoculation with BTV3, eight sheep were slaughtered, their blood collected aseptically and pooled. After clotting, the serum was removed, heat inactivated (1 hour at 56°C) and stored at -20°C. The BTV neutralising antibody titre and type specificity of this serum was established using the virus micro-neutralisation test.

7) **Measurement of Cell Surface Bound Anti-BTV Antibody.**

GBK cells in a 30mm plastic petri dish were infected with BTV at an MOI of 1. After incubation overnight cells were removed by vigorous pipetting, washed three times in Glasgow
modified Eagle's medium and finally resuspended in RPMI and adjusted to $10^5$ cells/ml. One ml cell suspensions were then added to an equal amount of serum (diluted 1/20) and the mixture left for 1 hour at 4°C. After centrifuging (200xg, 5 minutes at 4°C) the cell pellet was washed twice in PBS before resuspending in 2ml of Glasgow modified Eagle's medium. Two ml of a 1/2000 diluted peroxidase conjugated rabbit anti-sheep immunoglobulin (DAKO Immunoglobulins, Denmark) was added and the mixture incubated for 1 hour at 4°C before washing the cells three times in PBS and finally resuspending in 100μl of PSA:BSA. These cells were then added to a microtitre plate well (Flow Lab. Ltd.) and the plate centrifuged (400xg, 10 minutes at 4°C). The supernatant was removed and 100μl of OPD added to the well. After 10 minutes at room temperature 100μl of $\text{H}_2\text{SO}_4$ was added and the optical density was read using an automated ELISA scanner (Flow Lab. Ltd.).

8) Chromium Release Assays.

a) Murine Cytotoxic T Lymphocytes.

(i) Confluent monolayers of L929 and 3T3B cells in 90 mm plastic petri dishes were simultaneously incubated with 100μCi of Na$_2$(51Cr)O$_4$ (Radiochemical Centre, Amersham) and BTV at a MOI of 1. After one hour at 37°C, cells were washed twice in Eagle's medium, refed with fresh medium and left for twenty four hours. They were then removed from the dishes with trypsin and versene, washed three times in Eagle's medium and...
adjusted in RPMI-HEPES to $10^5$ cells/ml. These target cells were added in 100µl volumes ($10^5$cells/ml) to flat bottomed microtitre plates. Mouse splenocytes (section3, b) were then added in 100µl suspensions and plates were incubated at 37°C in a humidified incubator containing 5% CO$_2$ in air for seven hours. All assays were carried out in six well replicates using various effector to target ratios. Plates were then centrifuged (200xg, for 1 minute at 4°C) before half of the contents of each well was removed and used to estimate the release of ($^{51}$Cr) using a gamma counter (MR1032, Kontron Ltd.).

(ii) The percentage specific lysis of ($^{51}$Cr) was calculated as follows:-(Effector cell/target cell release minus target cell alone release) divided by (Total releasable chromium minus target cell alone release) multiplied by 100. Total releasable chromium was obtained by exposing target cells to 2.0 percent Triton X-100.

(iii) For in vitro cultures spleens were removed from mice inoculated at least fourteen days previously with inactivated or live virus. Fifteen ml of a spleen cell suspension adjusted to contain $10^6$ cells/ml in RPMI-HEPES plus $5 \times 10^{-5}$ M 2-mercaptoethanol was incubated in plastic flasks (Falcon flask number 3024F) with 1 ml of live or inactivated BTV. After 5 five days at 37°C in a humidified atmosphere containing 5% CO$_2$ cells were harvested, washed twice in Eagle's medium and resuspended in RPMI-HEPES. The number of viable cells was counted using trypan blue dye exclusion (approximately 30% of cultured cells), before used in chromium release assays as
effector cells against L929 infected BTV targets (section a).

b) 'Cold Target' Competitive Inhibition.

Details of this assay procedure have been described elsewhere (Zinkernagel and Doherty, 1975). Briefly unlabelled or 'cold' L929 cells infected with the appropriate BTV type were mixed with effector spleen cells. This was immediately followed by the addition of \(^{51}\text{Cr}\) labelled L929 cells infected with the same or different BTV types. Assays were then carried out as described in section a(i),(ii).

c) Ovine Cytotoxic T Lymphocytes.

A similar assay procedure as used previously for the study of murine CTL's (a(i),(ii)) was carried out. Ovine PBL's, obtained daily from animals following the inoculation of BTV, were used as effector cells. Third or fourth tissue culture passaged primary LT cells infected with BTV 24 hours previously and labelled overnight with \(^{51}\text{Cr}\) acted as target cells in a 7 hour chromium release assay.
d) Antibody Dependent Cell Mediated Cytotoxicity.

GBKs, LFK or LT cells were infected at a MOI of 1 with BTV or IBR and simultaneously labelled with \(^{51}\)Cr (section a(i)). Twenty four hours later these cells were used as target cells. Bovine udder macrophages or peripheral blood leucocytes, ovine udder macrophages, udder neutrophils or peripheral blood leucocytes were used as effector cells. Assays were set up with effector to target cell ratios of 100:1 plus the addition in 50\(\mu\)l amounts of BTV or IBR immune sera at a final concentration of 1:50. Appropriate controls were included on each plate before incubation for 7 hours. Plates were then treated as described in section a(i) and lytic activity calculated as shown in section a(ii).

9) Interferon Assay.

a) Interferon Induction.

A confluent layer of GBK cells was infected with BTV4 at a MOI of 1. After 24 hours the culture fluid was removed and centrifuged (400xg for 10 mins.). The supernatant was then treated at pH 2 for twenty four hours, a procedure known to inactivate BTV (Rinaldo et al., 1973). The pH was then returned to 7.2 by dialysis against PBS before finally storing the fluid in 1 ml amounts at \(-70^\circ\)C.
b) Interferon Assay.

A 1 ml amount of the putative interferon (diluted 1:20 in PBS plus 0.2% BSA) was added to confluent GBK monolayers grown in a 6 x 24 well plastic plate (Flow Lab. Ltd.) The plates were incubated overnight at 37°C in 5% CO₂. Monolayers were then washed twice in PBS before being infected with 0.2 ml of the appropriate dilution of BTV or VSV. After one hour absorption at 37°C, the virus was removed and 2 ml of overlay containing Eagle's supp and 0.5% agarose was added. Twenty four hours later in the case of VSV and 72 hours later in the case of BTV assay, 1 ml of a solution containing 1:10,000 methylene blue and 4% formaldehyde was added. Plaque counts were made the following day after removal of the overlay by washing. Wells containing cells not exposed to interferon were included as virus titration controls. The results were expressed as % reduction of plaque forming units between virus controls and interferon treated cells.

10) Cellular Adoptive Transfer Techniques.

a) Thoracic Duct Cannulation

Donor monozygotic animals were inoculated with 10⁶ TCID₅₀/ml of BTV and thoracic duct cannulation was carried out 7 or 14 days later. Animals were starved of food and water for twelve hours before induction of anaesthesia was obtained using sodium thiopentone (Intraval Sodium), intravenously injected
(1g/100kg body weight). Following intubation, anaesthesia was maintained using halothane in a closed circuit system.

Superficial blunt dissection of overlying tissue in the neck immediately above the prescapular area enabled the thoracic duct to be located at a point where it empties into the left jugular vein. Transparent polyvinyl tubing of internal diameter 1.35 mm (Portex Ltd.) was used to cannulate the duct and was secured in place by two sutures placed 8 mm apart along the thoracic duct. The tubing was exteriorised in the neck region and directed into a sterile plastic 200 ml bottle attached by a collar to the neck of the animal. The bottle contained 1,000 iu of heparin, 10,000 iu penicillin and 10 mg of streptomycin. When full, which took approximately 1 to 2 hours, the bottle was replaced until the required number of cells were collected.

Pooled collections of thoracic duct lymphocytes (TDL) from each animal were washed three times in RPMI-HEPES at 4°C. Cell viability was checked and found to be approximately 98%. TDL's and fluid were also examined for the presence of BTV and BTV antibodies.

b) **Cell Characterisation.**

TDL's were characterised using fluorescein conjugates of rabbit anti-sheep IgG (Miles-Yeda Ltd.) for identification of B cells and peanut agglutinin (Arachis hypogaea, Sigma Chemicals Ltd.) for T cell identification. In each case a 1 ml
amount of cell suspension (approximately $5 \times 10^5$ cells/ml) was mixed with an equal amount of a 1/10 dilution in PBS of the conjugate. The mixture was incubated for 1 hour at $4^\circ C$ and then washed three times in PBS before microscopic examination. The percentage of cells showing surface fluorescence in the cell suspension was obtained by the examination of at least 300 cells.

c) **T Cell Enrichment Procedures.**

i) **Anti-sheep immunoglobulin:** Normal sheep serum was precipitated using sodium sulphate (18% final concentration) (Hudson and Hay, 1976). The resulting precipitate was then redissolved in a small amount of PBS and dialysed against PBS overnight at $+4^\circ C$. This solution was adjusted to contain 2mg/ml of immunoglobulin and emulsified with an equal volume of Freund's incomplete adjuvant. Rabbits were inoculated once weekly for four weeks at multiple sites with 1ml amounts of the emulsified antigen. The rabbits were bled out 1 week later. The pooled rabbit serum was precipitated again using sodium sulphate and adjusted to contain 1mg/ml of immunoglobulin.

ii) **Coating of Plastic Plates With Anti-Ig:** Fourteen ml of rabbit anti-sheep Ig was added to each 140mm plastic petri dish (Sterilin Ltd. No. 305v) and incubated for 18 hours at $+4^\circ C$. Immediately prior to use of the plates the anti-Ig was drawn off carefully and the plates washed three times in 15ml
iii) Cell Enrichment:- TDLs were adjusted to contain $1.5 \times 10^7$ cells/ml in bicarbonate free culture medium containing 5% foetal calf serum. Twenty-one ml of TDLs were added to each labelled petri dish and incubated for 30 minutes at room temperature on a level table. After this time the plates were lightly agitated and incubated for an additional 30 minutes. The non-adherent cells were then removed by careful pipetting and these cells added to a fresh labelled petri dish and the procedure repeated. At each stage samples of adherent cells were removed and checked for cell composition using the specific B and T cell fluorescein conjugates.

d) Cell Transfer.

In each case the recipient monozygotic animal received approximately $6 \times 10^9$ of either the total or T cell enriched population of washed TDLs in a 20ml amount. Cells were given intravenously and animals were challenged with BTV 12 hours later.
Chapter Three.

HUMORAL IMMUNITY; VIRUS NEUTRALISATION STUDIES.

Introduction

This chapter describes the humoral response and clinical manifestations to single, serial and simultaneous virus inoculation in British breeds of sheep and cattle.

Classically serum neutralisation data has been used both as an indication of likely protection against disease and as an epidemiological tool to define the virus types present in a particular area. In the field animals are likely to be simultaneously or sequentially challenged by a number of virus types although the literature contains no information on the possible serological effect this might have. Hence this work was undertaken in the hope that this knowledge would allow a better analysis of field sera data and perhaps help in improving current vaccine design.

Experimental design

A summary of virus inoculations and challenges can be seen in Table 3a. At the time of each challenge virus inoculation
Table 3a: BTV inoculations carried out in sheep and cattle

<table>
<thead>
<tr>
<th>Exp. species</th>
<th>nos. primary inoculation</th>
<th>first nos. of challenge</th>
<th>nos. of controls</th>
<th>second nos. of challenge</th>
<th>nos. of controls</th>
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</thead>
<tbody>
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<td>(a) sheep</td>
<td>4 BTV5</td>
<td>BTV5 (43)\textsuperscript{a}</td>
<td>2</td>
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<tr>
<td>(b) sheep</td>
<td>8 BTV4</td>
<td>BTV3 (66)</td>
<td>4</td>
<td>BTV6 (176)</td>
<td>4</td>
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<tr>
<td>(c) sheep</td>
<td>4 BTV3</td>
<td>BTV6 (38)</td>
<td>2</td>
<td></td>
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<tr>
<td>(d) sheep</td>
<td>4 BTV4</td>
<td>BTV6 (44)</td>
<td>2</td>
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<td></td>
</tr>
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<td>(e) sheep</td>
<td>8 BTV2</td>
<td>BTV5 (112)</td>
<td>4</td>
<td>BTV6 (224)</td>
<td>4</td>
</tr>
<tr>
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<td>4 BTV4</td>
<td>BTV3 (120)</td>
<td>2</td>
<td>BTV6 (286)</td>
<td>2</td>
</tr>
<tr>
<td>(g) sheep</td>
<td>4 BTV3, 4 and 6</td>
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</table>

\textsuperscript{a} The figure in brackets is the number of days post primary virus inoculation that the challenge virus was administered.
animals not previously exposed to BTV were inoculated to act as single virus inoculation controls.

Results

Sequential inoculations; sheep

a) Inoculation and challenge of sheep with BTV5

1) Primary inoculation of BTV5

   a) Clinical response:- The four sheep inoculated with BTV5 showed an incubation period of five days followed by a pyrexia lasting five days (Fig 3a, Table 3b); the mean peak temperature for this group of animals was 40.3°C attained on day 6 pi. No other clinical abnormalities were observed. Virus was detected in the blood by day 2 pi in all four sheep and rose to peak levels between days 6 and 8. Viraemia levels declined slowly but virus continued to be recovered from the blood until day 23 in two of the four animals.

   b) Antibody response:- All four animals developed BTV group-specific precipitating antibodies which were first detected by day 10 pi and remained for the rest of the experiment. The presence of neutralising antibody to BTV5 was first detected by day 10 pi in three of the four sheep and by day 11 in the fourth animal. The level of neutralising antibody rose to maximum titres of around 1/120 by day 15.
### Table 3b: Clinical response to the inoculation of BTV in sheep and cattle

<table>
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<th>Exp. virus used</th>
<th>onset day of pyrexia</th>
<th>duration onset of pyrexia</th>
<th>peak day of pyrexia</th>
<th>peak day of viraemia</th>
<th>length of viraemia</th>
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<td>3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7</td>
<td>5</td>
<td>2</td>
<td>5.4&lt;sup&gt;b&lt;/sup&gt;</td>
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<td></td>
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<tr>
<td></td>
<td>BTV5</td>
<td>no pyrexic or viraemic response</td>
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<tr>
<td>(b) BTV4</td>
<td>3</td>
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<td>2</td>
<td>5.2</td>
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</tr>
<tr>
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<td>9 22</td>
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<td>5</td>
<td>5.2</td>
<td>11(3)</td>
<td>30(1) 45(3)</td>
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<tr>
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<td>3(3)</td>
<td>4(1)</td>
<td>2.5</td>
<td>7 31</td>
</tr>
<tr>
<td>(controls)</td>
<td>no pyrexic response</td>
<td>4</td>
<td>3.2</td>
<td>7</td>
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</tr>
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<td>3(3)</td>
<td>4(1)</td>
<td>2.5</td>
<td>7 31</td>
</tr>
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<td></td>
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<tr>
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<td>no pyrexic response</td>
<td>4</td>
<td>3.2</td>
<td>7</td>
<td>33</td>
</tr>
<tr>
<td>(controls)</td>
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<td>3/4 no viraemic response</td>
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<td>4</td>
<td>3.2</td>
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<td>33</td>
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<td>2.8(1)</td>
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<td>3/4 no viraemic response</td>
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<td>4</td>
<td>3.2</td>
<td>7</td>
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<tr>
<td>(controls)</td>
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<td>3.2</td>
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<tr>
<td>(f) BTV4,3 and 5</td>
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<td>3</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>8 31</td>
</tr>
</tbody>
</table>

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<sup>a</sup> Days post inoculation of virus

<sup>b</sup> Viraemia expressed as log<sub>10</sub> TCID50/ml

<sup>c</sup> Number in brackets represents number of animals in that group which gave the response. No brackets indicates that all animals in the group gave the same response.
Fig. 3a: Temperature and viraemia response of sheep following inoculation of BTV

- Rectal temperature °C
- Viraemia TCID₅₀/ml.

Results calculated as arithmetic mean of four animals.
2) **Challenge with BTV5 (day 43 pi)**

a) Clinical response: Following challenge with the homologous virus type no pyrexia was seen for the following 21 days and BTV could not be detected in the blood of these animals.

b) Antibody response: No significant increase in neutralising antibody occurred following challenge (Fig 3b). Sera from these animals were also examined for the presence of antibodies to the remaining 21 BTV types and no neutralising antibodies were detected against any of these.

b) **Inoculation of sheep with BTV4 followed by sequential challenge with types 3 and 6.**

1) **Primary inoculation of BTV4**

a) Clinical response: The blood virus levels and temperature responses were similar to those described above for BTV5 (Table 3b).

b) Antibody response: All eight animals produced precipitating antibodies to BTV by day 11 pi and these were maintained throughout the remainder of the experiment. The
Fig. 3b: Development of neutralising antibodies to BTV type 5.

Neutralising antibodies to BTV5. Titres calculated as geometric mean from four sheep of reciprocal $\log_{10} VN_{50}$ and expressed as neutralising antibody titre.
Fig. 3c: Development of neutralising antibodies to BTV types 4, 3 and 6 following the serial inoculation of these BTV types.

- Neutralising antibodies to BTV4

- Neutralising antibodies to BTV3

- Neutralising antibodies to BTV6

Time expressed as days after inoculation of BTV4; BTV3 inoculated day 66; BTV6 inoculated day 176. Antibody titre calculated as geometric mean from eight sheep of reciprocal $\log_{10}^{VN_{50}}$. 
development of neutralising antibodies to BTV4 can be seen in Fig. 3c. Examination for the presence of antibodies to the other 19 BTV types (nb. only this number of types were recognised at the time of this experiment) at day 24 pi gave negative results.

2) Challenge inoculation with BTV3 (day 66 pi)

a) Clinical response:— The temperature and viraemia responses were similar to that seen following the initial BTV4 inoculation and also no distinction could be made between the pyrexia and viraemia established in these animals when compared to a group of control sheep which received only BTV3 (Table 3b).

b) Antibody response:— The four control sheep showed a neutralising antibody response to type 3. The principals (those animals that had been previously inoculated with BTV.) also developed a type 3 neutralising antibody response together with an increase in the previously established type 4 neutralising antibody response (Fig 3c). Sera were also examined for the presence of neutralising antibodies to all other BTV types. The presence of non-specific neutralising substances in the sera of some animals (Klontz et al., 1962) makes it difficult to state clearly the lowest neutralising antibody titre to be considered as positive evidence of the animal having experienced BTV. However, in these experiments the lowest dilution at which sera were tested was a 1/20 final
dilution. In line with policy at the Animal Virus Research Institute, Pirbright, where sera with antibody levels of 1/15 or less are considered negative, reactions of a 1/20 dilution were scored as positive. Figures for group antibody levels were obtained by calculating group geometric means, thus animals with sera reacting below 1/20 were given the value of zero. This occasionally resulted in group means being below the lowest dilution at which individual animals were tested.

The cross reactivity of the sera produced was estimated by summing the number of virus types neutralised by a particular serum at levels equalling or exceeding 1/20, 1/30 or 1/40 (Fig 3d). At low serum dilutions (1/20) most sera neutralised a number of virus types to which these principal animals had not been exposed. This effect was not detectable before the inoculation of BTV and was not therefore due to non-specific activity in the sera. At these low sera dilutions, a greater number of virus types were neutralised than at higher dilutions and the extent of this cross reactivity increased with time up until day 24. The control animals which had received type 3 only were tested at a single dilution of 1/20; the response remained homologous except for a short period around day 24 pi. Thus when considering a serum positive for the presence of antibodies to a particular BTV type at a titre of 1/20, neutralising antibodies to far more BTV types were recorded in that serum than at a titre of 1/40. This could give rise to erroneous interpretations when screening sera at a single dilution for the presence of neutralising antibodies to BTV.
Fig. 3d: Number of BTV types against which neutralising antibodies were evoked following inoculation of BTV type 3 into sheep previously immunised with BTV type 4.

- ★ Number of types represented at a titre of >1/20
- ● Number of types represented at a titre of >1/30
- ★★ Number of types represented at a titre of >1/40
- ✪ Number of types represented at a titre of >1/20 in control animals receiving only BTV3

Points were derived by calculating the arithmetic mean of the results from eight animals.
Levels of neutralising antibodies to each of the 20 BTV types in the 77 day period following the challenge with BTV3 into sheep previously infected with BTV4, can be seen in Fig 3e. Before the challenge with BTV3, only antibodies to BTV4 were detected but seven days after this BTV3 challenge, group levels of 1/15 or higher were recorded to BTV2, 8 and 20 as well as types 4 and 3. The heterologous character of the response increased further but was transient and, by day 77, titres of 1/15 or higher were recorded only against BTV3, 4, 17 and 20. In terms of the next challenge it is pertinent to note that antibodies to BTV6 were detectable between the 9th and 74th days after this second virus infection, although sheep had not as yet been exposed to this virus type.

In the four control animals receiving BTV3 only the response was predominantly homotypic (Fig 3e), although antibodies at low levels, i.e. group means of 1/15, were recorded against BTV6 and 17 on day 19, and against BTV4, 6 and 20 on day 25. Only antibodies to BTV3 were recorded at levels above 1/15.

3) Rechallenge with BTV6 (day 176 pi of BTV4)

a) Clinical response:— Although controls demonstrated the typical temperature and viraemia responses to a single BTV serotype, the eight principal sheep developed neither a pyrexia nor viraemia after this inoculation (Table 3b).

b) Antibody response:— In the control group specific neutralising antibodies were first recorded around day 10 pi,
Fig. 3e: Level of neutralising antibodies to BTV types at various times after challenge with BTV type 3 in a group of eight sheep previously exposed to BTV type 4.

All titres calculated from geometric mean of reciprocal $\log_{10}^{VN_{50}}$ values and expressed as neutralising antibody titre. Sera with titres $>1/15$ scored as zero. Antibody levels in the control animals receiving BTV3 only are shown in hatching.
Table 3c: Development of neutralising antibodies to BTV6 after inoculation of BTV6 into sheep previously inoculated with BTV types 4 and 3

<table>
<thead>
<tr>
<th>Days post inoculation</th>
<th>0</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>10</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Principal sheep(^a)</td>
<td>-</td>
<td>-</td>
<td>1.25(^b)</td>
<td>1.23</td>
<td>1.68</td>
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<tr>
<td>Control sheep(^c)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.30</td>
<td>2.13</td>
</tr>
</tbody>
</table>

\(^a\) Previously exposed to BTV4 and 3

\(^b\) Neutralising antibody titre expressed as geometric mean of reciprocal log\(_{10}\) VN\(_{50}\)

\(^c\) Previously unexposed to BTV

- Neutralising antibody titre <1/15
and developed in a manner similar to all other primary inoculations (Table 3c). However in seven of the eight principals, levels above 1/20 against BTV6, were detectable by as early as day 6 following exposure to BTV6; by day 12, levels were similar in both groups.

The extent of the heterotypic antibody response following the sequential exposure of BTV4 immune sheep to BTV3 and 6 can be seen in Figs 3d, 3e and 3f. The levels of type-specific neutralising antibody during the 42 days following inoculation of BTV6 can be seen in Fig 3f. Again, the response was broadly heterotypic but at a slightly higher level and with more types being detected. A small heterotypic response also occurred in the BTV6 control group, with neutralising antibodies to BTV3, 6, 11 and 14 being detected but BTV6 apart, at levels of less than 1/10 and thus are not considered as significant.

Sera from all these experiments were also examined for the presence of neutralising antibodies to epizootic haemorrhagic disease of deer virus (New Jersey strain; EHDV1) and Ibaraki virus, with negative results.

It is possible that the protective response seen in the above series of inoculations may be due to either the inoculation of BTV4 followed by BTV6 or BTV3 followed by BTV6 and the experiment below examines the ability of the inoculation of either BTV4 or BTV3 to protect against challenge with BTV6.
Fig. 3f: Level of neutralising antibodies to BTV types at various times after inoculation of BTV type 6 in a group of eight sheep previously inoculated with BTV type 4 and challenge with BTV type 3.

All titres calculated from geometric mean of reciprocal log_{10}VN_{50} values and expressed as neutralising antibody titre. Sera with titres >1/15 scored as zero. Antibody levels in the control animals receiving BTV6 only are shown in hatching.
c) Inoculation of sheep with BTV3 or BTV4 followed by challenge with BTV6.

1) Primary inoculation of BTV3 or BTV4

a) Clinical response:– The patterns of temperature response and blood virus levels following the inoculation of BTV3 or BTV4 into sheep were similar to those described above for BTV5 (Table 3b).

b) Antibody response:– Both groups produced type antibody to the inoculated virus, in 7 out of the 8 animals by day 10 pi and by day 11 in the remaining animal.

2) Challenge with BTV6 (for BTV4 immune animals, day 44 pi; for BTV3 immune animals, day 38 pi.)

a) Clinical response:– The pattern of the temperature and viraemia response to the inoculation of BTV6 can be seen for both groups in Fig. 3g. No real difference was detected between animals receiving either BTV3 or BTV4 prior to the challenge with BTV6.

b) Antibody response:– The development of neutralising antibody to BTV6 can be seen in Fig. 3g.

The patterns of both responses were similar to those in the control animals in the previous experiment which had not been
Fig. 3g: Temperature, viraemia and neutralising antibody responses in groups of sheep inoculated with BTV type 3 or 4 and challenged with BTV type 6.

- Rectal temperature °C

- Viraemia TCID\(_{50}\) /ml.

- Neutralising antibodies to BTV6 (BTV 4 immune)

- Neutralising antibodies to BTV6 (BTV 3 immune)

Temperature and viraemia results calculated as arithmetic mean of four animals per group. Antibody titre calculated as geometric mean of reciprocal \(\log_{10} VN_{50}\).
exposed to BTV prior to the inoculation of BTV6. Thus the single inoculation of either BTV3 or BTV4 did not protect these animals from challenge with BTV6.

Having examined the ability of BTV4, 3 and 6 to evoke a heterotypic response it was felt necessary to examine other BTV types, to see if they too gave rise to similar responses.

d) Inoculation of sheep with BTV2 followed by sequential challenge with types 5 and 6.

1) Primary inoculation of BTV2

   a) Clinical response:- The temperature and viraemia response following the inoculation of BTV2 in eight animals was similar to other inoculation of a BTV type into fully susceptible animals (Fig. 3b).

   b) Antibody response:- Precipitating antibodies were first recorded by day 11 pi in six of the eight animals and by day 12 in the remaining two. These were maintained throughout the remainder of the experiment. The development of neutralising antibody to BTV2 can be seen in Fig. 3h. Antibodies were first detected on day 10 and rapidly rose to reach a mean group level of around 1/160. This titre was maintained until the inoculation of BTV5. Examination for the presence of antibodies to the other 21 BTV types in these animals gave
Fig. 3h: Development of neutralising antibodies to BTV types 2, 5 and 6 following the serial inoculation of these BTV types.

- Neutralising antibodies to BTV2
- Neutralising antibodies to BTV5
- Neutralising antibodies to BTV6

Time expressed as days after inoculation of BTV2; BTV5 inoculated day 112; BTV6 inoculated day 224. Antibody titre calculated as geometric mean from eight sheep of reciprocal $\log_{10}^{\text{VN} 50}$
negative results.

2) Challenge with BTV5 (day 112 pi)

   a) Clinical response:- The pattern of temperature response and viraemia obtained was similar to that above following the inoculation of BTV5 (Fig. 3b).

   b) Antibody response:- As was described previously following the inoculation of a second BTV type into sheep, a broad heterotypic response was observed (Fig. 3i and j) which was comparable with that observed following the sequential inoculation of BTV4 followed by BTV3. It is again pertinent to note that this response included antibodies to the next challenge virus BTV6 (Fig. 3h).

3) Rechallenge with BTV6 (day 224 pi BTV2)

   a) Clinical response:- Controls developed the typical response to a single BTV type (Table 3b). However the eight principals developed neither a pyrexia or viraemia.

   b) Antibody response:- As was observed previously (Table 3c) the pattern of development of antibodies to BTV6 in the principal sheep was dissimilar to that in the control animals (Table 3d). In this experiment BTV6 neutralising antibody was detectable as early as day 4 post challenge with BTV6 in the principal sheep, whilst in the control animals neutralising antibody was first detectable on day 10. However levels in the
Fig. 3i: Number of BTV types against which neutralising antibodies were evoked following inoculation of BTV type 3 into sheep previously immunised with BTV type 4.

- Number of types represented at a titre of >1/20
- Number of types represented at a titre of >1/30
- Number of types represented at a titre of >1/40
- Number of types represented at a titre of >1/20 in control animals receiving only BTV3

Points were derived by calculating the arithmetic mean of the results from eight animals.
Fig. 3j: Level of neutralising antibodies to BTV at various times after challenge with BTV type 5 in a group of eight sheep previously exposed to BTV type 2.

All titres calculated from geometric mean of reciprocal $\log_{10} VN_{50}$ values and expressed as neutralising antibody titre. Sera with titres $>1/15$ scored as zero. Antibody levels in the control animals receiving BTV3 only are shown in hatching.
Table 3d: Development of neutralising antibodies to BTV6 after inoculation of BTV6 into sheep previously inoculated with BTV types 2 and 5

<table>
<thead>
<tr>
<th>Days post inoculation</th>
<th>0</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>10</th>
<th>12</th>
<th>16</th>
<th>20</th>
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<tbody>
<tr>
<td>Principal sheep(^a)</td>
<td>-</td>
<td>0.8(^b)</td>
<td>0.6</td>
<td>0.8</td>
<td>1.2</td>
<td>1.3</td>
<td>1.4</td>
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<tr>
<td>Control sheep(^c)</td>
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<td>-</td>
<td>-</td>
<td>1.1</td>
<td>1.9</td>
<td>2.4</td>
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</table>

\(^a\) Previously exposed to BTV2 and 5  
\(^b\) Neutralising antibody titre expressed as geometric mean of reciprocal log\(_{10}\) VN\(_{50}\)  
\(^c\) Previously unexposed to BTV  
- Neutralising antibody titre <1/15
control animals rose rapidly to reach above 1/120 by day 20, whilst in the principal sheep levels remained low and did not rise above 1/40.

Once again a heterotypic antibody response occurred following this third BTV inoculation (Fig. 3k). However the overall response was not as great as with the previous triple inoculations (BTV4, 3 and 6).

Having observed these responses in sheep it was felt relevant to examine cattle for the ability to respond similarly.

**Sequential inoculations: cattle.**

e) **Inoculation of cattle with BTV4 followed by sequential challenge with types 3 and 6.**

1) **Primary inoculation of BTV4**

   a) Clinical response:- The outcome of the inoculation of BTV4 into cattle was dissimilar to that in sheep (Table 3b). No pyrexia could be detected and the onset of viraemia was not until day 5 pi. Further the viraemia was longer lasting and in three of the four animals was still present by day 45 pi.

   b) Antibody response:- Two of the four animals produced BTV group reactive precipitating antibodies by day 15 pi and the remaining two by day 17. These antibodies were maintained
Fig. 3k: Level of neutralising antibodies to BTV at various times after inoculation of BTV types 6 into a group of eight sheep inoculated with BTV type 2 and challenged with BTV type 5.

All titres calculated from geometric mean of reciprocal $\log_{10}^{\text{VN}_{50}}$ values and expressed as neutralising antibody titre. Sera with titres $\geq 1/15$ scored as zero. Antibody levels in the control animals receiving BTV6 only are shown in hatching.
throughout the remainder of the experiment. The development of neutralising antibodies to BTV4 can be seen in Fig. 31. Examination for the presence of antibodies to the other 21 BTV types in these animals gave negative results.

2) Challenge with BTV3 (day 120 pi)

a) Clinical response:— The viraemia obtained was similar to that following the BTV4 inoculation and no distinction could be made between this viraemia and that obtained in two BTV3 inoculated control animals (Table 3b).

b) Antibody response:— The two control animals showed a neutralising antibody response to only type 3. The principals also developed a type 3 neutralising antibody response together with an increase in the previously established type 4 neutralising antibody level. As with the sheep, following the inoculation of two BTV types, antibodies to the challenge type, BTV6 were detectable for a period (Fig. 31), but were transient.

3) Rechallenge with BTV6 (day 286 pi of BTV4)

a) Clinical response:— Although the two inoculation controls demonstrated the typical viraemia following the inoculation of a single BTV type (Table 3c), three of the four principal cattle did not develop a viraemia. The fourth animal
Fig. 31: Development of neutralising antibodies to BTV types 4, 3 and 6 following the serial inoculation of these BTV types in cattle.

- Neutralising antibodies to BTV4
- Neutralising antibodies to BTV3
- Neutralising antibodies to BTV6

Time expressed as days after inoculation of BTV4; BTV3 inoculated day 120; BTV6 inoculated day 286. Antibody titre calculated as geometric mean from four cattle of reciprocal $\log_{10}$ VN50.
(animal C) did develop a viraemia although it was at a lower level than in the control animals (Table 3e).

b) Antibody response:- The development of antibodies to BTV6 in both groups was similar and mean levels reached in the principal animals were comparable with those to BTV4 and BTV3 (Fig. 31).

The levels of neutralising antibody to types 4, 3 and 6 in individual animals can be seen in Table 3f. No real difference was observed in animal C when compared to the other three animals, until day 34 pi with BTV6. This would indicate that levels of neutralising antibody to BTV6 did not correlate with the ability to withstand a challenge by this virus, although the three to four fold increase in BTV6 neutralising antibody following this virus challenge indicated the presence of the viraemia.

Sera collected during this series of inoculations were examined for neutralising antibodies to all designated types of BTV. The extent of the heterotypic response following these sequential inoculations can be seen in Fig. 3m and was similar to those observed in sheep following the same virus inoculations.

Having established that the serial inoculation of various BTV types in both cattle and sheep gives rise to heterotypic antibodies and protection it is also necessary to see if a similar response would occur after the simultaneous inoculation of three BTV types. This latter procedure is
Table 3e: Levels of viraemia following the inoculation of BTV type 6 into animals previously exposed to BTV types 3 and 4.

<table>
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<tbody>
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<td></td>
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</tr>
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<td>-</td>
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<tr>
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<tr>
<td>24</td>
<td>-</td>
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<tr>
<td>30</td>
<td>-</td>
</tr>
</tbody>
</table>

Animals A, B, C and D cattle previously exposed to BTV 4 and 3.

Animals E and F cattle not previously exposed to BTV.

<sup>a</sup> Animals inoculated intradermally with 10<sup>6</sup> TCID<sub>50</sub> BTV6.

<sup>b</sup> Viraemia expressed as TCID<sub>50</sub>/ml of washed blood.

- Samples negative for the presence of BTV following examination of blood in both roller tubes and embryonated eggs.
Table 3f: The development of neutralising antibodies to BTV types 4, 3 and 6 following a) the serial inoculation BTV 4 and 3:

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Day 0&lt;sup&gt;a&lt;/sup&gt;</th>
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<th>Day 14</th>
<th>Day 21</th>
<th>Day 49</th>
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<tbody>
<tr>
<td></td>
<td>Type</td>
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<td>B</td>
<td>C</td>
<td>D</td>
</tr>
<tr>
<td>BTV4</td>
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<td>1/240</td>
<td>1/80</td>
<td>1/320</td>
<td></td>
</tr>
<tr>
<td>BTV3</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td>1/40</td>
</tr>
<tr>
<td>BTV6</td>
<td>-</td>
<td>-</td>
<td></td>
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<td>1/120</td>
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Table 3f (contd.):

b) the serial inoculation of BTV4, 3 and 6.

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<td>1/80</td>
<td>1/112</td>
<td>1/112</td>
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<td>1/112</td>
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<td>1/112</td>
<td>1/80</td>
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<td>1/80</td>
<td>1/320</td>
<td>1/1280</td>
<td>1/160</td>
</tr>
</tbody>
</table>

Neutralising antibody titre expressed as reciprocal \(\log_{10}VN_{50}\)

a Days post inoculation with the appropriate BTV's types
b Serum collected from animals A, B, C and D following serial inoculation with two or three BTV types.
Fig. 3m: Level of neutralising antibodies to BTV at various times after challenge with BTV type 3 in a group of four cattle previously exposed to BTV type 4.

All titres calculated from geometric mean of reciprocal $\log_{10}^{VN_{50}}$ values and expressed as neutralising antibody titre. Sera with titres $>1/15$ scored as zero. Antibody levels in the control animals receiving BTV3 only are shown in hatching.
similar to that used in vaccination schedules in BTV endemic areas.

**Simultaneous inoculations: sheep**

f) **Inoculation of sheep simultaneously with BTV3, 4 and 6.**

a) **Clinical response:** These animals developed a pyrexia and viraemia which was similar to that obtained previously following the inoculation of a single BTV type into susceptible animals (Table 3b).

To determine which of the inoculated virus types might be present, virus neutralisation tests were carried out on virus samples collected on day 7 pi. A number of pools of BTV type specific antisera (Table 3g) were used to establish if one or several virus types were present. To enable comparisons between these pooled sera to be made and thus make it possible to deduce which of the three viruses inoculated might be present in the blood of these animals at day 7, box neutralisation tests were carried out for each serum pool. Virus neutralisation regression lines were calculated for each serum pool against the day 7 virus isolate that were defined by the values A and B in the equation; $y = A + Bx$, where the intercept A represents the log$_{10}$ decrease in virus titre produced by undiluted serum, and B is the neutralisation slope: (Westaway, 1965). 'Area functions', were calculated from the regression line analysis, where area $= -1/2 \frac{A^2}{B}$.
Table 3g: Neutralising activity, expressed as an area function of various antisera against virus(s) isolated on day 7 post inoculation of four animals with BTV types 4, 3 and 6 simultaneously.

<table>
<thead>
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<th>Animal Number</th>
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<th>41</th>
<th>42</th>
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<td>40</td>
<td>41</td>
<td>42</td>
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<tr>
<td>Antisera</td>
<td>area</td>
<td>norm</td>
<td>area</td>
<td>norm</td>
</tr>
<tr>
<td>BTV3,4 &amp; 6</td>
<td>17</td>
<td>100%</td>
<td>10</td>
<td>100%</td>
</tr>
<tr>
<td>BTV3 &amp; 4</td>
<td>16</td>
<td>94%</td>
<td>9.6</td>
<td>96%</td>
</tr>
<tr>
<td>BTV3 &amp; 6</td>
<td>10.2</td>
<td>60%</td>
<td>7.7</td>
<td>77%</td>
</tr>
<tr>
<td>BTV4 &amp; 6</td>
<td>15.3</td>
<td>90%</td>
<td>2.7</td>
<td>27%</td>
</tr>
<tr>
<td>BTV3</td>
<td>11.9</td>
<td>70%</td>
<td>8.5</td>
<td>85%</td>
</tr>
<tr>
<td>BTV6</td>
<td>1.4</td>
<td>8.2%</td>
<td>-ve</td>
<td>0%</td>
</tr>
</tbody>
</table>

*a* Area function derived from the regression line equation \( y=A+bx \) and \( area=-1/2A^2/B \). \( A \) represents the log₁₀ decrease in virus titre produced by undiluted serum and \( B \) is the neutralisation slope (Westaway 1965).

*b* The normalised value has been obtained by assigning a value of 100 to the area function of the antisera pool against all three inoculated viruses and adjusting the functions in the other sera by proportion. The coefficient of correlation of the linear regression curve (ie from which \( B \) is obtained) was always >0.9
In order to allow direct comparisons between different antiserum/virus mixtures, the areas under the curves were normalised with respect to the pooled antiserum to all the BTV types inoculated.

From the results it can be seen that little difference was obtained when using a pool containing antiserum to BTV types 3, 4 and 6 and a pool containing only antiserum to types 3 and 4. Further, antiserum to BTV6 alone caused little or no neutralisation, whilst antiserum to BTV3, or a pool of antiserum to BTV6 and 4, or BTV3 and 6 resulted in levels of over 50% of normalised values. This would indicate that in all four animals on day 7, blood contained BTV types 3 and 4 but not BTV type 6.

b) Antibody response:- All four animals developed BTV group specific precipitating antibodies on day 13 and retained this response for the rest of the experiment. The development of neutralising antibody to the three inoculated virus types can be seen in Fig. 3n. Antibodies to both BTV types 4 and 3 were present in all four animals by day 15 post inoculation. Mean titres to BTV4 and 3 reached levels of 1/100 and were maintained. Antibodies to BTV6 could not be detected in any animal following these inoculations. This would add further credence to the conclusion above, that BTV6 failed to replicate in these animals.

The sera from these animals were also examined for neutralising antibodies to the other 19 designated BTV types (Table 3o). Antibodies were detected to BTV types 15 and 17
Fig. 3n: Development of neutralising antibodies to BTV types 3, 4 and 6 following the simultaneous inoculation of these virus types into four sheep

- Neutralising antibodies to BTV3

- Neutralising antibodies to BTV4

- Neutralising antibodies to BTV6

Antibody titre calculated as geometric mean from four animals of reciprocal log$_{10}^{VN50}$
Fig. 3o: Level of neutralising antibodies to BTV types at various times after the simultaneous inoculation of four sheep with BTV types 3, 4 and 6.

All titres calculated from geometric mean of reciprocal $\log_{10} VN_{50}$ values and expressed as neutralising antibody titres. Sera with titres < 1/15 scored as zero.
prior to inoculation and following inoculation, to BTV types 1, 20 and 22. However group mean levels were not greater than 1/10 except to BTV22. In contrast to the serial inoculation of BTV types 4, 3 and 6, a broad heterotypic antibody response was not observed following the simultaneous inoculation of these three BTV types into sheep.

Discussion.

Throughout this series of experiments, the only clinical manifestation following the inoculation of BTV was a pyrexic response and viraemia. No experimental animal developed characteristic bluetongue lesions (Bowne, 1971) or became noticeably ill. It has been consistently noted that a variety of apparently virulent field isolates fail to produce characteristic signs of disease in laboratory sheep or cattle (Luedke and Jochim, 1968) thus, the effect of BTV on laboratory animals had to be measured in terms of pyrexia, viraemia and evaluation of the immune response.

The homologous challenge of sheep previously infected with BTV5 did not give rise to pyrexia or a detectable viraemia. Thus it appears that previous exposure to BTV5 had protected these animals. The antibody response following homologous challenge remained monotypic and similar protection and monotypic antibody responses have been shown in homologous virus challenge with BTV2, 3 and 4 (Jeggo and Gumm, unpublished observations).
On the other hand, the outcome of heterotypic challenge was different. Using a variety of different BTV types, previous exposure to a single BTV type in both sheep and cattle failed to protect against heterotypic challenge in that in all these cases pyrexia and viraemia levels were indistinguishable from controls. However after the serial inoculation of two BTV serotypes not only were antibodies produced to both types but also appreciable levels of antibodies to a number of other types (Figs. 3d, 3e, 3j, 3k, 3o and 3p).

In direct contrast to these clinical observations on the inoculation of one or two BTV types, no pyrexia or viraemia was detected after the serial inoculation of a third BTV type in all sheep or three out of four cattle examined. Following inoculation of this third BTV a similar broad heterotypic response occurred (Figs. 3f and 3k) and with sequentially inoculated animals showing a more rapid development of antibodies to this third challenge type than in control animals (Table 3a and 3b). This accelerated immune response (Roitt, 1980) demonstrates the likely existence of memory cells to this third BTV type. These cells would probably have been evoked when antibodies were stimulated following the second BTV administration. It is of interest however to note that, the accelerated antibody response to this third type, in this case BTV6, did not occur with any other type. Although the reason for this is not clear, it is likely that shared antigens among this particular combination of three types led to the establishment of these memory cells.

This particular feature might mean that it is misleading to
try to use these combinations of sequential BTV types as being typical of the response of all other possible combinations. However, it clearly shows that the serial administration of two or three different BTV types gives rise to a broad antibody response although with the 22 BTV types known at present, the possible sequential permutations are enormous and would require much additional work to quantify.

The heterotypic response obtained in this series of experiments, however, means that caution must be exercised in the evaluation of field serological surveys. The presence, in a field serum, of antibodies to more than one serotype means that firm conclusions cannot be drawn as to which virus types may be present in that country or area, as has been previously attempted (Davies 1978; Hafez and Ozawa, 1981; Hafez et al., 1976). Recent work in Australia has alluded to the possibility of cross-reactive antibodies (Della-Porta et al., 1983) and the results above may go some way to explaining their findings.

The usefulness of this broad heterotypic antibody response must rest on the role neutralising antibodies play in protection and recovery from a BTV challenge. These experiments indicate that at least two sequential inoculations are needed before protection is seen, but whether this protection was mediated by neutralising antibody is not so easily determined. The role of neutralising antibody in other infectious diseases has been well documented (Onions, 1983) but in virus diseases the position is not clear (Rouse and Babuiik, 1978; Askonas et al., 1981). With bluetongue,
protection has been demonstrated in the absence of detectable neutralising antibody but in the presence of a cell-mediated immune response (Stott et al., 1979). So, the protective role of the accelerated humoral response seen after the third virus inoculation needs further study.

Neitz (1948) reported a series of classic studies demonstrating the existence of antigenic plurality among bluetongue viruses and the majority of the virus strains used in his experiments were later allotted to serotypes by Howell (1960). Neitz (1948) clearly showed that all the virus types he used produced a solid immunity to challenge with the same virus type but, when challenged with a different virus type, two different effects could occur. Either the second virus established a clinical infection in the sheep similar to that seen in fully susceptible sheep or, the extent of the clinical response to the heterologous infection was much less severe, despite the fact that there was no alteration to either the length of the incubation period or height of the ensuing pyrexia in the previously exposed sheep. These observations enabled Neitz (1948) to formulate a concept of "common or basic" immunity between strains. This present study was only able to confirm that sheep resist homologous challenge but not to confirm the observation of "basic" immunity after two virus inoculations, as no difference could be detected in the reaction of British sheep after only one virus inoculation. The reasons for this discrepancy are not entirely clear although it is possible that the different breeds of sheep used may have been important as large breed differences have
been shown to occur following the inoculation of BTV (Gorman and Taylor, 1982). It may also be possible that the procedure adopted by Neitz for virus isolation purity may not have been as reliable as they are today, or finally it is possible that the combination of virus types chosen may be responsible for the differing observations.

Neitz (1948) also carried out experiments in which BTV3, 4 and 8 were inoculated into sheep in sequence and in different combinations. He found no increase in the "basic" immunity after the first inoculation and sheep were just as susceptible after infection with two virus types as with one type. From this he concluded that polyvalent immunity could only be produced by simultaneous inoculation of a number of different strains, a concept that has been fundamental to the use of live vaccines ever since. Again this study draws a completely different conclusion in that, within the limitations of the test system, it was shown that prior exposure to two virus types abrogates the viraemia and pyrexia following administration of a third BTV type. Again the same reasons described above could apply for this discrepancy.

More recently, Thomas and Trainer (1971), Della-Porta et al., (1981) and Gorman et al., (1981) have reported antigenic cross reactivity between virus types. Although studies on virus proteins giving rise to type specificity have been restricted by problems associated with virus purification (Huismans and Howell, 1973), Huismans and Erasmus (1981) and Gumm and Newman (1982) have shown that the outer capsid polypeptide P2 of is the main type specific antigen, whereas
the core polypeptide P7 is responsible for group specificity. Although the exact proteins responsible for the plurality of the neutralising response after sequential inoculations remains to be determined, the use of these purified proteins may help to define more clearly the serological relationships within the BTV group. It may be that sequential administration of type specific proteins instead of whole virus would result in only that type specific antibody being produced without extending the heterogenicity of the response. However the use of such viral subunits as vaccines would only be of use in areas in which one or two BTV types are to be expected, as broad heterotypic responses are highly desirable in areas where many BTV types exist.

The simultaneous inoculation in sheep of three BTV types gave rise to a viraemia and pyrexia indistinguishable from that following a single virus inoculation. Examination of the viraemia on day 7 post inoculation revealed only the presence of two BTV types and in direct contrast to the serial inoculation of BTV types, examination of the neutralising antibody response showed the development of type specific antibodies to only those two viruses and did not show the presence of a broad heterotypic antibody response. The reason for this may revolve around variations in the replication cycles of different BTV types and variations in their ability to induce other immune mechanisms. Work with live polyvalent virus vaccines, particularly in polio (Roca-Garcia et al. 1964), influenza (Askonas et al., 1981) and foot and mouth disease (Andrew, 1930) have shown that following this type of
immunisation protocol humoral immune responses occur to all inoculated viruses, although at differing levels. However no clear evidence exists demonstrating the replication of all inoculated viruses and evidence of interference both between homotypic and heterotypic polio viruses has been shown (Pohjanpelto and Cooper, 1965). As will be described later in this thesis, BTV induces cross-reactive CTL's and both the level of CTL's and susceptibility to the action of these cells varies with different serotypes. Further BTV induces high levels of interferon in vitro but appears to be resistant to its actions in vitro and this too may vary within serotypes. Hence it may be that BTV3 and 4 induce CTL's and/or interferon to which they are not as susceptible as BTV type 6.

Unfortunately time did not permit the further challenge of these animals although these results and those presented above would strongly argue against the present system of vaccination in countries such as South Africa, where live attenuated pentavalent vaccines are given three times at three week intervals (Howell 1979). Since the serial inoculation of only two BTV types gives rise to a broad heterotypic antibody response and heterotypic immunity whereas simultaneous inoculation did not, the vaccination procedure could be simplified in areas in which protection from a number of BTV types is required by using sequential inoculation of only two BT types.

However several key questions still remain. Firstly, to what extent do these observations occur throughout the BTV serotypes and which types might give the broadest level of
heterotypic immunity? Secondly, although the simultaneous inoculation of three BTV types resulted in the replication of only two of them with little or no heterotypic antibody production, would challenge of these animals produce an improved heterotypic response which was longer lasting than that following the single serial inoculation of virus? Thirdly, is the failure of replication of one of several viruses on simultaneous inoculation a phenomenon common to any virus combination or was it just a feature of this particular combination? Obviously further studies along the lines adopted in these experiments should provide this information and such work merits serious consideration in areas in which broad heterotypic protection is required.
CHAPTER FOUR

CELLULAR IMMUNITY; CYTOTOXIC T LYMPHOCYTE STUDIES

Introduction.

In the previous chapter the humoral response to the inoculation of BTV in sheep and cattle was described. It has recently been suggested that a cell-mediated immune (CMI) response is involved in protection from BTV re-infection and that sheep produce such a response on administration of an inactivated BTV vaccine preparation. (Stott et al., 1979). Hence in this chapter the ability of BTV to evoke a cellular immune response in mice and sheep is examined.

In other virus infections a number of T cell subsets involved in the CMI response have been investigated (Allison 1974, Bloom and Rager-Zisman, 1975) and attention has focussed on CTL's which have the ability to destroy virus infected cells early in infections and so prevent viral spread (Gilliland and Francis, 1974; Zinkernagel and Althage, 1977). Although this killing is defined by genetic and viral phenotype restrictions recent work has demonstrated that within some virus groups CTL's cause cross-reactive lysis (Rosenthal and Zinkernagel, 1980. Gajdowa et al., 1980; Webster and Askonas, 1980). Furthermore with influenza virus,
cross-reactive CTL's only occur following certain immunisation procedures (Webster and Askonas, 1980). Because of the technical problems associated with such work in large animals an initial investigation of CTL induction by BTV was studied in a mouse model with special reference to the ability of live and inactivated preparations to produce CTL's in vivo or on secondary stimulation in vitro. Demonstration of cross-reactive CTL's to different types of BTV and a role for them in protection from disease could lead to improved vaccine procedures. This possibility was investigated in mice using a number of BTV types.

Observations in the mouse model however, proved of such interest that an attempt to investigate ovine CTL's were carried out in ovines by examining target and effector cells from the same animal, thus avoiding problems associated with genetic restriction (Zinkernagel and Doherty, 1975).

Results

a) Induction of Cytotoxic T Lymphocytes in mice.

1) Primary Induction

Infection of C3H mice with BTV4 gave rise to the development of cytotoxic spleen cells which caused the maximum lysis of target L929 cells between 7 and 8 days post
infection, dropped to undetectable levels by 18-20 days after infection (Fig. 4a) and was dependent on the effector-to-target ratio (Table 4a). Uninfected cells were also lysed by spleen cells harvested between 2 and 7 days after infection but the level of lysis was always very much lower than that seen in infected cells (Fig. 4a). Although after 7 days lysis of uninfected cells was normally less than 2.5%, on some occasions higher levels did occur (Table 4a) and the reason for this variability is not known.

Similar levels of lysis were produced whether mice were infected with virus from mouse brain or from BHK cells (Table 4b) and so virus grown in BHK cells was used routinely. Unlike previous work (Oellerman et al., 1979), it was found (Table 4c) that BHK or mouse-adapted virus could be recovered from the heart or spleen of mice following intraperitoneal inoculation. The titres of virus reached a peak between 3 and 4 days after infection, the time at which CTL activity appeared (Fig 4a); by 8 days virus was no longer recovered. However, in spite of this replication, the development of CTL's was shown to depend on the immunising dose of virus given (Table 4b) although mice inoculated with virus inactivated with heat or BPL did not produce CTL's (Table 4b).

2) Secondary Induction.

Spleen cells from mice infected at least 14 days previously with live virus developed high levels of cytotoxicity upon secondary stimulation in vitro with either live or inactivated
Fig. 4a. Development of murine CTL's\(^a\) against bluetongue virus type 4 infected L929 cells.

- **- - -** Difference between infected and uninfected L929 cells
- **- - -** uninfected L929 cells
- **- - -** infected L929 cells

\(^a\) C\(_3\)H mice spleen cells. Mice immunised with 0.5 \(\times 10^6\) TCID\(_{50}\) BTV4. % specific lysis obtained using chromium release assay.
Table 4a. Primary induction of anti-bluetongue CTL using various effector to target cell ratios.

<table>
<thead>
<tr>
<th>Effector Target cell ratio</th>
<th>Infected (%)</th>
<th>Non-infected (%)</th>
<th>Difference (%)</th>
</tr>
</thead>
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<tr>
<td>100:1</td>
<td>43.6</td>
<td>13.3</td>
<td>30.3</td>
</tr>
<tr>
<td>50:1</td>
<td>28</td>
<td>8.3</td>
<td>19.7</td>
</tr>
<tr>
<td>25:1</td>
<td>16</td>
<td>7.4</td>
<td>8.6</td>
</tr>
</tbody>
</table>

*a* C₃H spleen cells immunised 7 days previously with 0.5x10⁶.8 TCID₅₀/ml BTV4

*b* L929 cells + BTV4

Table 4b. Primary induction of anti-bluetongue virus CTL using various effector cell populations.

<table>
<thead>
<tr>
<th>Effector cell population</th>
<th>L cells+BTV L cells alone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immune C₃H spleen cells</td>
<td></td>
</tr>
<tr>
<td>10⁶.8 BTV4 mouse brain b</td>
<td>35.3</td>
</tr>
<tr>
<td>10³.0 BTV4 BHK</td>
<td>35.7</td>
</tr>
<tr>
<td>10³.0 BTV4 BHK</td>
<td>9.6</td>
</tr>
<tr>
<td>10².0 BTV4 BHK</td>
<td>7.0</td>
</tr>
<tr>
<td>10⁶.8 BTV4 BPL inactivated</td>
<td>0.0</td>
</tr>
<tr>
<td>10⁶.8 BTV4 56°C 60 mins</td>
<td>0.0</td>
</tr>
</tbody>
</table>

*a* Mean of 6 assays; assay time 7 hours. Effector to target cell ratio 100:1. Standard error less than 6%

*b* Mice inoculated 7 days prior to assay with 0.5ml of virus. Minimum of 2 mice per experiment. Lysis expressed as a mean of the individual mouse results.
### Table 4c: Level of virus in tissues following inoculation of C3H mice with bluetongue virus type 4

<table>
<thead>
<tr>
<th>Days post inoculation</th>
<th>BTV4 BHK&lt;sup&gt;b&lt;/sup&gt;</th>
<th>BTV4 Mouse brain&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Heart&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Heart</td>
</tr>
<tr>
<td></td>
<td>Spleen&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Spleen</td>
</tr>
<tr>
<td>2</td>
<td>$10^2.1f$</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>$10^2.2$</td>
<td>$10^2.4$</td>
</tr>
<tr>
<td>4</td>
<td>$10^3.2$</td>
<td>$10^1.2$</td>
</tr>
<tr>
<td>5</td>
<td>$10^1.8$</td>
<td>$10^1.8$</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td>11</td>
<td>N.T</td>
<td>0</td>
</tr>
</tbody>
</table>

- **a** 0.5ml of virus injected intra-peritoneally. 2 mice per protocol
- **b** BTV4 passaged 6 times in BHK cells prior to inoculation
- **c** BTV4 passaged 3 times in suckling mice prior to inoculation
- **d** Whole heart ground up with sterile sand and diluted out using log dilutions in PBS+0.2 % BSA
- **e** Spleen cell suspension (containing approx. $1 \times 10^6$ spleen cells), diluted out as d.
- **f** Virus titrations: Expressed as TCID<sub>50</sub>/ml using 5 tubes per dilution

- **0** No virus detected
- **Trace** 1/5 tubes using undiluted heart/spleen
- **N.T.** Not tested
Table 4d. Secondary induction of cytotoxic T lymphocytes by in vitro stimulation of primed spleen cells

<table>
<thead>
<tr>
<th>Effector cells&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Target cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>In vivo stimulant</td>
<td>In vitro stimulant</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>BTV4</td>
<td>none</td>
</tr>
<tr>
<td>BTV4</td>
<td>inactivated BTV&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>BTV4</td>
<td>live BTV&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

inactivated

| BTV4 | live BTV | 25.7 | 6.3 | 19.4 | |

---

<sup>a</sup> Spleen cells removed from mice infected 14 days with BPL inactivated or live virus (10<sup>6.8</sup><sub>TCID<sub>50</sub></sub>) pooled preparations incubated in vitro for 5 days prior to the assay

<sup>b</sup> Carried out after 7 hours incubation of effector and target cells, 6 well duplicates. Standard error < 7%

<sup>c</sup> L cells + BTV4 25:1 effector to target cell ratio

<sup>d</sup> BTV4 inactivated for 1 hour at 56°C, added to an in vitro spleen cell culture 5 days prior to the assay

<sup>e</sup> BTV4 (10<sup>6.8</sup><sub>TCID<sub>50</sub></sub>) added to 15ml of spleen cell culture 5 days prior to assay
BTV4 (Table 4d). At an effector-to-target cell ratio of 25:1, cells stimulated by live virus showed a specific cytotoxicity of 64.8%, whereas the previous use in vivo of similar numbers of stimulation effector cells at this level had never produced lytic levels above 10% (Table 4b). Even though the numbers of effector cells in primary assays (Table 4b) were increased four-fold such levels of cytotoxicity were not matched. Spleen cells from mice inoculated with BPL-inactivated BTV4 and secondarily stimulated in vitro with live BTV4 also produced CTL's. Hence, although inactivated preparations do not produce primary CTL induction, they did produce specific BTV memory cells.

3) T Cell Dependence.

To substantiate the fact that the lysis observed in primary assays was mediated by T cells, procedures aimed at enriching T cell numbers in spleen cell suspensions were carried out. The passage of spleen cell suspensions over nylon wool columns led to an increase in lysis from 24.3% to 35.3% (Table 4e). Treatment of spleen cell suspensions with anti-thymocyte serum and complement completely prevented lysis of infected target cells (Table 4e). Following the use of anti-IgG serum, an increase in lysis occurred from 25.7% to 37.5% (Table 4e). This increase may reflect the removal of humoral mediated suppression of CMI (Hellstrom and Hellstrom, 1974. Lagrange et al., 1974) or an actual increase in the percentage of CTL's in the spleen cell suspensions. Taken together however, this
### Table 4e: Effects of various treatments on anti-blue tongue CTL populations

<table>
<thead>
<tr>
<th>Effector cell population</th>
<th>L cells + BTV</th>
<th>L cells</th>
<th>3T3B</th>
<th>L cells pseudorabies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-immune</td>
<td>0</td>
<td>0.7</td>
<td>0</td>
<td>0.2</td>
</tr>
<tr>
<td>Immune Balb c spleen</td>
<td>2</td>
<td>0</td>
<td>21.3</td>
<td>0.1</td>
</tr>
<tr>
<td>Immune C3H spleen cells</td>
<td>25.7</td>
<td>0.4</td>
<td>0.8</td>
<td>1.3</td>
</tr>
<tr>
<td>Nylon wool non-adherent</td>
<td>35.9</td>
<td>0.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antithymocyte 1.2 serum</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ complement</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti IgG+complement</td>
<td>37.4</td>
<td>0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complement</td>
<td>26.9</td>
<td>0.2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

---

*\(^a\) C\(^3\)H mice spleen cells. Immunized 7 days with 0.5 X 10^6 TCID\(_{50}\) BTV4*

*\(^b\) Means of six assays. Assay time 7 hours. Effector to target ratio 100:1 standard error less than 4%. Minimum of 2 mice per experiment. Lysis expressed as a mean of the individual mouse results

---

### Table 4f. Lysis by anti-blue tongue type 4 CTLs against various target cells

<table>
<thead>
<tr>
<th>Effector cell population</th>
<th>Target cell infection</th>
<th>Target cell (Infected targets - uninfected control cells)</th>
<th>% specific lysis(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immune C(^3)H</td>
<td>BTV4</td>
<td>L292</td>
<td>27.0</td>
</tr>
<tr>
<td>Immune C(^3)H</td>
<td>BTV3</td>
<td>L292</td>
<td>9.6</td>
</tr>
<tr>
<td>Immune B(\alpha)l b c</td>
<td>BTV4</td>
<td>3T3B</td>
<td>14.9</td>
</tr>
<tr>
<td>Immune B(\alpha)l b c</td>
<td>BTV2</td>
<td>3T3B</td>
<td>5.1</td>
</tr>
</tbody>
</table>

---

*\(^a\) Mice spleen cells inoculated 7 days previously with 0.5 X 10^6 of BTV4 intra-peritoneally. Minimum of 3 mice per experiment*

*\(^b\) 7 hour assay time. 100:1 effector to target cell ratio. Minimum of 2 mice per experiment. Lysis expressed as a mean of the individual mice results*
evidence strongly suggests that the observed lysis was T cell
mediated.

Although similar experiments were not carried out on
secondarily stimulated CTL's it is also assumed that these
cells are cytotoxic T cells and not in vitro generated NK
cells. Apart from direct evidence in other systems that this
is the case (Zweerink et al., 1977) mouse NK cell reactivity
has been shown to be relatively labile in culture and hence
after 5 days would not be expected to survive (Herberman and
Holden, 1978; Paige et al., 1978; Stutman et al., 1978).

4) Viral and Target Cell Specificity.

To demonstrate the fact that this lysis was genetically
restricted, a feature which discriminates between CTL and
natural killer cell (NK) activity, spleen cells from Balb C
and C3H immune spleen cells were used as effector cells
against non-histocompatible targets and no lysis occurred
(Table 4e).

To demonstrate virus specificity, L929 or 3T3B cells were
infected with pseudorabies virus and again no lysis occurred
(Table 4e).

Hence the characteristic viral and target cell specificity
noted in other murine T cell mediated anti-viral
cytotoxicities (Doherty et al., 1976; Zinkernagel and Welsh,
1976; Hapel et al., 1978; Lawman et al., 1980) were also seen
in the BTV assay.
However, L929 cells infected with BTV3 or 3T3B cells infected with BTV2 were susceptible to lysis by histocompatible BTV4 immunised mouse spleen cells, although the level of this activity was less than that observed in the homologous virus system (Table 4f).

b) Generation of Murine CTL's Following various Immunisation Procedures.

Experiments had already shown that mice inoculated with live BTV produced a CTL response. However a second inoculation of live virus 21 days later failed to increase the level of this lysis (Table 4g). BPL inactivated virus preparations, whether given in a single or double inoculation or with adjuvant, failed to induce lysis (Table 4g). After inactivation of whole virus infected cells with glutaraldehyde lysis was observed but only at a low levels (Table 4h). Two inoculations of a preparation of similarly inactivated cells without adjuvant resulted in the highest level of lysis, although this was considerably below that observed with live virus preparations. Adjuvanted preparations produced even lower levels of lysis and the reason for this remains to be elucidated.

The ability of live or BPL inactivated virus to induce memory cells is shown in Table 4i. Live virus induced memory cells were activated by homologous or heterologous bluetongue types to produce CTL's. The degree of lysis stimulated in secondary assays varied and depended on the virus type used. Inactivated preparations also induced memory cells which, on
Table 4g. Induction of CTL's in C₃H mice following inoculation with live and BPL inactivated bluetongue virus.

<table>
<thead>
<tr>
<th>Immunisation procedure</th>
<th>Infected</th>
<th>Uninfected</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 x Live virus</td>
<td>100</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>2 x live virus</td>
<td>92</td>
<td>4</td>
<td>88</td>
</tr>
<tr>
<td>1 x inactivated virus</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2 x inactivated virus</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1 x inactivated virus in F.I.C.A.</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2 x inactivated virus in F.I.C.A.</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1 x inactivated virus in F.C.A.</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2 x inactivated virus in F.C.A.</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

a C₃H mice inoculated 0.5ml I/P either once and tested 7 days later, or twice, the two inoculations separated by 2 weeks and then tested 7 days later.

b Effector cells—C₃H mice spleen cells. Target cells—L cells infected 24 hours prior to the assay with BTV4 and labelled with (⁵¹Cr.) 7 hour release assay. 100:1 effector to target cell ratio.

c BTV4 10⁶.8 TCID₅₀

d inactivated by BPL (30 mins. at 37°C, 18 hours at 4°C, 0.3% BPL)
e Freunds incomplete adjuvant
f Freunds complete adjuvant

d inactivated by 0.15% glutaraldehyde (35 mins. at 4°C)

e and f as Table 7

Table 4h. Induction of CTL's in C₃H mice following inoculation with live and glutaraldehyde inactivated bluetongue virus infected cells.

<table>
<thead>
<tr>
<th>Immunisation procedure</th>
<th>infected</th>
<th>uninfected</th>
<th>difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 X live virus</td>
<td>100</td>
<td>1.1</td>
<td>98.9</td>
</tr>
<tr>
<td>2 X live virus</td>
<td>97</td>
<td>7</td>
<td>90</td>
</tr>
<tr>
<td>1 x inactivated virus</td>
<td>28</td>
<td>6</td>
<td>22</td>
</tr>
<tr>
<td>2 x inactivated virus</td>
<td>59</td>
<td>9</td>
<td>50</td>
</tr>
<tr>
<td>1 x inactivated + F.I.C.A.</td>
<td>5</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>2 x inactivated + F.I.C.A.</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1 x inactivated + F.C.A.</td>
<td>24</td>
<td>14.7</td>
<td>9.3</td>
</tr>
<tr>
<td>2 x inactivated + F.C.A.</td>
<td>35.7</td>
<td>13.9</td>
<td>18.2</td>
</tr>
</tbody>
</table>

a, b, and c as Table 7

d inactivated by 0.15% glutaraldehyde (35 mins. at 4°C)
e and f as Table 7
Table 4i. Production of specific CTL's by secondary \textit{in vitro} stimulation of spleen cells from C3H mice after various immunisation procedures.

<table>
<thead>
<tr>
<th>Primary \textit{in vitro} stimulant</th>
<th>secondary \textit{in vitro} stimulant</th>
<th>\textsuperscript{51}Cr release assay\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>infected targets</td>
<td>uninfected targets</td>
</tr>
<tr>
<td>Live BTV4\textsuperscript{d}</td>
<td>live BTV4</td>
<td>55.8g</td>
</tr>
<tr>
<td>&quot; &quot;</td>
<td>live BTV10</td>
<td>63.8</td>
</tr>
<tr>
<td>&quot; &quot;</td>
<td>live BTV3</td>
<td>54.5</td>
</tr>
<tr>
<td>&quot; &quot;</td>
<td>inact. BTV4</td>
<td>49.6</td>
</tr>
<tr>
<td>&quot; &quot;</td>
<td>none</td>
<td>38</td>
</tr>
<tr>
<td>BPL inact. BTV4\textsuperscript{e}</td>
<td>live BTV4</td>
<td>15.3</td>
</tr>
<tr>
<td>&quot; &quot;</td>
<td>live BTV10</td>
<td>27.3</td>
</tr>
<tr>
<td>&quot; &quot;</td>
<td>live BTV3</td>
<td>20.7</td>
</tr>
<tr>
<td>&quot; &quot;</td>
<td>inact. BTV4</td>
<td>25</td>
</tr>
<tr>
<td>&quot; &quot;</td>
<td>none</td>
<td>16.8</td>
</tr>
<tr>
<td>Glutaraldehyde inactivated BTV4\textsuperscript{f}</td>
<td>live BTV4</td>
<td>11</td>
</tr>
<tr>
<td>&quot; &quot;</td>
<td>inact. BTV4</td>
<td>7.1</td>
</tr>
<tr>
<td>&quot; &quot;</td>
<td>none</td>
<td>5.1</td>
</tr>
</tbody>
</table>

\textsuperscript{a} 7 hour \textsuperscript{51}Cr release assay. Effector to target cell ratio of 25:1. Target cells-L929. Cells infected 24 hours previously with BTV4. Effector cells-5 day old \textit{in vitro} grown C\textsubscript{3}H mice spleen cells

\textsuperscript{b} C\textsubscript{3}H mice injected with 0.5ml I/P of preparation. Killed 7 days later and spleen cells harvested and maintained on RPMI in falcon flasks

\textsuperscript{c} Stimulant added on day 1 to \textit{in vitro} cultured spleen cells

\textsuperscript{d} \textsuperscript{10} \textsuperscript{6} TCID\textsubscript{50} of live BTV4

\textsuperscript{e} 0.3\% BPL, 30 minutes at 37\degree C overnight at 4\degree C

\textsuperscript{f} 0.15\% gluteraldehyde, 35 minutes at 4\degree C

\textsuperscript{g} % specific lysis. Mean value of at least two seperate experiments. S.D. less than 7%
secondary *in vitro* stimulation, were able to effect specific lysis (Table 4i). However, as was observed with the primary stimulation, the level of lysis was much lower than that caused by live virus preparations.

The antibody response as measured by the group specific ELISA test, following administration of live and inactivated virus is shown in Fig 4b. Clearly, two inoculations of live virus were most effective in inducing a high antibody response while inactivated virus without adjuvant was least effective.

c) **Generation of Cross-reactive Murine Cytotoxic T Lymphocytes.**

Reciprocal mouse and target priming with six different types of live BTV revealed a pattern of complete and variable cross-reactivity (Table 4j). Non-immunised mice and uninfected target cells exhibited low levels of specific lysis. Although a varied cross-reactivity occurred, the following points emerge: (i) there was no clear preference for the homologous interaction; (ii) certain BTV type raised effector cell populations which were better able to lyse the six BTV type infected target cells. In particular those produced by BTV types 1, 6 and 10; (iii) certain BTV type infected targets were more readily lysed by the various effector cells, e.g. BTV types 1 and 10. (iv) BTV16 induced effector cells and infected target cells gave low levels of specific lysis against the other five types examined.
Fig. 4b. Measurement of the serum antibodies produced in C₃H mice following the intraperitoneal injection of various bluetongue virus preparations, using the group specific ELISA test. (Pooled sera from two mice/preparation). Optical density values are minus negative control values.

X 1 - single intraperitoneal injection of 0.5ml of preparation.
X 2 - double intraperitoneal injection of 0.5ml of preparation.
sera collected 10 days after final injection.

Live - BTV4 4 × 10⁷.⁴ TCID₅₀

Inact. - BTV4 inactivated by 0.3% BPL for 30 mins. at 37°C and overnight at 4°C.
F.C.A. inact. - inactivated as above and emulsified with an equal volume of Freunds complete adjuvant.
F.I.C.A. inact. - inactivated as above and emulsified with an equal volume of Freunds incomplete adjuvant.
Table 4j. Percent specific lysis of various bluetongue virus type induced CTL's\(^a\) against various bluetongue virus type infected target cells\(^b\)

<table>
<thead>
<tr>
<th>Effector cell types</th>
<th>Target cell types</th>
<th>Mean % lysis across 6 uninfec. targets</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>One</td>
<td>three</td>
</tr>
<tr>
<td>One</td>
<td>33(^c)</td>
<td>23</td>
</tr>
<tr>
<td>Three</td>
<td>14</td>
<td>9</td>
</tr>
<tr>
<td>Four</td>
<td>19</td>
<td>15</td>
</tr>
<tr>
<td>Six</td>
<td>31</td>
<td>19</td>
</tr>
<tr>
<td>Ten</td>
<td>29</td>
<td>20</td>
</tr>
<tr>
<td>Sixteen</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>Control(^d)</td>
<td>1</td>
<td>5</td>
</tr>
</tbody>
</table>

\(^a\) C\(_3\)H mice spleen cells. C\(_3\)H mice inoculated 7 days previously with 10\(^6\) TCID\(_{50}\) BTV intraperitoneally

\(^b\) L929 cells Infected 24 hours previously with 5 x 10\(^6\) TCID\(_{50}\) of BTV

\(^c\) % specific lysis. 7 hour assay. 100:1 effector to target cell ratio. Minimum of 3 separate assays, 2 mice per assay. Standard error less than 7%

\(^d\) Uninfected C\(_3\)H mice spleen cells
1) **In vitro secondary stimulation.**

In *vitro* secondary stimulation of BTV4 primed spleen cells with BTV types 4, 10, and 3 gave rise to specific lysis (Table 4k) against BTV4 infected targets. Secondary stimulation with BTV type 10 and 4 gave similar levels of lysis against BTV4 targets, whilst BTV3 gave a lower but significant degree of lysis (Table 4l). In *vitro* secondary stimulation of BTV4 primed spleen cells with BTV4 produced CTL's which lysed BTV type 10 and 3 infected target cells (Table 4l), although again a lower level of lysis occurred against BTV3 infected targets whilst BTV10 infected targets gave the highest level of lysis. Although secondary stimulation gave rise to cell populations which produced levels of lysis approximately twice those seen in primary assays, the ratio of the same target/effector cell combination was similar, e.g. primary assays of BTV4 effectors on BTV4 infected targets gave 20% specific lysis (SL) (Table 4k), BTV4 memory cells stimulated with BTV4 and tested on BTV4 infected targets gave 36.7% SL (Table 4l) ratio 1:1.84: BTV4 effectors on BTV10 infected targets gave 28% SL (Table 4k), BTV4 memory cells stimulated with BTV4 and tested on BTV 10 infected targets gave 53% SL (Table 41) ratio 1:1.89. Thus, secondary stimulation in *vitro* of BTV4 memory cells with various BTV types produces CTL's which will lyse various BTV types infected targets and the pattern of this cross-reactivity is similar to that found in the primary in *vitro* stimulation assays.
stimulation with various bluetongue virus types\textsuperscript{a} of bluetongue type 4 memory cells\textsuperscript{b}.

<table>
<thead>
<tr>
<th>In vitro stimulant of memory cells</th>
<th>target cells\textsuperscript{c}</th>
</tr>
</thead>
<tbody>
<tr>
<td>BTV4 (10\textsuperscript{6.8} TCI50\textsubscript{50})</td>
<td>44.3\textsuperscript{d}</td>
</tr>
<tr>
<td>BTV4 (10\textsuperscript{8} TCI50\textsubscript{50})</td>
<td>43.3</td>
</tr>
<tr>
<td>BTV10 (10\textsuperscript{6} TCI50\textsubscript{50})</td>
<td>44.4</td>
</tr>
<tr>
<td>BTV3 (10\textsuperscript{6} TCI50\textsubscript{50})</td>
<td>17.9</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Effector cells stimulated and held for 5 days in upright falcon flasks prior to the assay. 25:1 effector to target cell ratio. 7 hour assay at 37\textdegree C.

\textsuperscript{b} C\textsubscript{3}H mice spleen cells. Mice inoculated 3 weeks previously with 10\textsuperscript{6.8} TCI50\textsubscript{50} given intraperitoneally.

\textsuperscript{c} L929 cells infected with BTV4.

\textsuperscript{d} % specific lysis difference between infected and uninfected L929 cell values.

Table 41. Percent specific lysis against various bluetongue virus infected target cells following bluetongue virus in vitro stimulation of BTV4 memory cells.

<table>
<thead>
<tr>
<th>In vitro stimulant of memory cells\textsuperscript{a}</th>
<th>Target cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>BTV4 \textsuperscript{b}</td>
<td>BTV3</td>
</tr>
<tr>
<td>10\textsuperscript{6} TCI50\textsubscript{50} BTV4</td>
<td>36.7\textsuperscript{b}</td>
</tr>
<tr>
<td>none</td>
<td>8.8</td>
</tr>
</tbody>
</table>

\textsuperscript{a} C\textsubscript{3}H mice spleen cells. Mice inoculated 3 weeks earlier with 10\textsuperscript{6.8} TCI50\textsubscript{50} BTV4 given intraperitoneally.

\textsuperscript{b} Percent specific lysis. 7 hour assay at 37\textdegree C. 25:1 effector to target cell ratio.
2) **Double in vivo immunisation procedures.**

Following the immunisation of C$_3$H mice with two inoculations of BTV, the CTL population and antibodies induced were examined. Mice responded to BTV4 immunisation by producing specific antibody; one inoculation produced sera which gave optical density readings on an ELISA of 0.4 which increased to 0.85 after two inoculations.

CTL production in mice initially immunised with BTV4 and inoculated 2 weeks later with either BTV4, BTV10 or pseudorabies virus caused only low levels of lysis against L929 cells infected with BTV types 4, 10 or 16. However, BTV4 immunised mice inoculated with BTV16, evoked CTL's which caused higher levels of lysis against all three BTV types infected targets (Table 4m). This lysis is all the more significant when compared with that induced by a single inoculation of BTV16 (Table 4n).

A probable explanation for these results is that BTV4 immunised mice will produce antibodies which will neutralise both the homologous type and type 10 as it is known that some degree of antibody cross-neutralisation occurs between types 4 and 10 (B.J. Erasmus, personal communication). This neutralisation could then affect the ability of virus to induce further CTL production. However, following BTV16 inoculation, no neutralisation occurs and an enhanced CTL response follows. A similar explanation has been used to explain analogous experiments with the influenza type A viruses (Effros et al., 1977) and VSV (Rosenthal and
Table 4m. Percent specific lysis of various bluetongue virus type infected targets by CTL's induce following inoculation of BTV4 primed mice with various BTV types \textit{in vivo}.

<table>
<thead>
<tr>
<th>Effector cells\textsuperscript{a}</th>
<th>target cells\textsuperscript{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Second in vivo inoculation</td>
<td>BTV4</td>
</tr>
<tr>
<td>BTV4</td>
<td>9\textsuperscript{c}</td>
</tr>
<tr>
<td>BTV10</td>
<td>8</td>
</tr>
<tr>
<td>BTV16</td>
<td>23</td>
</tr>
<tr>
<td>Pseudorabies virus</td>
<td>7.5</td>
</tr>
</tbody>
</table>

\textsuperscript{a} C\textsubscript{3}H mice spleen cells. Mice inoculated 2 weeks prior to second \textit{in vivo} inoculation with 10\textsuperscript{6.8} TCID\textsubscript{50} BTV4 given intraperitoneally. Second inoculation 10\textsuperscript{6} TCID\textsubscript{50} of virus intraperitoneally.

\textsuperscript{b} L929 cells infected with various BTV types.

\textsuperscript{c} Percent specific lysis following 7 hour \textsuperscript{51}Cr release assay. 100:1 effector to target cell ratio.

---

Table 4n. Percent specific lysis of BTV1 and BTV16 induced CTL's against various orbivirus infected target cells.

<table>
<thead>
<tr>
<th>Effector cells\textsuperscript{a}</th>
<th>BTV1</th>
<th>BTV16</th>
<th>target cells\textsuperscript{b}</th>
<th>Ibaraki</th>
<th>E.H.D.\textsuperscript{c}</th>
<th>Corriparta</th>
</tr>
</thead>
<tbody>
<tr>
<td>BTV1</td>
<td>24\textsuperscript{d}</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>BTV16</td>
<td>11</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

\textsuperscript{a} C\textsubscript{3}H mice spleen cells. Mice immunised 7 days previously with 10\textsuperscript{6.8} TCID\textsubscript{50} BTV type 1 or 16.

\textsuperscript{b} L929 cells infected with 5 \times 10\textsuperscript{6} TCID\textsubscript{50} of the appropriate virus. 7 hour \textsuperscript{51}Cr release assay. 100:1 effector to target cell ratio.

\textsuperscript{c} Epizootic Haemorrhagic Disease virus.

\textsuperscript{d} \% specific lysis difference between infected and uninfected L cell values.
3) 'Cold target' competitive inhibition assay.

Relationships between BTV types 10, 16 and 6 were examined by interposing unlabelled virus infected L929 cells between effectors and targets (so-called 'cold target' competition assays). Optimum results were obtained at a ratio of cold targets to $^{51}$Cr labelled targets of 8:1 (Fig 4c). In the case of BTV10 evoked effector cells, BTV10 infected cold targets successfully inhibited homologous lysis, whereas type 6 cold targets caused less inhibition. If CTL subsets are produced against group and type antigens homologous cold targets will compete against both causing the maximum inhibition. Hence when using BTV 10 cold targets, BTV10 effectors may have recognised both group and type antigens and these targets caused the greatest degree of inhibition. Using BTV6 cold targets with BTV10 effectors, a reduced effect occurred whilst BTV16 cold targets had little or no effect. Hence when using BTV6 cold targets the BTV10 effector cells only recognised group antigens on these cold targets and a smaller degree of inhibition of labeled targets occurred. The low activity of BTV16 cold targets in this assay reflects similar observations in the primary assay (Table 4j) where BTV16 only evoked low levels of CTL's and where type 16 infected targets show low levels of specific lysis in the presence of heterologous effectors. In the homologous BTV6 test, however, where BTV6 cold targets might be expected to
Fig. 4c. Inhibition of immune spleen cell effectors (100:1) in a 7 hour assay using different ratios of cold, unlabelled competitor cells. The competitors were normal L929 cells, or L 929 cells infected with bluetongue virus types 10,6 or 16. Both cold and labelled L 929 cells were added to the effector cells at the same time.

- Cold targets BTV10
- Cold targets BTV6
- Cold targets BTV16
- Uninfected targets

Level of lysis (X) with no cold targets shown on vertical axis.
cause the greatest inhibition, type 10 targets again resulted in the highest level of inhibition. These results further demonstrate the cross-reactive nature of BTV evoked CTL's and again reflect the ability of certain BTV types to cause more cross-reactions than other types.

4) Relation with other orbiviruses.

Ibaraki, Epizootic Haemorrhagic Disease (EHD) and Corriparta virus infected L929 cells were not lysed by either BTV1 or BTV16 induce CTL's (Table 4n). Serologically EHD and Ibaraki are considered closely related to BTV (Della-Porta et al., 1979). The lack of cross-reaction between these viruses again indicates that the antigens concerned with protective serological and CMI responses are different and that the cross-reactive CTL-evoking antigen which is present in BTV's is different from that present on other orbiviruses.

d) Induction of Ovine Cytotoxic Lymphocytes.

The development of ovine anti-BTV cytotoxic cells following the intradermal inoculation of BTV into sheep can be seen in Fig. 4d. Only low levels of lysis occurred prior to day 9 p.i. of BTV, with peak levels occurring around day 15 p.i. and a rapid decline 3 to 4 days later. Lysis against uninfected targets was consistently low (mean value over 22 days in four animals 4.3±6.6). PBL's from an animal not inoculated with BTV
Fig. 4d. Development of ovine CTL's against bluetongue virus infected testis cells.

* - * Difference between infected and uninfected targets.

*-------* Uninfected testis cells.

·---------· Infected testis cells.

a Sheep PBL's collected at various days post inoculation with 10^6 TCID_{50} BTV4. % specific lysis obtained using chromium release assay. Minimum of 6 wells/sample. Results mean of 4 sheep.

b Testis cells derived from the same animal as effector PBL population. Infected 24 hours prior to assay with BTV4.
failed to lyse BTV infected target cells. The effects of various treatments on these cells can be seen in Table 4a. Passage of these cells over nylon wool increased levels of lysis, whilst treatment with anti-ovine IgG serum with or without complement had little effect. However since anti-ovine thymocyte serum was not available it was not possible to confirm that the lytic activity was T cell-mediated. Immune PBL's from two animals using allogeneic targets failed to give rise to lysis. This indicates the possibility that lysis in these animals was MHC restricted and hence suggests that it was T cell-mediated (Zinkernagel and Doherty, 1975).

Discussion

The characteristics of the cytotoxicity response of mice to BTV4 infection provides evidence that it is T cell-mediated and conforms to the pattern of virus and genetic restriction described for many other murine systems (Doherty et al., 1976; Hapel et al., 1978; Lawman et al., 1980; Quinlan et al., 1978; Blanden et al., 1976). However at two to five days after infection, spleen cell suspensions caused significant lysis of non-infected target cells (Fig. 4a). The early appearance of this activity, the fact that it was only observed in spleen cell suspensions from virus infected mice and that it was also observed against non-infected targets argues that this part of
Table 4o. Effects of various treatments on ovine anti-bluetongue virus CTL populations.

<table>
<thead>
<tr>
<th>Effector cell</th>
<th>% specific lysis&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>testis cells</td>
</tr>
<tr>
<td>(infected BTV) (uninfected BTV)</td>
<td></td>
</tr>
<tr>
<td>Non-immune PBL's&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0</td>
</tr>
<tr>
<td>Immune PBL's&lt;sup&gt;c&lt;/sup&gt;</td>
<td>42</td>
</tr>
<tr>
<td>Nylon wool non-adherent</td>
<td>57</td>
</tr>
<tr>
<td>PBL's + anti-ovine IgG + complement</td>
<td>42</td>
</tr>
<tr>
<td>PBL's + complement</td>
<td>49</td>
</tr>
<tr>
<td>Unrelated immune PBL's&lt;sup&gt;d&lt;/sup&gt;</td>
<td>5</td>
</tr>
<tr>
<td>Unrelated immune PBL's</td>
<td>8.7</td>
</tr>
</tbody>
</table>

<sup>a</sup> Means of 6 assays. 7 hour <sup>51</sup>Cr release assay. Effector to target cell ratio 100:1. Standard error less than 6%

<sup>b</sup> Derived from same animal as target cell prior to inoculation with BTV

<sup>c</sup> Derived from same animal as target cell. Collected 15 days post-inoculation with 10<sup>6</sup> TCID<sub>50</sub> BTV

<sup>d</sup> Derived from different animal from which the target cells were obtained but similarly inoculated with BTV
the activity was due to natural killer cell activity (Rodda and White, 1978; Welsh, 1978; Welsh, 1981). BTV is known to be highly effective at inducing interferon (Rinaldo et al., 1973) and this acts as a powerful augmentor of NK cell activity (Trinchieri and Santoli, 1978; Gidlund et al., 1978).

The maximum lysis observed by the CTL's was considerably lower than that described for some other systems and it is possible that some regulating event occurs in vivo to curtail lytic activity as has been observed in mice infected with herpes simplex virus (Lawman et al., 1980).

It was not possible, however, to demonstrate primary CTL induction with inactivated preparations (Tables 4a and 4g) even though the preparations were immunogenic as shown by the induction of antibody (Fig. 4b). This is important at two levels. Firstly there are considerable differences in the ability of various live and inactivated viruses to produce a CMI response (Braciale and Yap, 1978; Ertyl et al., 1977; Kirschner et al., 1978). Thus, live and inactivated vaccinia (Hapel et al., 1978) and Sendai virus (Schrader and Edelman 1977) preparations are equally effective in promoting a CTL response, whereas some authors (Braciale and Yap, 1978; Lawman et al., 1980) have shown that inactivated herpes and influenza virus fail to elicit CTL production. It has been argued (Hapel et al., 1978) that some inactivated viruses do not fuse into the membrane of stimulator cells to produce the necessary interaction with antigens of the Ir gene to elicit CTL production. Work with herpes simplex virus (Rouse and Lawman, 1980; Schmid and Rouse, 1983) has investigated this facet of
CTL production and emphasised the importance of antigen triggering of helper cells to produce a factor which acts on precursor cells. BTV infected fixed cells preparations did induce a primary response which was improved by a second inoculation. It may well be that BTV presentation in this system is adequate and hence even though the preparation is inactivated CTL production ensues.

Secondly, it has been shown in sheep, using adjuvanted inactivated BTV vaccines (Stott et al., 1979), that such preparations can mediated protection against infection, apparently by stimulating a CMI response, as measured by a lymphocyte transformation test. Although it was not possible in mice, to demonstrate primary CTL production by inactivated virus (Table 4a and 4g) BTV inactivated preparations did induce memory T cell production which on in vitro culture gave large numbers of CTL's (Tables 4d and 4i). If this situation could be shown with BTV in sheep, then rapid CTL production upon challenge could limit virus multiplication and clinical signs and be the mechanism by which inactivated vaccines protect sheep against live virus challenge (Stott et al., 1979).

Evidence that the presentation of virus or induced antigens are responsible for the induction of an antibody or CTL response highlights the need for improved purified BTV preparations so that the proteins responsible for the immune effects can be identified and used to produce improved vaccines. However if CTL production is found to have clinical significance and inactivated vaccines to be a necessity then
fixed cell preparations appear to be the only vaccines worth pursuing.

Turning now to the specificity of recognition of the BTV evoked CTL's, the results show that no clear preference exists for the homologous type (Table 4j). In contrast to the lack of discrimination on the part of the T-cell response on the inoculation of one BTV type, the humoral response to BTV appears to be type specific (Howell, 1963) and until recently (Stott et al., 1979) the production of antibodies either by attenuated or killed BTV vaccines was the criterion by which such vaccines were assessed. A similar situation had existed with influenza virus, but here the discovery of cross-reactive T cells (Effros et al., 1977; Zweerink et al., 1977) followed by functional assays which have shown their importance in heterotypic challenge (Webster and Askonas, 1980), has meant that vaccine procedures can now be functionally assessed in terms of both humoral and cell-mediated immunity (Webster and Askonas 1980). Apart from these two viruses, cross-reactive T cells have also been described for vesicular stomatitis virus (Rosenthal and Zinkernagel, 1980) and flaviviruses (Gajdowa et al., 1980) and the possibility exists that this phenomenon may play a role in heterotypic immunity in other virus groups.

Although previous work has suggested that the antigens recognised by both T and B cells are similar (Binz and Wigzell, 1975) the results with the BTV types (Table 4j) and between other orbiviruses (Table 4n) together with the influenza and vesicular stomatitis virus work suggest that distinct and different antigens are being recognised by the
humoral and cellular immune systems. This particular facet of influenza immunology has received much attention (Zweerink et al., 1977; Effros et al., 1977) and a number of different explanations have been argued. With the advent of monoclonal antibodies the immunodominance of the influenza haemagglutinin molecule for antibody responses and its strict type response is in no doubt. Although there is evidence that T cells may also recognise part of the haemagglutinin molecule (Askonas and Webster, 1980; Koszinowski et al., 1980; Braciale et al., 1981), it has been suggested that the internal RNP and M protein may account for this cross-reactivity (Biddison et al., 1977; Reiss and Schulman, 1980). With BTV, such elegant analysis of purified viral proteins has not been done, although sites on type-specific proteins 2 and 5 and the group protein 7 (Huismans and Howell, 1973) are possible candidates for T-cell recognition.

Examining the functional importance of these cells the results in mice are similar to parallel studies with influenza virus where data has accumulated indicating the importance of cell-mediated immunity (Larson et al., 1978) in protection in both man and mice (Webster and Askonas, 1980). The fact that BTV is not lethal for mice, including nude and irradiated animals (unpublished observations) and that viraemias are of low levels and short durations (Table 4c) makes the assessment of the functional importance of CTL's in BTV infected mice difficult. If however, functionally important cross-reactive ovine CTL's are shown to exist then this would have a number of implications on vaccine policy. Firstly, live
virus or inactivated virus preparations on cell membranes appears to be a prerequisite for a primary CTL response.

Secondly, it is apparent that with the types tested certain virus types are more effective at inducing CTL's than others and thus if a broad heterotypic immunity is advantageous then these should have priority as vaccine types. Thirdly, multiple vaccinations may be contra-indicated as neutralising antibody appears to decrease the cross-reactive response. It is also of interest to note that the original protection work of Neitz in the 1940's, which showed far fewer groups than subsequent in vitro neutralisation tests (Howell, 1963), i.e. more cross-protection between isolates, might well reflect the importance of this heterotypic response and not "be merely a demonstration of the fallibility of cross-protection tests" (Howell 1970).

As has been indicated the full value of observations in the murine system rest on the demonstration of similarly functional CTL's in sheep. However problems of genetic restriction in ovines (Van Dam 1981) and suitable T cell markers (Higgins 1981) limit the degree to which ovine CTL's can be characterised. The use of target cells derived from the same animal as effector cells in functional CTL assays avoids the problems associated with MHC restriction and cytolysis. From the results it appears that sheep on inoculation of BTV produce cells capable of lysing infected targets (Fig. 4d). Further it would seem that these cells will not lyse non-histocompatible targets and are not functional dependent on the presence of antibody (Table 4o). These results would support
the notion that the lysis is neither due to natural killer cells, whose activity is not genetically or virologically restricted, or to killer cells whose activity is dependent on the presence of specific anti-BTV antibody. Unfortunately it was not possible to investigate the viral specificity of this ovine cell-mediated lysis due to lack of suitable numbers of target cells.

To confirm that this lysis is carried out by specific cytotoxic T cells would require the use of anti ovine cell markers. However, at present these are not available. Although peanut agglutinin (Arachis hypogea, Sigma Chemicals Ltd.) has been described as a T cell marker (Reisner et al., 1979) in humans it does not seem to distinguish between T cell subsets (Newman and Delia 1983) and no data exists on its usefulness in ovines.

Thus although not all the characteristics of the murine CTL (Zinkernagel and Doherty, 1975; Zinkernagel and Welsh, 1976) have been described for this ovine cell which mediates lysis it would appear that it probably belongs to the cytotoxic T cell subset. If this is indeed the case several interesting points can be made. Firstly, the activity of ovine CTL's was detectable initially around day 8 pi of virus. This corresponds with a major fall in the level of viraemia and the return of temperatures to normal (chapter 3, Fig. 3a). The picture is similar in mice with the reduction in viraemia in these animals (Table 4c) occurring at the same time as the detection of anti-BTV CTL activity. Thus it is possible that in a primary BTV infection CTL's act at this early stage to
bring about recovery from a BTV infection. However in both species antibody as measured by the in vitro neutralisation test is also first detected within 2 days of this abrogation of the pyrexia and drop in viraemia (chapter 3 Fig. 3c); Ollerman et al., 1976). It is not therefore feasible at this stage to define the precise role of either neutralising antibody or CTL activity in recovery from a primary BTV infection.

Secondly, memory cells will have been created at the same time as primary ovine CTL induction and thus on subsequent BTV challenge, these memory cells could rapidly proliferate to give high numbers of specific CTL's early on in this infection and it could be this process that is responsible for protection observed in homologous virus challenge (Chapter 3 exp. (a)). Thirdly, if, as in the murine system a proportion of the BTV induced CTL's are cross-reactive, a challenge with a second BTV type would increase further the number of these cells and hence the ability of sheep to withstand a third BTV challenge.
CHAPTER FIVE

OTHER IMMUNE MECHANISMS; ANTIBODY DEPENDENT CELL-MEDIATED
CYTOTOXICITY AND INTERFERON

a) Antibody Dependent Cell-mediated cytotoxicity (ADCC)

Introduction

ADCC occurs when an effector cell, equipped with an Fc receptor binds through an antibody molecule to virus antigen on infected cells (MacLennan, 1972). Although the first in vitro demonstration of ADCC was reported ten years ago (Bloom and Rager-Zisman, 1975) and as an in vitro phenomenon described to occur with a great number of different virus infected cells it is only recently that an in vivo role for ADCC has been clearly shown using adoptive transfer of xenogeneic cells in mice (Kohl and Loo, 1982).

This form of cytotoxicity is exquisitely sensitive in vitro and requires only very low levels of antibody, often one or more orders of magnitude less than that required to mediate antibody complement lysis or virus neutralisation (Shore et al., 1974). Consequently ADCC is important especially early in recovery when levels of antibody are low (Rouse and Babiuk, 1978).
Complement has been shown to enhance ADCC (Rouse et al., 1977) and this is particularly effective at limiting conditions, such as low effector to target cell ratios, low antibody concentrations and short term assays (Rouse et al., 1977). Furthermore, IgM antibody, the earliest form of antibody to appear after infection but which cannot mediate ADCC, can participate in ADCC complement facilitated reactions. Thus this form of defence mechanism could also be important early in recovery when the predominant antibody is IgM, levels of antibody are low and effector cells are few.

The work in this section examines the possible role of ADCC in BTV infections in mice, sheep and cattle.

Results

Using a variety of effector cells, target cells and sera no significant ADCC lysis was detected (Table 5a). The addition of complement to the system also failed to cause lysis of BTV infected target cells. Because of this inability to detect ADCC it was decided that the technical aspects of the assay should be tested by using a virus system which had been shown previously to give good levels of ADCC activity (Wardley et al., 1976). Bovine udder macrophages and buffy coat cells which had previously failed to kill BTV infected GBK cells gave significant lysis against GBK cells infected with IBR virus (Table 5b). The level of this lysis and the kinetics of its development were similar to those observed previously against other herpes
Table 5a: Development in cattle and sheep of ADCC against BTV infected cells as measured by a chromium release assay

<table>
<thead>
<tr>
<th>Effector</th>
<th>Target Cells</th>
<th>Assay</th>
<th>Serum</th>
<th>% Specific Lysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+ Infected</td>
<td>Time</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Virus</td>
<td>(hrs)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>a) Bovines</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Udder macrophages</td>
<td>GBK + BTV4</td>
<td>7</td>
<td>murine antiBTV4</td>
<td>0</td>
</tr>
<tr>
<td>Udder macrophages</td>
<td>GBK + BTV4</td>
<td>7</td>
<td>bovine antiBTV4</td>
<td>0</td>
</tr>
<tr>
<td>PBL's</td>
<td>GBK + BTV4</td>
<td>7</td>
<td>bovine antiBTV4</td>
<td>0</td>
</tr>
<tr>
<td>Udder macrophages</td>
<td>GBK + BTV4</td>
<td>2</td>
<td>bovine antiBTV4</td>
<td>+ complement 0</td>
</tr>
<tr>
<td>Udder macrophages</td>
<td>GBK + BTV4</td>
<td>7</td>
<td>bovine antiBTV4</td>
<td>+ complement 0</td>
</tr>
<tr>
<td><strong>b) Ovine</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Udder macrophages</td>
<td>GBK + BTV4</td>
<td>7</td>
<td>ovine antiBTV4</td>
<td>0</td>
</tr>
<tr>
<td>PBL's</td>
<td>GBK + BTV4</td>
<td>7</td>
<td>ovine antiBTV4</td>
<td>0</td>
</tr>
<tr>
<td>PBL's</td>
<td>LFK + BTV4</td>
<td>7</td>
<td>ovine antiBTV4</td>
<td>0</td>
</tr>
<tr>
<td>PBL's</td>
<td>LFK + BTV3</td>
<td>7</td>
<td>ovine antiBTV4</td>
<td>0</td>
</tr>
<tr>
<td>PBL's</td>
<td>LT + BTV3</td>
<td>7</td>
<td>ovine antiBTV3</td>
<td>0</td>
</tr>
<tr>
<td>PBL's</td>
<td>LT + BTV4</td>
<td>7</td>
<td>ovine antiBTV3</td>
<td>0</td>
</tr>
<tr>
<td>Udder neutrophils</td>
<td>LFK + BTV3</td>
<td>7</td>
<td>ovine antiBTV3</td>
<td>0</td>
</tr>
<tr>
<td>Udder neutrophils</td>
<td>LFK + BTV3</td>
<td>18</td>
<td>ovine antiBTV3</td>
<td>3</td>
</tr>
<tr>
<td>Udder neutrophils</td>
<td>LFK + BTV3</td>
<td>24</td>
<td>ovine antiBTV3</td>
<td>2.6</td>
</tr>
<tr>
<td>Udder neutrophils</td>
<td>LFK + BTV3</td>
<td>36</td>
<td>ovine antiBTV3</td>
<td>1.7</td>
</tr>
</tbody>
</table>

Effector to target cell ratio 100:1. % specific lysis obtained from a minimum of 6 well duplicates.

GBK - Georgia bovine kidney cells; LFK - primary lamb foetal kidney cells; LT - primary lamb testis cells.

Serum tested at three dilutions: neat, 1/50, 1/500.

PBL's - peripheral blood leukocytes.

50µl of a 1/4 dilution of fresh guinea pig complement added to each well containing test serum.
Table 5b: Development in cattle of ADCC against IBR infected cells as measured by a chromium release assay

<table>
<thead>
<tr>
<th>Effector cells</th>
<th>targets cells(^b) + infecting virus</th>
<th>Serum titre(^c)</th>
<th>% specific lysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Udder macrophages</td>
<td>GBK</td>
<td>1/50</td>
<td>2.3</td>
</tr>
<tr>
<td>Udder macrophages</td>
<td>GBK + IBR</td>
<td>1/50</td>
<td>62</td>
</tr>
<tr>
<td>Udder macrophages</td>
<td>GBK + IBR</td>
<td>1/500</td>
<td>14</td>
</tr>
<tr>
<td>PBL's(^d)</td>
<td>GBK + IBR</td>
<td>1/50</td>
<td>60</td>
</tr>
</tbody>
</table>

\(^a\), \(^b\), and \(^d\) as Table 5a

\(^c\) mouse antIIBR serum.

Assay time 7 hours
viruses (Kohl et al., 1979; Rouse et al., 1976b). These results (Table 5b) indicate the technical ability to perform the test and the adequacy of the effector cell population. The reason for the lack of activity in the BTV system could therefore be a property of the target cell. Thus to confirm that the BTV antigens were being presented on the target cell surface at the time at which they were being used in the ADCC assays and in a form recognised by the antibody, an ELISA system was used to measure cell surface antigen. Serum from sheep prior to and 40 days after inoculation of BTV gave similar optical density readings (mean values 0.31 and 0.33 nm respectively) when reacted with uninfected GBK cells, but, when BTV infected GBK cells were used, 0 day sera gave readings of 0.30 nm whereas post-inoculation sera gave readings of 0.55 nm. This indicates that specific sheep anti-BTV antibody present in the 40 day pi serum, was attaching to infected cells and that the failure to detect ADCC against BTV was not due to lack of presentation of virus or viral antigens on cell surfaces.

Although it has been shown that certain classes of antibody are unable to mediate ADCC (Rouse and Babiuk, 1978) the sera examined (Table 5a) included samples from animals at various times during a BTV infection and should thus have been expected to contain the immunoglobulin classes able to mediate ADCC.
Fig. 5a: Development of ADCC against IBR infected cells using a chromium release assay.

(a) % specific lysis obtained using various anti-IBR sera dilutions and at various effector to target cell ratios. Effector cells - bovine udder macrophages; target cells - GBK cells infected 24 hours previously with IBR. 7 hour assay time.

(b) % specific lysis obtained using a variety of assay times.

* - * Infected targets
• — — • Uninfected targets
Discussion

Attempts to demonstrate ADCC following BTV infection in mice, sheep and cattle consistently failed. The reason for this is not entirely apparent as the technical aspects of the assay were checked in an IBR system. It thus appears most unlikely that the functional elements of this test were in any way defective. Therefore, either the parameters described for ADCC in other systems are entirely different, or this method of virus control is not operative in BTV infected animals. Descriptions of ADCC assays certainly relate that a very wide number of cell types are capable of effecting lysis (Rouse et al., 1976a; Zighelboim et al., 1973) and that different antibody classes and subclasses are involved (Rouse and Babiuk, 1978). However, the effector cell populations that were used should have contained any putative effector cell and the wide range of anti-BTV sera tested would again be likely to have contained any functionally active antibody. The ELISA test further substantiates the reactivity of the antibody populations which should then allow effector cells to lyse BTV infected targets.

By accepting the functional integrity of the system the only possible conclusion is that ADCC does not operate in BTV infections. The reasons for this are not clear but are most likely to hinge on the immunologically specific part of the reaction ie the binding of the anti-BTV antibody to the infected cells. It would appear that an element of this reaction is missing and hence the triggering of the lytic
event does not occur. Two further possibilities exist which need investigation. It may be that BTV infected cells show increased Fc receptor production, as has been described in other systems (Adler et al., 1978) and that these receptors 'mop' up the Fc of the antibody and hence prevent it from reacting with the effector cells. It is also possible that the multiplication of BTV in reticulendothelial cells affects their function as ADCC mediating effectors. This could be tested by measuring their activity to another virus during a BTV infection.

The fact that no ADCC activity could be found to BTV infected cells is contrary to all other reported virus systems (Rouse and Babiuk, 1978). If indeed BTV is unique in this respect how might it affect the course of the infection? The importance of the mechanism resides in the fact that only small amounts of antibody are required to trigger it and thus ADCC has the potential of destroying cells before virus maturation is complete. The pattern of BTV replication in its host is not dissimilar to many other viruses which would argue against a vital role for ADCC in viral infections. On occasions however, virus persists at low levels in the blood of infected animals (Luedke et al., 1983) and it may be this deficit in removing intracellular virus which contributes to this phenomenon.
b) Induction of interferon and a study of its in vivo anti-viral activity against BTV

Introduction

Of the many immunologically non-specific substances involved in anti-viral immunity, interferon has received the most attention. Soon after its discovery in the late fifties by Issacs and Lindenman (1957), considerable data was assembled indicating its role in recovery from virus infections. Agents containing ds RNA are reported to be among the most potent inducers of interferon (Dubovi and Akers, 1971; Grossberg, 1972; Tytell et al., 1967) both in vitro and in vivo. These include members of the reovirus family, especially BTV (Jameson et al., 1978). The ability of BTV to induce interferon has been studied in mice (Jameson et al., 1978) and a number of cell lines (Fulton and Pearson, 1982), although there are no reports on the susceptibility of BTV to interferon or whether sheep and cattle infected with BTV produce interferon. These questions are pertinent when trying to ascribe a role for interferon during a BTV infection and this section deals with this aspect of the ruminants response to BTV.
Results

a) Induction of interferon in BHK cells

The addition of BTV4 to GBK cells resulted in a cell produced anti-viral substance whose properties can be seen in Table 5c. The following results would indicate this substance to be a interferon (McNeil, 1981).

a) It was not inactivated by treatment at pH 2 (see materials and methods)

b) It displayed anti-viral activity against unrelated viruses i.e. VSV and aphtho virus.

c) Its activity was inhibited by incubation with trypsin for one hour at 37°C.

d) Its activity was inhibited by pretreatment of the cells with 5μg/ml of actinomycin D for one hour at 37°C.

b) Anti-BTV activity

No anti-viral activity of this preparation could be demonstrated against BTV, although both VSV and aphtho virus were inhibited by the addition of this BTV induced interferon (Table 5c).

c) BTV induction of interferon in sheep and cattle

In an attempt to measure the induction of interferon in sheep and cattle following the inoculation of BTV, serum was collected from animals hourly, for 24 hours and then twice daily for ten days. The anti-viral activity of the serum was
Table 5c: Characterisation and antiviral activity of BHK grown, BTV induced interferon.

<table>
<thead>
<tr>
<th>Virus</th>
<th>virus dilution (log_{10})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-1</td>
</tr>
<tr>
<td>VSV (alone)^a</td>
<td>+</td>
</tr>
<tr>
<td>VSV + interferon^c</td>
<td>+</td>
</tr>
<tr>
<td>% reduction</td>
<td>0%</td>
</tr>
<tr>
<td>Interferon (alone)</td>
<td>0</td>
</tr>
<tr>
<td>VSV + interferon + trypsin</td>
<td>+</td>
</tr>
<tr>
<td>% reduction</td>
<td>0%</td>
</tr>
<tr>
<td>VSV + interferon + actin.D^e</td>
<td>+</td>
</tr>
<tr>
<td>% reduction</td>
<td>0%</td>
</tr>
<tr>
<td>Aphtho virus^f (alone)</td>
<td>+</td>
</tr>
<tr>
<td>Aphtho virus + interferon</td>
<td>+</td>
</tr>
<tr>
<td>% reduction</td>
<td>0%</td>
</tr>
<tr>
<td>BTV4 (alone)</td>
<td>+</td>
</tr>
<tr>
<td>BTV4 + interferon</td>
<td>+</td>
</tr>
<tr>
<td>% reduction</td>
<td>0%</td>
</tr>
</tbody>
</table>

^a Vesicular stomatitis virus (Indiana strain).

^b Number of plaques per well. Typical results of experiments repeated on at least 2 occasions. Means of minimum of 4 well per dilution. + = too many plaques present per well to count separately.

^c Interferon diluted 1/20

^d Interferon incubated for 1 hr. at 37°C with 500ug/ml trypsin before use.

^e Cells pretreated with 5ug/ml of actinomycin D for 1 hr. at 37°C before the addition of interferon.

^f Aphtho virus (type 0)
assayed using the VSV plaque reduction system. On occasions serum was tested on the same day and at other times stored at -70°C before use. At no time could any anti-viral activity be detected.

Discussion

As has been shown by other workers (Jameson et al., 1978) BTV was able to induce BHK cells to produce a substance with all the properties of interferon when tested in conventional assays. However this substance was ineffective in curtailing BTV cytopathology. In contrast in vivo BTV did not appear to stimulate detectable interferon production.

The literature contains many examples of the differing stimulatory potential and susceptibility of viruses to interferon (Gresser et al., 1976; Ho, 1973). Interferon has been viewed primarily as an anti-viral agent that activates enzymes which interfere with viral replication in host cells (Lengyel, 1981). In the case of rotaviruses their relative lack of sensitivity has been ascribed to a lack of activation of the cellular enzymes which cause the anti-viral activity (La Bonnardiere et al., 1978). Recent work has suggested that priming of cells in vitro enhances both the interferon inducing properties and susceptibility of rotaviruses to interferon (McKimm-Breschkin and Holmes, 1982) through the activation of 2-5a synthetase and other enzymes. The structural similarity between BTV and rotaviruses which are
both dsRNA viruses with two virion shells, suggests that such studies should perhaps be performed with BTV.

The inability to detect interferon in the serum of BTV infected sheep and cattle may be due to the extreme lability of ruminant interferon (McNeil, 1981; Letchworth, personal communications) as other workers have shown in mice, that BTV will induce serum interferon (Jameson et al., 1978). Further the importance of interferon might lie beyond its direct effect on viral multiplication, as increasing evidence points to interferon as a regulator of a multitude of cell-mediated immune responses (Friedman, 1981). In particular it augments natural killer (NK) cell activity and the reported development of interferon in BTV infected mice correlates well with the pattern of BTV induced murine NK cell activity reported in this thesis (Chapter 4, Fig 4a). Further this corresponds to the short lived BTV viraemia pattern observed in mice (Chapter 4, Table 4a). It could be that in the mouse, interferon is induced following the inoculation of BTV and this, along with certain cell-mediated immune mechanisms, in particular NK cell activity, curtails the infection. This phenomenon has been argued for a number of infections both in humans and mice (Djeu et al., 1982; Lanza and Djeu, 1982; Jacobson et al., 1981; Sethi et al., 1983). In ruminants the apparent inability of BTV to induce interferon may therefore, in part, be responsible for the extended primary infections seen in these species. In contrast, however, the observation that the serial inoculation of two BTV types results in protection from challenge with a third type, may be partly due to the effects
of interferon produced by immune T lymphocytes, induced during
the previous exposures to BTV.
CHAPTER SIX

PASSIVE PROTECTION; ANTIBODY MEDIATED.

Introduction

When both cattle and sheep are sequentially inoculated with two BTV types they are protected against challenge with a third BTV and this serial inoculation of two or more BTV types gave rise to a broad heterotypic antibody response (Chapter 3). Similarly BTV induces CTL's which in mice at least, are capable of cross-reactive lysis (Chapter 4). These observations suggest that the broad heterotypic immunity required to protect against a number of types may be achieved with only one or two BTV inoculations rather than the multivalent vaccines used at present. Despite these observations however, the relative importance of the antibody or T cell response, in terms of recovery and protection, is still unclear, and before an assessment can be made of how such a modified vaccine might be used it is first necessary to examine the roles of humoral and cellular immunity in protection and recovery from bluetongue.

The work in this chapter attempts to evaluate the role of humoral immunity by challenging animals which had previously received anti-BTV serum, colostrum or a monoclonal antibody.
Experimental Design

1) Passive antibody transfer studies. Four animals not given immune serum, were inoculated with BTV3 to act as virus controls (Group A). Eight sheep were inoculated intraperitoneally with 300 ml of BTV3 immune sera which had an homologous neutralising antibody titre of 1/180. Three days later four of these animals were challenged with BTV3 (Group B) and four with BTV4 (Group C).

2) Colostrum immunity studies. Twin lambs born to and suckling from a BTV2 immune ewe whose colostrum had a neutralising antibody titre of 1/30 against BTV2, were inoculated with BTV2 seven days after birth. A lamb from a ewe known not to have experienced BTV infection was similarly inoculated to act as a viral control.

3) Monoclonal antibody studies. A murine hybridoma, 6C2A.4.2, which produced antibody that specifically neutralised BTV17 (Wyoming strain), was grown in the peritoneal cavity of mice (Letchworth and Appleton, 1983). Ascitic fluid from these mice, having a neutralising antibody titre of 1/40 against BTV117 (Wyoming strain), was inoculated intravenously at a dosage of 0.03% of body weight into one sheep. This animal and an uninoculated control were challenged six hours later with BTV17 (Wyoming strain).
Results

1) Passive antibody transfer studies

The inoculation of BTV3 into the four unimmunised control animals (Group A) produced a pyrexia, viraemia (Table 6a) and neutralising antibody to BTV3 (Fig. 6a)

Three of the four animals inoculated with BTV3 immune sera and challenged with BTV3 (Group B), developed neither pyrexia nor viraemia (Table 6a). Neutralising antibodies to BTV3 could only be detected in one of these three animals on the day of challenge and by four days after inoculation in the other two animals. Titres of neutralising antibody then rose only slowly in these three sheep and remained below those of the control animals (Fig. 6a)

The fourth animal in this group, number 76, although immunised intraperitoneally with BTV3 immune sera developed a pyrexia and viraemia similar to that seen in the controls. However, in this animal antibodies to BTV3 were not detectable until day 10 p.i. and rose rapidly to reach levels similar to those in the control. The fact that this animal reacted in a similar manner to the controls indicated that the hyperimmune serum may have been injected into the gut lumen and that the animal was not passively immunised.

Sheep inoculated with BTV3 immune serum and challenged with BTV4 (Group C) also developed a typical viraemia and pyrexia (Table 6a). Antibodies to BTV4 were detectable in the four animals by day 10 after challenge. Titres rose rapidly to
Table 6a: Viraemia levels in groups of sheep challenged with BTV following passive immunisation with BTV immune serum

<table>
<thead>
<tr>
<th>Animal Groups</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment with immune serum</td>
<td>none</td>
<td>300 ml</td>
<td>300 ml</td>
</tr>
<tr>
<td>Virus challenge $^a$</td>
<td>BTV3</td>
<td>BTV3</td>
<td>BTV4</td>
</tr>
<tr>
<td>Days post inoculation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.9$^b$</td>
<td>-ve$^c$</td>
<td>-ve</td>
</tr>
<tr>
<td>4</td>
<td>4.2</td>
<td>-ve</td>
<td>1.5</td>
</tr>
<tr>
<td>6</td>
<td>4.4</td>
<td>-ve</td>
<td>2.9</td>
</tr>
<tr>
<td>8</td>
<td>4.7</td>
<td>-ve</td>
<td>3.9</td>
</tr>
<tr>
<td>10</td>
<td>4.1</td>
<td>-ve</td>
<td>2.8</td>
</tr>
<tr>
<td>13</td>
<td>1.6</td>
<td>-ve</td>
<td>1.2</td>
</tr>
<tr>
<td>15</td>
<td>2.3</td>
<td>-ve</td>
<td>0.3</td>
</tr>
<tr>
<td>19</td>
<td>2.3</td>
<td>-ve</td>
<td>1.2</td>
</tr>
<tr>
<td>23</td>
<td>2.1</td>
<td>-ve</td>
<td>0.3</td>
</tr>
<tr>
<td>27</td>
<td>0.3</td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td>30</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
</tr>
</tbody>
</table>

$a$ 10$^6$TCID$_{50}$ of BTV intradermally.

$b$ Viraemias expressed as log$_{10}$TCID$_{50}$/ml.

$c$ Samples from group B titrated with negative results in embryonated eggs.

Results calculated as arithmetic means of individual animals/group.
Fig. 6a The development of neutralising antibody to BTV3 and BTV4 following inoculation of these virus types into passively immunised animals.

🌟 Neutralising antibodies to BTV3, controls (Group a)

🌟🌟 Neutralising antibodies to BTV3 following passive immunisation with BTV3 immune serum and challenge with BTV3 (Group b)

🌟🌟🌟 Neutralising antibodies to BTV3 following passive immunisation with BTV3 immune serum and challenge with BTV4 (Group c)

●●● Neutralising antibodies to BTV4 following passive immunisation with BTV3 immune sera and challenge with BTV4

Time expressed as days post inoculation with BTV. Antibody titre calculated from geometric means of reciprocal $\log_{10} VN50$ with 4 animals in each group.
reach approximately 1/1280 in all four animals (Fig. 6a).
Antibodies against BTV3 were detectable at low titres on the
day 0 in these animals (Fig. 6a) and changed little following
the virus challenge. The animals were also examined for the
antibodies to all other BTV types, with negative results.

2) Colostral immunity studies

The lamb born to a ewe previously unexposed to BTV
developed a typical viraemia after inoculation with BTV2
(Table 6b). It showed a pyrexia peaking at seven days after
inoculation and was anorexic and recumbent from seven to nine
days following inoculation. Neutralising antibody to BTV2 was
first detected by day 12 (Fig. 6b), rose rapidly and levelled
out at a titre of around 1/160. Twin lambs born to a ewe
previously inoculated with BTV2 did not develop a pyrexia and
did not become anorexic or recumbent. Although viraemia was
detected in both lambs it was short lived and reached only low
levels (Table 6b). Colostrum from this ewe had a neutralising
antibody titre to BTV2 of 1/30 and neutralising antibody to
BTV2 was detected in serum from these lambs at the time of
BTV2 challenge (Fig. 6b); these levels rose one or two fold,
were maintained for approximately 30 days, and then declined.

3) Monoclonal antibody studies

After inoculation of BTV17 (Wyoming strain) into a sheep
which had received monoclonal antibody, the animal developed a
Table 6b: Viraemia levels in lambs following inoculation of BTV2

<table>
<thead>
<tr>
<th>Days post Inoculation</th>
<th>Animal Number</th>
<th>Days post Inoculation</th>
<th>Animal number</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>64 c</td>
<td>65 c</td>
<td>81 d</td>
</tr>
<tr>
<td>0</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td>2</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td>4</td>
<td>-ve</td>
<td>1.8</td>
<td>16</td>
</tr>
<tr>
<td>5</td>
<td>1.4 b</td>
<td>1.0</td>
<td>2.0</td>
</tr>
<tr>
<td>6</td>
<td>0.6</td>
<td>0.8</td>
<td>2.2</td>
</tr>
<tr>
<td>7</td>
<td>2.0</td>
<td>2.0</td>
<td>5.6</td>
</tr>
<tr>
<td>8</td>
<td>0.4</td>
<td>0.6</td>
<td>2.2</td>
</tr>
</tbody>
</table>

\(10^6\text{TCID}_{50}\) of BTV intradermally

Viraemias expressed as \(\log_{10}\text{TCID}_{50}/\text{ml}\); virus isolation carried out in BHK cells

Received colostrum from ewe immunised 2 years previously with BTV2

Received colostrum from ewe free from contact with BTV
Fig. 6b The development of neutralising antibody to BTV2 following inoculation of this virus into three lambs.

*----* Neutralising antibody titre to BTV2 in two lambs designated 64 and 65 and born from BTV2 immune dam

○----○ Neutralising antibody titre to BTV2 in lambs designated number 81 and born from a ewe fully susceptible to BTV

Time expressed as days post inoculation with BTV2
viraemia but no pyrexia. However, the viraemia was of shorter
duration and of a lower titre than that seen in the control
(Table 6c). A difference was also seen in the antibody
response in that, although no neutralising antibody was
detected after administration of the monoclonal antibody, it
appeared four days prior to that in the controls (Fig. 6c).
This may have been due to the earlier availability of free
antibody because of the additive effect of the passively
acquired antibody with that actively induced. It is also
possible that passively acquired antibody and challenge virus
formed complexes which then more effectively stimulated
antibody production (Klaus et al., 1980).

Discussion

From the above results it can be seen that passive transfer
of BTV immune serum is capable of abrogating the pyrexia and
viraemia that occurs following the inoculation of BTV into
adult sheep. This antibody-mediated protection was also shown
to be type-specific since animals challenged with a BTV type
different from that used to raise the BTV immune sera showed a
typical BTV viraemia and pyrexia.

There are several possible ways by which antibody can
prevent and aid in the recovery from viral infections. It can
neutralise extracellular virus to interact with complement and
or cellular components in the lysis of virus infected cells
(Rager-Zisman and Allison, 1973; Rouse and Babiuk, 1978;
Cooper, 1979; Griffin and Johnson, 1977). The present studies
Table 6c: Viraemia levels in sheep following inoculation of BTV17 (Wyoming strain)

<table>
<thead>
<tr>
<th>Animal Number</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monoclonal Antibody to BTV17</td>
<td>3 ml i/v none</td>
<td>3 ml i/v none</td>
</tr>
<tr>
<td>Days post Inoculation</td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td>0</td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td>2</td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td>3</td>
<td>-ve</td>
<td>0.4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>0.4</td>
<td>2.8</td>
</tr>
<tr>
<td>5</td>
<td>1.6</td>
<td>4.0</td>
</tr>
<tr>
<td>7</td>
<td>2.8</td>
<td>3.0</td>
</tr>
</tbody>
</table>

Days post Inoculation<sup>a</sup> | 8 | 1.0 | 2.8 |
| 9 | -ve | 0.4 |
| 11 | -ve | -ve |
| 13 | -ve | -ve |
| 15 | -ve | -ve |
| 17 | -ve | -ve |
| 23 | -ve | -ve |

<sup>a</sup> 10<sup>6</sup> TCID<sub>50</sub> of BTV intradermally

<sup>b</sup> Viraemias expressed as log<sub>10</sub> TCID<sub>50</sub>/ml; virus isolation carried out in BHK cells
Fig. 6c The development of neutralising antibody following the inoculation of BTV17 (Wyoming strain) into sheep

+---+---+---+---+---+---+---+---+---+---+---+---+---+---+---+---+---+

Neutralising antibody titre in a sheep receiving 3ml of a monoclonal antibody against BTV prior to the inoculation of BTV17

Neutralising antibody titre in a sheep not receiving antibody prior to the inoculation of BTV17

Time expressed as days post inoculation with BTV17 (Wyoming strain)
have similarities to the situation which arises after an animal has recovered from a primary infection and is subsequently challenged. However, in passively immunised animals there are no anti-BTV memory cells and hence it should be possible to delineate more accurately the role which antibodies play in protection.

In BTV infections other studies have indicated that after entry primary virus replication occurs in the local draining lymph node followed by dissemination through the blood and a second phase of generalised virus replication which appears responsible for the rapid increase in viraemia levels (Lawman 1979).

Passively administered antibody could act by neutralising inoculated virus and preventing the first replication cycle or lyse virus-infected cells at the primary replication site. If passively acquired antibody did not act successfully in reducing virus titres at this initial stage, it is unlikely that it would have a significant effect later. Thus in the first experiments it would appear that in the passively immunised adult sheep, virus was either prevented from entering the local lymph node in an infectious state or its replication was effectively curtailed at that point. However, in the lambs clinical signs became evident indicating that the colostrally derived antibody was insufficient to neutralise the challenge virus. It would thus seem unlikely that the lower levels of antibody obtained after the intraperitoneal passive transfer of immune serum in the adult sheep was capable of neutralising the virus. So what role could other
factors be playing? The immaturity of the lambs may mean that
virus is able to multiply more effectively in cells of the
reticulo-endothelial system compared to the adult and hence
even though more antibody is present it is not sufficient to
prevent a viraemia. It is also likely that neutralising
antibodies are not the only anti-BTV antibodies responsible
for the curtailment of virus multiplication. The ewe to which
the BTV challenged lambs were born had recovered from a BTV
infection some 2 years previously, whereas the antibody used
in the passive transfer came from sheep which had been
infected two months previously. It could be that the anti-BTV
antibodies of these sera were functionally different and that
an important protective population is relatively short lived
and hence was not transferred in the colostrum. It is also
possible that antibody absorption through the gut is selective
(Brambell 1970) and that the important antibody population
does not reach protective levels within the lamb's serum.

Whatever the explanation, there is little correlation
between the in vitro virus neutralisation titres and
protection. Other studies (Neitz, 1948; Osburn et al., 1978)
have examined passive transfer of BTV antibody in foetal and
neonatal lambs. Neitz (1948) showed that immune colostrum
would prevent the clinical response to BTV inoculation in 4 to
8 day old lambs but he did not examine viraemia or antibody
levels. Osburn, Sawyer, Moe and Cordy (1978) found that
passive immunisation of foetuses failed to protect against
intrauterine challenge; in one foetus virus was present and in
all cases lesions associated with BTV were found in the brain
tissues. Evidence from other arbovirus infections also shows that there is a poor correlation between serum neutralisation levels and protection and it has been suggested that antibody dependent cell mediated cytotoxicity may be the mechanism through which antibody acts to reduce virus levels (Griffin and Johnson, 1977; Mathews and Roehrig, 1982; Schmaljohn et al., 1982). However it has not been possible to detect such a mechanism acting against BTV infected cells (Chapter 5).

The inability to detect neutralising antibody in sheep for six days after inoculation of monoclonal antibody may reflect the insensitivity of the in vitro assay as in other systems it has been found that, because of the high specificity of monoclonal antibodies against critical neutralisation sites only low levels are needed to afford protection (Mathews and Roehrig, 1982). Other work using this monoclonal (Letchworth and Appleton, 1982) also demonstrated protection against BTV in both mice and sheep, but again only low levels of neutralising antibody developed.

These experiments indicate that BTV antibody has a vital role to play in protection and recovery from bluetongue but that neutralisation of virus by antibody as measured in vitro may not be the process whereby antibody protects in vivo.
CHAPTER SEVEN

PASSIVE PROTECTION; CELL-MEDIATED.

Introduction

Although passive antibody transfer techniques have given a clearer understanding of the role of humoral immunity in prevention from re-infection, similar approaches concerned with the role of cellular components have been limited by the need to transfer syngeneic lymphocytes. Work in laboratory rodents has readily demonstrated the importance of cellular mechanisms but similar approaches has been restricted in other animals (Emery, 1981) by the availability of major histocompatibility complex (MHC) compatible donors and recipients. Lack of mutual reactivity between leucocytes from chimeric bovine co-twins enabled studies on cellular immunity in Theileria Parva infections to be conducted (Emery, 1981; Emery and McCullagh, 1980) and the recent availability of ovine monozygotic twins (Willadsen, 1979; Williams et al., 1982) offer a method for the further study of cell-mediated immunity in domestic animal viral infections.

In an attempt to clarify the position further and examine in more detail the role of cell-mediated immunity in BTV infections, monozygotic animals were used in cellular adoptive...
transfer experiments involving thoracic duct lymphocytes.

Experimental design.

The demonstration that peak ovine CTL activity occurred 14 days pi of BTV (chapter 4) indicated that this would most likely be the time at which a transferred TDL population would give the maximal protective effect. Hence the first experiment, using half the available monozygotic animals, was carried out with the cell from the donor animals transferred at 14 days pi with BTV.

1) Adoptive transfer of TDL's, 14 days after inoculation of the donor with BTV; homologous virus challenge.

Three sheep (the donors) were inoculated with BTV3 and 14 days later the thoracic duct of each animal cannulated and approximately 1 litre of thoracic duct fluid collected. After separating and washing, the cells were inoculated intravenously into the other monozygotic twin, the recipient. Twelve hours later the recipients were challenged with BTV3. A control sheep, not inoculated with BTV was similarly cannulated, thoracic duct fluid collected and the TDL's inoculated into its monozygotic twin. This animal was also challenged 12 hour later with BTV3

2) Adoptive transfer of TDL's, 7 days after the inoculation of the donor with BTV; homologous virus challenge.
The donor was inoculated with BTV3 and 7 days later the thoracic duct cannulated, 1 litre of fluid collected, the cells separated, washed and then transferred to the recipient. Twelve hours later the recipient was challenged with BTV3.

3) Adoptive transfer of a T cell enriched population of TDL's, 14 days after inoculation of the donor with BTV; homologous virus challenge.

The donor was inoculated with BTV3 and 14 days later the thoracic duct cannulated and the thoracic duct fluid collected. The cells were separated, washed and after 'panning' using anti-sheep IgG coated plates, to T cell enrich the TDL population, these cells were inoculated into the the recipient. Twelve hours later it was challenged with BTV3.

4) Adoptive transfer of TDL's, 14 days after the inoculation of the donor with BTV; heterologous virus challenge.

The donor was inoculated with BTV3. Fourteen days later the thoracic duct was cannulated, approximately 1 litre of fluid collected and the cells separated and washed prior to inoculation into the recipient. Twelve hours later the recipient was inoculated with BTV4. The recipient in this experiment had been infected with BTV3 six weeks previously and had reacted with a typical pyrexia, viraemia and neutralising antibody response. A control animal, which did not receive TDL's but had been similarly inoculated with BTV3 six weeks previously, was also challenged with BTV4 at this time.
Results

1) Adoptive transfer of TDL's, 14 days after inoculation of the donor with BTV; homologous virus challenge.

The inoculation of BTV3 into the three donor sheep produced a typical pyrexic, viraemic and antibody response (Figs. 7a, 7b and 7c). Peak temperature and viraemia levels were obtained on day 7 post inoculation and virus continued to be isolated for at least 20 days (Fig. 7b).

The amount and composition of the thoracic duct fluid on day 14 post virus inoculation can be seen in Table 7a. Although it did not contain BTV, neutralising antibody to BTV3 was present at low levels (1/20 in each case). The uninoculated control animal (animal number 1, Table 7a) only retained the cannula for four hours and hence only 500cc of fluid was collected. This resulted in less cells being transferred to the twin of this animal.

The transfer of cells and challenge of all four recipient animals resulted in the temperature and viraemia response shown in Figs. 7a, b, and c. The control animal receiving TDL's from a uninoculated donor developed a response typical to that shown in fully susceptible animal (Fig. 7d) and the level and duration was indistinguishable from that following the inoculation of BTV3 into other donor animals.

The three recipients given TDL's from a donor inoculated 14
Fig. 7a: Elevation of temperature following the inoculation of BTV into monozygotic animals. Thoracic duct lymphocyte transfer at 14 days pi.

- Donor(s)
- Recipient(s)

Rectal temperature °C. (i) Mean of individual animals results (ii), (iii) and (iv) individual animal results from animal numbers 2, 3 and 4 respectively.
Fig. 7b Viraemia in monozygotic animals following the inoculation of BTV. Thoracic duct lymphocyte transfer at 14 days pi.

* - * Donor(s)

Recipient(s)

Viraemia TCID\textsubscript{50}/ml. (i) Mean of individual animals results (ii), (iii) and (iv) individual animal results from animal numbers 1, 2 and 3 respectively.
Fig. 7c: Neutralising antibody to BTV3 in monozygotic animals following the inoculation of this BTV type. Thoracic duct lymphocyte transfer at day 14 pi.

Donor(s)

Recipient(s)

(i) Mean of individual animal results (ii), (iii) and (iv) individual animal results from animal numbers 1, 2 and 3 respectively.
Table 7a: Nature of thoracic duct fluid collected following cannulation fourteen days after the inoculation of BTV into sheep.

<table>
<thead>
<tr>
<th>Animal amount</th>
<th>time taken</th>
<th>cell count</th>
<th>% of % of transferred &lt;sup&gt;a&lt;/sup&gt; B cells &lt;sup&gt;b&lt;/sup&gt; T cells &lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 500cc</td>
<td>4 hrs.</td>
<td>8.5 X 108</td>
<td>20cc 36% 26%</td>
</tr>
<tr>
<td>2 1300cc</td>
<td>16 hrs.</td>
<td>6 X 109</td>
<td>20cc 42% 37%</td>
</tr>
<tr>
<td>3 1300cc</td>
<td>8 hrs.</td>
<td>4 X 109</td>
<td>20cc 46% 17%</td>
</tr>
<tr>
<td>4 1000cc</td>
<td>16 hrs.</td>
<td>4.6 X 109</td>
<td>20cc 31% 22%</td>
</tr>
</tbody>
</table>

<sup>a</sup> Inoculated intravenously into recipient monozygotic twin

<sup>b</sup> Identified using fluoroscein conjugated rabbit anti-sheep IgG

<sup>c</sup> Identified using fluoroscein conjugated peanut agglutinin
Fig. 7d: Temperature and viraemia responses following the inoculation of BTV into a recipient receiving thoracic duct lymphocytes from a BTV free donor animal.

* - Donor

- - - - - Recipient

Rectal temperature °C. Viraemia TCID₅₀/ml
days prior to transfer with BTV3, showed a reduction in both
the pyrexic and viraemic responses. The mean of the results
for the three recipients are compared to the donors in Figs.
7a and 7b. The peak temperature response was obtained 2 days
earlier and the mean level was 1°C below that of the donor
animals. The viraemia in the recipient group was also
different to that in the donors. Peak viraemias were obtained
between 3 and 4 days pi, compared to 7 days and then fell
rapidly, such that by 7 days pi levels were 99.8% below that
of the donor group. In one recipient BTV could not be
recovered from the blood beyond day 8, whilst in the other
two, although virus was recovered for at least 14 days post
inoculation, the viraemia was at considerably lower levels
than that in the donor animals (Fig. 7b).

Examination of the sera of both donor and recipient animals
for neutralising antibodies to BTV3 gave the results seen in
Fig. 7c. In the donor animals, as has been observed previously
(Fig 3b) neutralising antibody was first detected around day
10 and rose rapidly to reach plateau levels of around 1/1000
by 13 days pi. In the recipients a different pattern was
observed. Antibodies to BTV3 were first detected around day 5,
rose rapidly to reach levels around 1/1000 by day 8 and then
fell to around 1/40 by day 16. This pattern was consistent in
all three recipients receiving TDL's from BTV inoculated
donors. The other recipient receiving TDL's from an uninfected
animal developed antibodies in a similar manner to that of the
donor animals. Neutralising antibodies to the other 21 BTV
types were not detected in the sera of any of these animals.
These results indicate that TDL's from a BTV immune animal can reduce both the pyrexia and viraemia normally associated with BTV infections in sheep. The earlier appearance of neutralising antibody in the recipient animals indicates that either this antibody was produced by B cells transferred in the TDL population (approximately 40% of the TDL's were B cells, (Table 7b) or that T helper cells were able to more quickly prime the B cell response of the recipient and hence antibody was produced more quickly.

2) Adoptive transfer of TDL's, 7 days after the inoculation of the donor with BTV. Homologous virus challenge.

The temperature, viramias and neutralising antibody response, in both the donor and recipient following the inoculation of BTV3 can be seen in Figs. 7e. The donor animal showed a pyrexia and viraemia typical of that found following the inoculation of BTV into fully susceptible animals. Following thoracic duct cannulation on day 7 post virus inoculation, 1200cc of thoracic duct fluid was collected and 6 X 10^9 cells were transferred to the recipient. Examination of this fluid for BTV revealed 10^{2.4} TCID_{50}/ml and 10^{1.4} TCID_{50}/ml of BTV was still present in the washed cells. Hence approximately 10^{2.4}TCID_{50} of virus was transferred along with the thoracic duct lymphocytes. No neutralising antibody could be demonstrated in this fluid.

The pyrexia obtained following the inoculation of BTV3 into the recipient was similar in pattern to that observed in the
Fig. 7e: Temperature, viraemia and neutralising antibody response in monozygotic animals following the inoculation of BTV. Thoracic duct lymphocyte transfer at 7 days pi.

- Donor
- Recipient

Rectal temperature°C viraemia TCID₅₀/ml
recipients in the previous experiment. However the viraemia was unlike that of either the donor animal, or the recipients receiving TDL's from animals inoculated 14 days previously with virus. In this animal virus was isolated from the blood on day 0, levels fell by $10^2$ TCID$_{50}$ in 24 hours and then rapidly rose to peak levels 2 days later. These levels were maintained for 5 days but by day 8 had begun to fall. Following this the viraemia continued in the same manner as that of the donor animal (Fig. 7e).

Examination of sera from both donor and recipient for the presence of neutralising antibodies to BTV3 was similar and consistent with the inoculation of BTV into a fully susceptible animal.

These results indicate that TDL's collected 7 days after the inoculation of BTV are not as effective in curtailing the course of a BTV infection as 14 day old BTV induced TDL's. The early detection of BTV in the blood of the recipient animal reflects the presence of virus in the transferred cells and was of a similar pattern to that observed following intravenous inoculation of BTV (W.P. Taylor, personal communication).

3) Adoptive transfer of a T cell enriched population of TDL's, 14 days after the inoculation of the donor with BTV.

Homologous virus challenge.

The inoculation of BTV into the donor produced the typical pyrexia and viraemia seen in other donor animals (Figs. 7f).
Fig. 7f: Development of temperature and viraemia response following the inoculation of BTV in monozygotic twins; TDL's 'panned' prior to transfer at 14 days pi.

- Donor
- Recipient

Rectal temperature °C. Viraemia TCID_{50}/ml
Table 7b: Cell content of thoracic duct fluid prior to and after 'panning'\textsuperscript{a} to remove B lymphocytes.

<table>
<thead>
<tr>
<th>Number of cells</th>
<th>% B cells\textsuperscript{b}</th>
<th>% T cells\textsuperscript{c}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before 'panning'</td>
<td>6.3 X 10^9</td>
<td>34%</td>
</tr>
<tr>
<td>After 'panning'</td>
<td>1.4 X 10^9</td>
<td>2.3%</td>
</tr>
</tbody>
</table>

\textsuperscript{a} 'Panning' carried out in plastic petri dishes coated with rabbit anti-sheep IgG. Cells panned twice.

\textsuperscript{b} Identified using fluorescein conjugated rabbit anti-sheep IgG serum.

\textsuperscript{c} Identified using fluorescein conjugated peanut agglutinin.
The cell composition of the thoracic duct fluid collected following cannulation at 14 days pi can be seen in Table 7b. 'Panning', using anti-sheep IgG coated plates to remove B cells, resulted in a 75% loss of cells with the percentage of B cells falling from 34% to 2.3% and that of T cells increasing from 18% to 49%. It is highly likely that the remaining 48.7% of unlabelled cells are not all 'null' cells as the T cell markers used (peanut agglutinin) is thought not to label all T cell subsets (Dumont and Nardelli, 1979).

The inoculation of these enriched T cells and challenge twelve hours later with BTV3 into the recipient animal resulted in the viraemic and pyrexic response shown in Figs. 7f. Although the pyrexia is similar to that of the donor, a 90% reduction in the viraemia was obtained. Virus could not be detected in the blood of the recipient 13 days after the inoculation of BTV3, whilst in the donor virus was isolated for up to 28 days.

In contrast to the result obtained where panning did not occur prior to the TDL transfer (Experiment 1, Fig. 7d), the development of neutralising antibody was similar in both donor and recipient (Table 7c). Hence despite the reduction in the number of B cells in the transferred TDL population which appeared to abrogate the early neutralising antibody response (Fig 7d) the observed level of protection was similar to that seen in other 14 day recipients, indicating that the mechanism responsible for this protection is not antibody mediated.
Table 7c: Development of neutralising antibodies to BTV3 in monozygotic twins after the inoculation of BTV3.

<table>
<thead>
<tr>
<th>Days post inoculation&lt;sup&gt;a&lt;/sup&gt;</th>
<th>0</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>10</th>
<th>12</th>
<th>16</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>1.6</td>
<td>1.8</td>
<td>1.5</td>
<td>2.1</td>
</tr>
<tr>
<td>Recipient&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>1.3</td>
<td>1.9</td>
<td>1.6</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> \(10^6\text{TCID}_{50}\) BTV3

<sup>b</sup> Cannulated day 14 post inoculation with BTV3. \(1.36 \times 10^9\) T-cell enriched TDL's transferred to recipient 12 hours prior to virus challenge

<sup>c</sup> Neutralising antibody titre expressed as reciprocal \(\log_{10}\text{VN50}\). -ve neutralising antibody titre < 1.15
4) Adoptive transfer of TDL's, 14 days after the inoculation of BTV into the donor. Heterologous virus challenge.

The inoculation of BTV3 into the donor animal and BTV4 into the control animal (which had previously been inoculated with BTV3) produced the typical pyrexia and viraemia (Figs 7g) seen in other animals.

In the recipient, however, which had been inoculated with BTV3 previously and received TDL's from the donor inoculated with BTV3, the inoculation of BTV4 produced neither pyrexia nor viraemia (Fig 7g).

Both the recipient and control animal had antibodies to BTV3 prior to the inoculation of BTV4 and following BTV4 inoculation both produced neutralising antibodies to this type (Fig 7h). The antibody response in the BTV3 homologously challenged donor animal remained monotypic. Both the recipient and control animals were examined for antibodies to other BTV types and the heterologous nature of the response can be seen in Fig. 7i.

Thus the transfer of immune TDL's from an animal inoculated with BTV3 completely protected a previously BTV3 primed recipient from challenge with BTV4. Antibodies to BTV4 were induced in the recipient indicating a serological reaction to the inoculation of BTV4 although no evidence of a viraemia was obtained. As with the experiments (a) and (c) transferred cells at day 14 pi did not contain detectable virus.
Fig. 7g: Development of temperature and viraemia response in monozygotic animals following the inoculation of BTV; Heterologous virus challenge.

Rectal temperature °C. Viraemia TCID<sub>50</sub>/ml
Both recipient and control animal inoculated with BTV3 six weeks previously. Recipient received TDL's from BTV3 immune donor prior to challenge of both recipient and control with BTV4. Time expressed as days pi with BTV4.
Fig. 7h: Development of neutralising antibodies to BTV types 3 and 4 following the inoculation of monozygotic twins with these BTV types. Heterologous virus challenge.

* --- * Donor; Neutralising antibodies to BTV3
○ --- ○ Recipient; Neutralising antibodies to BTV4
* --- * Recipient; Neutralising antibodies to BTV3
* --- * Control; Neutralising antibodies to BTV4

Donor inoculated with BTV3. Time expressed as days pi of BTV3 for this animal. Both recipient and control animals inoculated with BTV3 six weeks previously. Recipient received TDL's from donor animal prior to challenge of both recipient and control with BTV4. Time expressed as days pi with BTV4 for these two animals.
Fig. 7i: Development of neutralising antibodies to BTV types following the inoculation of BTV types 3 and 4 into a recipient and control animal.

Both recipient and control animal inoculated with BTV3 six weeks previously. Recipient received TDL's from BTV3 immune donor prior to challenge of both recipient and control with BTV4. Time expressed as days pi with BTV4.
Although both recipient and control animals had antibodies to BTV3 prior to challenge with BTV4, it has been shown previously (Chapter 3, b and d) and also again in the control animal, that these antibodies do not decrease susceptibility to a second BTV type challenge. Hence the complete protection observed in the recipient as compared to the diminished response seen in donors challenged with homologous virus, required the presence of the immune TDL population and the presence of heterotypic antibody.

The inoculation of a second BTV type evoked neutralising antibodies to a large number of BTV types in both the control and recipient animals (Fig7i, Chapter 3, Figs 3b, d and e). This happened despite there being no evidence of virus replication, a situation similar to that seen when animals are normally challenged with a third BTV type.

Discussion.

These results indicate that the inoculation of BTV into sheep produces a population of TDL's which when transferred to another animal is capable of reducing the pyrexia and viraemia normally associated with a BTV infection. Further the activity of these cells is very little if transferred at 7 days pi of virus but is clearly demonstrable by 14 days pi. The transfer of the total cell population results in an early antibody response which is abrogated by the removal of B cells without affecting the degree of protection observed. This would
indicate that this protection is mediated by T cell through a mechanism which does not rely on antibody production. CTL's could obviously work in this way and the pattern of their in vivo appearance in sheep, being barely detected at 7 days pi but having maximal activity by 14 days, correlates well with this cell transfer data. Moreover, in the presence of heterotypic antibody, these cells are capable of completely abrogating both the pyrexia and viraemia associated with a second heterotypic challenge of BTV. Hence, although an animal inoculated with one BTV type is not usually protected from challenge with a second BTV type, the transfer of recently induced TDL's to another type prior to challenge prevents the expected viraemia and pyrexia.

These observations augment and confirm previous results obtained in this thesis. They indicated the functional importance of the previously identified ovine BTV induced CTL's and support the hypothesis proposed by work carried out in mice that this cell is capable of cross-reactive lysis. Thus BTV also appears to induce ovine cross-reactive CTL's which although short-lived are capable of protecting animals from heterologous challenge. However, it appears that a prerequisite to this heterologous protection is previous exposure to BTV and the exact nature of the co-operation between these short-lived cross-reactive CTL's and other previously evoked immune mechanisms is not clear.

Previous work has highlighted three major mechanisms whereby T lymphocytes mediate their antiviral effects (Allison, 1972; Notkins et al., 1970; Rouse and Babiuk, 1974a and b, 1975 and
1978). These are a) direct cytotoxicity, b) the release of interferon type II, and c) the recruitment of macrophages. In chapter 5 it was found that BTV in bovine cells was resistant to the direct effect of interferon and Lawman (1979) showed that the virus can replicate in macrophages during a BTV infection. Hence direct cytotoxicity would appear to be the protective mechanism in operation during a BTV infection. Recent work has investigated the ability of helper T cells to amplify both B and T cell responses and have shown that different helper T cell subsets exist which trigger specific immune response (Friedman and Thompson, 1983). Other work further argues that the requirement of antigen recognition occurs at the level of the interaction between helper T cells and accessory cells. This interaction would generate all the soluble factors needed for turning precursor CTL's into effective killer cells (Bellagrau, 1983). This activation of CTL precursors requires two signals, a) recognition of viral antigen and b) a soluble product from a T helper cell and that the specificity of this reaction is determined more by the T helper cell/antigen interaction than that of the CTL (Smid and Rouse, 1983). It may be therefore that in the transferred TD population cross-reactive helper T cell subsets seeded into a bed of anti-BTV memory cells induces more rapid development of direct cytotoxicity which brings about the observed heterologous protection.

Thus it would appear that the host's response to BTV infection involves both the cellular and humoral immune systems with protection from reinfection with the homologous
virus type most likely being antibody mediated, whereas, protection from heterologous challenge appears to rely on cell mediated effects. Repeated challenges with BTV will augment both effects through the amplifying effect of memory cells and the increasing cross-reactivity of the response.

These observations have parallels to studies carried out with influenza virus in mice and humans (Zweerink et al., 1977). These indicate that protection from homologous virus challenge is most effectively mediated by antibody even though the level of protection does not correlate well with haemagglutination inhibition titres (McMichael et al., Laver, 1982). Protection from heterologous virus challenge, however, has been shown to be a function of cross-reactive CTL's (McMichael et al., 1982).

Although the complete recovery from influenza infection is undoubtably due to many immunological factors, several recent reports have emphasized the crucial role of these cells. Wells and Ennis (1981) have shown that transfer of CTL's from mice during primary influenza infection and after secondary stimulation in vitro results in a decrease in pulmonary virus titres in recipient nude mice. The fact that these recipient mice showed no increase in serum antibody suggests that the T cells were responsible for viral clearance. Yap and Ada (1978) have reported similar findings, showing that adoptively transferred CTL's can protect mice from death following a lethal dose of influenza. Also, a cloned anti-influenza CTL line, has been shown to protect influenza infected mice from death (Lu and Askonas, 1980). Similar studies on the
importance of T cells in mice on recovery from ectromelia virus infection (Blanden, 1971) and herpes simplex virus (Kapoor et al., 1982) have also been reported.

More recent work however, indicates that the CTL alone may not be responsible for the heterotypic immunity and observed protection in vivo. With influenza cross-reactive T-helper cells which induce antibodies may also play a role (McMichael et al., 1982), whereas, in herpes simplex virus type I infections in mice, elevated serum interferon levels observed in the recipients of virus specific and H-2 compatible CTL's, may also contribute to the protective mechanism. This could be, either by increasing the susceptibility of infected cells to the action of CTL's, augmenting NK cell activity, or by interfering with the assembly of infectious virus (Sethi et al., 1983). The fact that complete virus clearance may depend upon a number of different responses is also illustrated by the heterologous BTV challenge. The antibody alone would not have affected virus titres although in conjunction with presumptive CTL's and helper cells appears to completely prevent virus replication. The action of this synergism is not clear although with herpes simplex virus, CTL clones and a non-neutralising antibody can similarly protect mice (Nash, personal communications). The possibility exists that this antibody reacts with the T cells in a way which has yet to be characterised, or that perhaps it improves antigen recognition and processing so that the recipients response is more vigorous and on top of the transferred cells, is sufficient to prevent virus replication.
Although it is highly pertinent to work in the host animal, work in ruminants is limited by the lack of suitable reagents to define the system. Hence although the transfer of TDL's gave rise to observable effects, the lack of suitable numbers of monozygotic animals, specific T cell subset markers and cell purification techniques in ruminants has meant that the analysis of these results is not always clear cut. Further detailed work may necessitate a return to a mouse model where the availability of well defined systems will enable a more exacting study to be carried out. It must be pointed out that these studies were carried out in sheep because of the obvious relevance of working in the correct host animal and because of the lack of response of mice to BTV infection and thus the inability to measure protection in the mouse. However improvements in virus isolation techniques may make it feasible to return to this model.

However, it is still advantageous to relate present findings to current BTV vaccines. It would appear that as with influenza vaccine policy, if a broad immunity is required against challenge from a number of virus types then a vaccine schedule should be used that will induce the greatest cellular immune response and this would preclude the use of inactivated preparations. However if homotypic protection is desired, then vaccines capable of inducing high levels of protective antibody should be used.

The risks associated with the use of live attenuated vaccines are many (Stott et al., 1979) but in areas requiring protection from a number of BTV types as in South Africa,
these may be outweighed by the ability of such preparations to induce broad protection, whilst the use of inactivated preparations would be most suitable in areas under the threat of challenge by one BTV type ie Cyprus.
1) The serial inoculation of two BTV types gives rise to a broad heterotypic antibody response. This response is transient but broadens following the inoculation of a third BTV type. These observations are seen in both cattle and sheep and with different combinations of BTV's.

2) The serial inoculation of two BTV types also gives protection from challenge with a third type. This occurs in both cattle and sheep and with different combinations of BTV types.

3) The simultaneous inoculation of three BTV types does not necessarily result in the replication of all three virus types and does not give rise to a broad heterotypic antibody response.

4) Immune sera has a role to play in protection from reinfection with BTV. However this protection is type specific and the degree of protection does not correlate with levels of neutralising antibody.

5) ADCC appears to have no part to play in immunity to BTV.
infections and although interferon does not appear to have a
direct effect on the curtailment of a BTV infection it may
play a part as an accessory factor in the immune response.

6) The inoculation of live BTV into mice gives rise to CTL's
whose activity peaks around day 7 pi.

7) The level of the murine CTL response is dependent on the
BTV type and does not occur when inactivated virus is used.

8) Anti-BTV murine CTL's are capable of varying cross-reactive
lysis within the BTV group.

9) The inoculation of BTV into sheep gives rise to ovine CTL's
whose activity peak around day 14 pi.

10) Ovine BTV induced CTL's are capable of reducing the
pyrexia and viraemia normally associated with a BTV infection.
In certain situations these cells can completely protect
against heterologous BTV challenge.

11) Cellular immunity, particularly the action of CTL's has a
part to play in recovery from a BTV infection and is
particularly important in providing short term heterotypic
immunity and protection from reinfection with a heterologous
BTV challenge.
These conclusions have implications in terms of BTV immunopathology and the control of BTV by vaccination.

One problem encountered throughout this work and that of others engaged in BTV research, is that of virus purification. Evidence continues to mount of cross-reactions between BTV serotypes and between BTV and other orbiviruses (Della-Porta et al., 1979). Until purified preparations of whole virus and virus antigens are available improved understanding in this area will be slow. The recent use of anti-BTV monoclonal antibodies (Letchworth and Appleton, 1983) in studies of virus structure and immunity to BTV infection, indicates the value of further work in this direction and indeed the use of monoclonals to help purify the virus and its components might be highly rewarding.

In contrast to the specificity of the antibody response, the anti-BTV CTL may offer a chance to reclassify the BTV's into functionally significant groups. The degree of cross-reaction found in this work suggests that only four or five major groups would emerge and this would lead to simplified vaccine schedules. Although work in ovine monozygotic sheep was highly rewarding, the existence of so many BTV serotypes and the lack of ovine cell markers necessitates a return to the mouse model. Here recent advances in cell cloning techniques and the availability of monoclonal antibody cell markers will enable rapid advances to be made in the study of BTV cell immunology. The recent use of cloned virus specific CTL lines (Braciale et al., 1981; Sethi et al., 1983) has enabled rapid advances to be made in understanding the role of
CMI in other virus infections and similar approaches should be undertaken with BTV.

There exists a need to rethink BTV vaccination, particularly in multi-type endemic areas. Clearly the serial inoculation of one or two BTV types should merit serious consideration. The use of different inactivants and of purified preparations in liposome carriers (Hackett et al., 1983) are areas worthy of further study.

Finally the vexing problem of virus persistence and overwintering mechanisms (Gibbs et al., 1979) must be addressed. Since it most likely revolves around the intracellular survival of the virus, mechanisms of cell lysis examined in this work should be pertinent. Defects in the immune response, such as the failure of ADCC to operate in BTV infections and the lack of susceptibility of the virus to interferon suggest that further studies on cellular immunity to BTV are most likely to provide solutions in this important area and its accompanying relevance to international trade restrictions.
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Summary. Mice immunized with a single bluetongue (BT) virus type were shown to produce cytotoxic T lymphocytes (CTL's) which cross-reacted with a number of BT virus types. These cross-reactive CTL's could be induced by both primary in vivo and secondary in vitro stimulation. A varying degree of cross-reactivity occurred with the six BT types examined. Aspects of the character of this cross-reactivity were examined and its role in protection from disease and vaccination strategy is discussed.

INTRODUCTION

Bluetongue is an infectious, non-contagious viral disease of ruminants, transmitted by insects and characterized by congestion, oedema and haemorrhage especially in sheep. The causative agent is classified as an orbivirus in the family Reoviridae. The viruses within this genus have now been separated on the basis of their in vitro serological reactions (Howell, 1963). Complement fixation has been used as the group test and serum neutralization as the type test (Boulanger & Frank, 1975). Based on this there now exist at least twenty known BT virus types.

Apart from these humoral responses bluetongue (BT) virus has been shown to induce a cell-mediated response in mice following the administration of live virus (Jeggo & Wardley, unpublished observations). These animals produce CTL's whose induction conforms to the patterns of H-2 restriction and virus specificity as demonstrated previously in other murine systems. A role for cell-mediated immunity (CMI) in protection from bluetongue disease in sheep, where protection has been demonstrated in the absence of neutralizing antibodies, has been inferred from work with inactivated BT virus vaccines (Stott, Osburn, Barber & Sawyer, 1979).

The importance of CMI for recovery from many viral infections has received much attention especially the role of CTL's. Recent work has demonstrated that within some virus groups these cells cause cross-reactive lysis (Rosenthal & Zinkernagel, 1980; Gajdowa, Mayer & Oravec, 1980; Webster & Askonas, 1980) and furthermore, in the influenza viruses, cross-reactive CTL's only occur following certain immunization procedures (Webster & Askonas, 1980).

Demonstration of cross-reactive CTL's to different types of bluetongue and a role for them in protection from disease could lead to improved vaccine procedures. This work investigates this possibility in mice using a number of BT virus types.

MATERIALS AND METHODS

Viruses
Bluetongue virus types were obtained originally from
the Veterinary Research Institute, Onderstepoort. They were passaged once or twice in egg embryos before adaption to BHK cells (passage numbers given following the BT number). BT4 was obtained as an isolate from the 1969 Cyprus bluetongue outbreak and designated ASOT 1. It was passaged three times in BHK cells. Virus stocks were prepared by growth in BHK cells and held at —70° before use. The following BT virus types were used: BT4 (ASOT 1), BT1 (E3BHK7), BT3 (E3BHK5), BT6 (E3BHK4), BT10 (E3BHK7), BT16 (E2BHK6). Titrations of virus were carried out in roller tubes using BHK cells.

Pseudorabies virus was kindly supplied by the Central Veterinary Laboratories, Weybridge, and was grown in renal swine cells (RS-2).

Ibaraki virus (BHK3) was obtained from the National Institute of Animal Health, Tokyo, Japan, Epizootic Haemorrhagic Disease, New Jersey strain (BHK5) was supplied by K. Herniman (A.V.R.I.) and Corriparta (BHK6) by Miss J. Taylor (Queensland Institute for Medical Research, Australia).

Mice
C3H(H-2K) mice were supplied by the Laboratory Animal Centre, Carshalton. Six to eight week old mice were immunized by intraperitoneal injection of stock viruses. For primary CTL assay the mice were killed by cervical dislocation 7 days later and the spleens removed aseptically. In double immunization procedures, mice were immunized 14 days apart and then killed 7 days after the second inoculation. For secondary in vitro studies mice were killed at least 14 days after immunization.

Cells
L929 cells were obtained from Flow Laboratories and maintained on Eagle's medium containing 10% ox serum together with penicillin (110 i.u./ml) and streptomycin (10 mg/ml).

Mouse spleen cell suspensions were prepared using standard techniques (Zweerink, Courtneidge, Skehel, Crumpton & Askonas, 1977), and finally resuspended in RPMI 1640 medium containing 10% foetal calf serum, 2 mM HEPES buffer and antibiotics at the above levels (RPMI-HEPES). For in vitro stimulation these suspensions were cultured at 10^6/ml in 10 ml volumes in upright Falcon Flasks (number 3024F). After 5 days, cells were harvested, washed twice in RPMI 1640 and viable cells used as effectors in the cytotoxic T-cell assay.

Cytotoxic T-cell assay
Primary and secondary in vitro assays were carried out as described previously (Jeggo & Wardley, submitted for publication). Briefly, 10^6 effector cells obtained from the spleens of mice which had undergone infection with BT virus were added in 100 μl amounts to flat-bottomed microtitre plates. BT virus and 51Cr labelled L929 cells acted as target cells and were added at 10^4 cells per well in 100 μl amounts. Plates were incubated at 37° in a humidified incubator containing 5% CO2 in air for 7 hr and centrifuged at 200 g for 1 min before half the contents of each well were harvested. Percentage specific release of 51Cr was calculated as follows: % specific lysis (SL) = [(effector cell/target cell release — target cell alone release)/ (total releasable 51Cr—target cell alone release)] x 100. Total releasable 51Cr was calculated from release in the presence of 1% Triton x 100.

‘Cold target’ competitive inhibition assay
Details of this assay procedure have been described elsewhere (Zinkernagel & Doherty, 1975). Briefly, unlabelled or ‘cold’ L cells infected with the appropriate BT type were mixed with effector spleen cells. This was immediately followed by the addition of 51Cr labelled L cells infected with a different bluetongue type, the rest of the assay was then carried out as for the primary assay.

Antibody titrations
Sera from mice infected with bluetongue virus were titrated for the presence of antibodies to BT virus by the group specific ELISA test (Hubschle, Lorenz & Matheka, 1981).

RESULTS
Reciprocal mouse and target cell priming with six different types of live BT virus revealed a pattern of complete and variable cross-reactivity (Table 1). Non-immunized mice and uninfected target cells exhibited low levels of specific lysis. Although a varied cross-reactivity occurred, the following points emerge: (i) there was no clear preference for the homologous interaction; (ii) certain BT type raised effector cell populations which were better able to lyse the six BT type infected target cells. In particular those produced by BT types 1, 6 and 10; (iii) certain BT type infected targets were more readily lysed by the various effector cells, e.g. BT types 1 and 10 infected targets. (iv) BT16
**Cross-reactive BT virus CTL's**

Table 1. Percentage specific lysis of various bluetongue virus type induced CTL’s* against various bluetongue virus type infected target cells†

<table>
<thead>
<tr>
<th>Effector cell types</th>
<th>Target cell types</th>
<th>Mean (%)</th>
<th>Lysis across six targets</th>
<th>Uninfected</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>One Three Four Six Ten Sixteen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>One</td>
<td>33† 23 24 31 47 8</td>
<td>27-6</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Three</td>
<td>14 9 8 11 13 0 9-2</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Four</td>
<td>19 15 20 7 28 2</td>
<td>15-1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Six</td>
<td>31 19 17 25 42 7</td>
<td>23-5</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Ten</td>
<td>29 20 21 20 42 4</td>
<td>22-6</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Sixteen</td>
<td>8 2 5 3 14 0</td>
<td>5-3</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Control§</td>
<td>1 5 5 3 1 0</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

* C3H mice spleen cells: C3H mice inoculated 7 days previously with approximately $10^6$ TCID$_{50}$ BT virus intraperitoneally.
† L929 cells infected approximately 24 hr previously with $5 \times 10^5$ TCID$_{50}$ of BT virus.
‡ Percentage specific lysis: 7 hr assay; 100:1 effector to target cell ratio. Minimum of three separate assays, two mice per assay. Standard error less than 7%.
§ Uninfected C3H mouse spleen cells.

induced effector cells and infected target cells gave low levels of specific lysis against the other five types examined.

**In vitro secondary stimulation**

**In vitro** secondary stimulation of BT type 4 primed spleen cells with BT types 4, 10 and 3 gave rise to specific lysis (Table 2) against BT 4 infected targets. Secondary stimulation with BT type 10 and type 4 gave similar levels of lysis against BT virus type 4 targets, whilst BT type 3 gave a lower but significant degree of lysis (Table 2). **In vitro** secondary stimulation of BT type 4 primed spleen cells with BT type 4 produced CTL’s which lysed BT types 4, 10 and 3 infected target cells (Table 3), although again a lower level of lysis occurred against BT 3 infected targets, whilst BT type 10 infected targets gave the highest level of lysis. Although secondary stimulation gave rise to cell populations which produced levels of lysis approximately twice those seen in primary assays, the ratio of the same target/effecter cell combinations was similar, e.g. in primary assay BTV4 effector on BTV4 infected targets = 20% SL (Table 1) BTV4 memory cells stimulated with BTV4 and tested on BTV4 infected targets 36.7% SL (Table 3) ratio 1:1.84. BTV4 effectors on BTV10 infected targets 28% SL (Table 1) BTV4 memory cells stimulated with BTV4 and tested on BTV10 infected targets 53% SL (Table 3) ratio 1:1.89. Thus, secondary stimulation **in vitro** of BT virus type 4 memory cells with various bluetongue virus types produces CTL’s which will lyse various BT type infected targets and the pattern of this cross-reactivity is similar to that found in the primary **in vivo** stimulation assay.

Table 2. Percentage specific lysis following **in vitro** secondary stimulation with various bluetongue virus types* of bluetongue type 4 memory cells†

<table>
<thead>
<tr>
<th>In vitro stimulant of memory cells</th>
<th>Target cells‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 ml BT4 (10$^6$ TCID$_{50}$/ml)</td>
<td>44-35</td>
</tr>
<tr>
<td>1 ml BT4 (10$^5$TCID$_{50}$/ml)</td>
<td>43-3</td>
</tr>
<tr>
<td>1 ml BT10 (10$^6$TCID$_{50}$/ml)</td>
<td>44-4</td>
</tr>
<tr>
<td>1 ml BT3 (10$^6$TCID$_{50}$/ml)</td>
<td>17-9</td>
</tr>
</tbody>
</table>

* Effector cells stimulated and held for 5 days in upright falcon flasks before assay. 25:1 Effector to target cell ratio, 7 hr assay at 37°.
† C3H mice spleen cells. Mice inoculated 3 weeks previously with $10^8$ TCID$_{50}$ BT4 given intraperitoneally.
‡ L929 cells infected with BT type 4.
§ Percentage specific lysis difference between infected and uninfected L-cell values.
Table 3. Percentage specific lysis against various bluetongue virus infected target cells following bluetongue virus in vitro stimulation of BT4 memory cells

<table>
<thead>
<tr>
<th>In vitro stimulant of memory cells*</th>
<th>Target cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>10⁸TCID₅₀/ml BT4</td>
<td>BT4  BT3  BT10</td>
</tr>
<tr>
<td>None</td>
<td>36-7† 15 53</td>
</tr>
<tr>
<td></td>
<td>8-8  6 16</td>
</tr>
</tbody>
</table>

* C3H mice spleen cells. Mice inoculated 3 weeks earlier with 10⁶ TCID₅₀ BT4 given intraperitonally.
† Percentage lysis using ⁵¹Cr release assay. Seven hour assay at 3⁷, 25:1 effector to target cell ratio.

Table 4. Percentage specific lysis of various bluetongue virus infected targets by CTL's induced following inoculation of BT type 4 primed mice with various BT virus in vitro

<table>
<thead>
<tr>
<th>Effector cells* second in vivo inoculation</th>
<th>Target cells†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BT4  BT10  BT16  Uninfected</td>
</tr>
<tr>
<td>BT4</td>
<td>9†  8  8  2</td>
</tr>
<tr>
<td>BT10</td>
<td>8  2  5  2-5</td>
</tr>
<tr>
<td>BT16</td>
<td>23  19  24-5  0</td>
</tr>
<tr>
<td>Pseudorabies virus</td>
<td>7-5  9  3-5  0</td>
</tr>
</tbody>
</table>

* C3H mice spleen cells. Mice inoculated 2 weeks before second in vivo inoculation with 10⁶ TCID₅₀ BT4 given intraperitonally. Second inoculation approximately 10⁶ TCID₅₀ of virus intraperitonally.
† L929 cells infected with various BT virus types.
†† Percentage specific lysis following 7 hr ⁵¹Cr release assay, 100:1 effector to target cell ratio.

Double in vivo immunization procedures

Following the immunization of C3H mice with two inoculations of BT virus, the CTL populations and antibodies induced were examined. Mice responded to BT virus type 4 immunization by producing a specific antibody. One inoculation produced sera which gave optical density readings on an ELISA of 0-4 which increased to 0-85 on two inoculations.

CTL production in mice initially immunized with BT type 4 and inoculated 2 weeks later with either BT type 4, BT type 10 or pseudorabies virus caused only low levels of lysis against L cells infected with BT types 4, 10 or 16. However, BT type 4 immunized mice inoculated with BT type 16 evoked CTL's which caused higher levels of lysis against all three BT type infected targets (Table 4). This lysis is all the more significant when compared with that induced by a single inoculation of BT type 16 (Table 1).

A probable explanation for the results from the double immunization procedure is that BT type 4 immunized mice will produce antibodies which will neutralize both types 4 and 10 as from the work of Erasmus (personal communication) some degree of antibody cross neutralization occurs between types 4 and 10 but not 16 and thus these secondary inoculated mice fail to produce high levels of CTL's. However, following BT type 16 inoculation, no neutralization occurs and an enhanced CTL response follows. A similar explanation has been used to explain analogous experiments with the influenza type A viruses (Effos, Doherty, Gerhard & Bennink, 1977) and VSV (Rosenthal & Zinkernagel, 1980).

‘Cold target’ competitive inhibition assay

Relationships between BT virus types 10, 16 and 6 were examined by interposing virus infected unlabelled L cells between effectors and targets. Optimum results were obtained at a ratio of cold targets to ⁵¹Cr labelled targets of 8:1 (Fig. 1). In the case of BT virus type 10 evoked effector cells, BT type 10 infected cold targets successfully inhibited homologous lysis, whereas type 6 cold targets caused less inhibition. If CTL subsets are produced against group and type antigens homologous cold targets will compete against both causing the maximum inhibition, whereas heterologous cold targets will only compete at the group level causing a less marked inhibition. Using BT virus type 6 cold targets with 10 effectors, a reduced effect occurred whilst BT virus type 16 cold targets had little or no effect. The low activity of BT virus type 16 cold targets in this assay reflects similar observations in the primary assay (Table 1) where BT virus type 16 only evokes low levels of CTL's and where type 16 infected targets show low levels of specific lysis in the presence of heterologous effectors. In the homologous BT virus type 6 test, however, where cold type 6 targets might be expected to cause the greatest inhibition, type 10 targets again resulted in the highest level of inhibition. These results further demonstrate the cross-reactive nature of BT virus evoked CTL's and again reflect the ability of certain BT types to cause more cross-reaction than other types.
Cross-reactive BT virus CTL's

Figure 1. Inhibition of immune spleen cell effectors (100:1) in a 7 hr assay using different ratios of cold, unlabelled competitor cells. The competitors were normal L cells, or L cells infected with Bluetongue types 10, 6 or 16. Both cold and labelled L cells were added to the effector cells at the same time. Cold targets BT 10 (O--O); BT 6 (●--●); BT 16 (*--*); uninfected (□--□). Level of lysis (X) with no cold targets shown on vertical axis.

Relations with other orbiviruses

Ibaraki, Epizootic Haemorrhagic Disease (EHD) and Corriparta virus infected L cells were not lysed by either BT 1 or BT 16 induced CTL's (Table 5). Serologically EHD and Ibaraki are considered closely related to BT virus (Borden, 1981). The lack of cross-reactive CTL's between these viruses again indicates that the antigens concerned with protective serological and CMI responses are different and that the cross-reactive CTL-evoking antigen which is present on BT viruses is different from that present on other orbiviruses.

Table 5. Percentage specific lysis of BT1 and BT16 induced CTL's against various orbivirus infected target cells

<table>
<thead>
<tr>
<th>Target cells†</th>
<th>Effector cells*</th>
<th>BT1</th>
<th>BT16</th>
<th>Ibaraki</th>
<th>EHD†</th>
<th>Corriparta</th>
</tr>
</thead>
<tbody>
<tr>
<td>BT1</td>
<td>24§</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>BT16</td>
<td>11</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* C3H mice spleen cells. Mice immunized 7 days previously with 10^6.8TCID_{50} BT virus type 1 or 16.
† L929 cells infected with appropriate virus. Approximately 5 × 10^6TCID_{50} 7 hr 51Cr release assay. 100:1 Effector to target cell ratio.
‡ Epizootic Haemorrhagic Disease virus.
§ Percentage specific lysis difference between infected and uninfected L-cell values.

DISCUSSION

The specificity of recognition by the BT virus evoked cytotoxic T cells was shown to have no clear preference for the homologous type (Table 1).

In contrast to this lack of discrimination on the part of the T-cell response, the humoral response to BTV appears to be type specific (Howell, 1963) and until recently (Stott et al., 1979) the production of antibodies either by attenuated or killed BT virus vaccines was the criteria by which such vaccines were assessed. A similar situation had existed with influenza virus, but here the discovery of cross-reactive T cells (Effros et al., 1977; Zweerink et al., 1977) followed by functional assays which have shown their importance in heterotypic challenge (Webster & Askonas, 1980),
has meant that vaccine procedures can now be functionally assessed in terms of both humoral and cell mediated immunity (Webster & Askonas, 1980). Apart from these two viruses cross-reactive T cells have also been described for vesicular stomatitis virus (Rosenthal & Zinkernagel, 1980) and flaviviruses (Gajdowa et al., 1980) and the possibility exists that this phenomenon may play a role in heterotypic immunity in other virus groups.

The level of responsiveness of both T and B cells to BTV is presumably regulated by both T-helper and suppressor cells. Although previous work has suggested that the receptor repertoire is similar for both (Binz & Wigzell, 1975) the results between the BT virus types (Table 1) and between other orbiviruses (Table 5) together with the influenza and vesicular stomatitis virus work suggest that distinct antigens are being recognized by the humoral and cellular immune systems. This particular facet of influenza immunology has received much attention (Zweerink et al., 1977; Effros et al., 1977) and a number of different explanations have been argued. With the advent of monoclonal antibodies the immunodominance of the influenza haemagglutinin molecule for antibody responses and its strict type response is in no doubt. Although there is evidence that T cells may also recognize part of the haemagglutinin molecule (Askonas & Webster, 1980; Koszinowski, Allen, Gething, Waterfield & Klenk, 1980; Braciale, Andrew & Braciale, 1981), it has been suggested that the internal RNP and M protein may account for this cross-reactivity (Biddison, Doherty & Webster, 1977; Reiss & Schulman, 1980). With BT virus, such elegant analysis of purified viral proteins has not been done, although sites on type-specific proteins 2 and 5 and the group antigen protein 7 (Huismans & Howell, 1973) are possible candidates for T-cell recognition.

Our results in mice are similar to parallel studies with influenza virus. With influenza virus, data have also accumulated indicating the functional importance of cell-mediated immunity (Larson, Tyrrell, Bowker, Potter & Schild, 1978) in protection in both man and mice (Webster & Askonas, 1980). The fact that BT virus is not lethal for mice, including nude and irradiated animals (unpublished observation) and that viraemias are of low levels and short duration (Jeggo & Wardley, unpublished observations) make the assessment of the functional importance of CTL's in BT virus infected mice difficult. Further, virus-specific CTL's remain to be identified in sheep. If, however, functionally important cross-reactive CTL's are shown to exist then this would prompt a further look at present vaccine policy. Firstly, we have previously shown that live virus is a prerequisite for a primary CTL response (Jeggo & Wardley, unpublished observations). Secondly, it is apparent that with the types tested certain BT virus types are more effective at inducing CTL's than others and if a broad heterotypic immunity may be advantageous then these should have priority as vaccine types. Thirdly, multiple vaccinations may be contraindicated if broad cross-protection is wanted as neutralizing antibody appears to decrease the cross-reactive CTL response. It should be stressed that the importance of these factors will only be fully realized if sheep CTL's can be demonstrated to have a protective effect. However, in recent work at this Institute, sheep infected with type 4 followed by type 3 have resisted challenge with type 6, although no neutralizing antibody to this type was demonstrable at the time of challenge (Jeggo, unpublished observation) perhaps indicating a cross-protective CTL response.

It is also of interest to note that the original protection work of Neitz in the 1940s, which showed far fewer groups than subsequent in vitro neutralization tests (Howell, 1963), i.e. more cross-protection between isolates, might well reflect the importance of this heterotypic response and not 'be merely a demonstration of the fallibility of cross-protection tests' (Howell, 1963).

ACKNOWLEDGMENTS

The authors would like to thank Mr. J. Anderson, Miss P. Hildred and Mr. T. Skeet for their technical assistance.

REFERENCES


Cross-reactive BT virus CTL's


Production of murine cytotoxic T lymphocytes by bluetongue virus following various immunisation procedures

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The induction of bluetongue virus specific cytotoxic T lymphocytes (CTLs) in C3H mice by various live and inactivated bluetongue virus preparations was studied. Live virus preparations were shown to induce good levels of CTLs; however, inactivation of virus preparations either by beta propiolactone or glutaraldehyde induced only a low level response. The use of Freund's adjuvants and double immunisation procedures failed to improve the response of the inactivated preparations. These findings are discussed in relationship to protection from bluetongue disease with various bluetongue virus vaccines.

BLUETONGUE virus (BTV) is transmitted to sheep by an insect vector and its control by means other than vaccination can present problems. However, even in endemic areas the use of live attenuated vaccines has drawbacks (Stott et al 1979), and it might be preferable to use inactivated preparations. At present it is not clear which host effector mechanism is responsible for protection and vaccines cannot yet be prepared to induce particular effector responses. Stott et al (1979) showed that an AEI inactivated vaccine preparation conferred protection in the absence of neutralising antibodies but in the presence of a cellular immune response as measured by blastogenesis. Again, in mice, BTV has been shown to be capable of inducing BTV specific cross-reactive cytotoxic T lymphocytes (CTLs) (Jeggo and Wardley 1982). These findings suggest that the cell mediated response to BTV may be important in protection. An investigation was therefore undertaken to investigate the ability of live and inactivated preparations to produce a cell mediated response as measured by the induction of CTLs.

Materials and methods

Viruses

BTV types 10 and 3 (obtained originally from the Veterinary Research Institute, Onderstepoort) were passaged once or twice in egg embryos before adaptation to baby hamster kidney (BHK) cells (passage numbers are given following the bluetongue number). BT type 4 was obtained as an original isolate from the 1969 Cyprus bluetongue outbreak, and was designated ASot 1. This was plaque purified and used as BT4 (BHK4). Bluetongue virus titrations were carried out in BHK cells using roller tubes and the titres expressed as the tissue culture infective dose for 50 per cent of tubes infected (TCID50).

Virus inactivation

Beta propiolactone (BPL). — BPL inactivation was carried out as described previously (Parker et al 1975). To summarise, 0·3 per cent BPL and virus were allowed to react for 30 minutes at 37°C and then overnight at 4°C.

Glutaraldehyde inactivation. Virus infected fixed cell vaccines were prepared according to the method of Powell (1975). In summary, BTV infected L929 cells expressing maximal surface membrane viral antigen were reacted with 0·15 per cent glutaraldehyde for 30 minutes, a time known to inactivate BTV. These fixed cells were then emulsified with equal volumes of Freund's complete or incomplete adjuvant.

Mouse immunisation

C3H mice were immunised by 0·5 ml intraperitoneal inoculation of live or inactivated virus preparation with or without adjuvant. Single inoculation was followed by spleen cell harvest seven days later. In animals inoculated twice the second inoculation was 14 days after the first. Spleen cell harvest then occurred seven days after this second inoculation.

Preparation of spleen cells

Single cell suspensions of mouse spleen cells were prepared in accordance with the method of Lawman et al (1980). These cells were either used in primary
cytotoxicity assay or cultured in vitro for secondary assays.

**In vitro culture and infection of spleen cells**

Spleen cells were adjusted to a cell concentration of $2 \times 10^6$/ml and cultured in upright Falcon Flasks (Flow Laboratories) at a cell density of $10^6$/cm$^2$ in 12 ml of RPMI 1640 with 10 per cent fetal calf serum (inactivated for 30 minutes at $56^\circ C$), 2 mM glutamine, 100 units/ml penicillin, 0.2 mg/ml streptomycin, 25 mM HEPES buffer and $5 \times 10^{-5}$ M 2-mercaptoethanol. One ml of live or BPL inactivated virus was added and flasks incubated for five days in humidified 5 per cent carbon dioxide at $37^\circ C$. Cells were then harvested, washed and counted for use in the cytotoxicity assay.

**Cytotoxicity assay**

These were performed according to the method of Lawman et al. (1980). Briefly, spleen cells were suspended in RPMI 1640 containing 7 per cent fetal calf serum buffered with HEPES, and added to flat bottomed microtitre wells containing $51$-chromium labelled L929 cells as targets, to give a final effector to target cell ratio of 100:1 for primary assays and 25:1 for secondary assays. These target cells had been infected 24 hours previously with BTV. Assays were carried out at $37^\circ C$ for seven hours and 50 per cent of the well content harvested for radioactive counting. Controls included target cells alone and unimmunised mouse spleen cells.

Per cent specific chromium release was calculated as follows:

\[
\% \text{ specific lysis} = \frac{\text{cpm release from test} - \text{cpm release in controls}}{\text{total releasable counts} - \text{cpm release in controls}} \times 100
\]

**Antibody estimation**

Mouse serum antibody was titrated using the group specific ELISA test (Hübschle et al. 1981).

**Results**

Mice inoculated with live BTV produced a CTL response as measured by the $51$-chromium release assay (Table 1). A second inoculation of live virus 21 days later failed to increase the level of this lysis (Table 1). BPL inactivated virus preparations, whether given in a single or double inoculation or with adjuvant, failed to induce lysis (Table 1). After inactivation with glutaraldehyde, however, lysis was observed but only at a low level (Table 2). Two inoculations of a preparation of fixed cells without adjuvant resulted in the highest level of lysis, although this was considerably below that observed with live virus preparations. Adjuvanted preparations produced even lower levels of lysis and the reason for this remains to be elucidated.

The ability of live or BPL or glutaraldehyde inactivated bluetongue virus infected cells

<table>
<thead>
<tr>
<th>Immunisation procedure</th>
<th>Target cell lysis expressed as % of value obtained with live virus</th>
</tr>
</thead>
<tbody>
<tr>
<td>live virus</td>
<td>100  0  100</td>
</tr>
<tr>
<td>inactivated virus</td>
<td>92   4  96</td>
</tr>
<tr>
<td>1 x inactivated virus</td>
<td>0    0  0</td>
</tr>
<tr>
<td>2 x inactivated virus</td>
<td>0    0  0</td>
</tr>
<tr>
<td>1 x inactivated virus in FICA</td>
<td>0  0  0</td>
</tr>
<tr>
<td>2 x inactivated virus in FICA</td>
<td>0  0  0</td>
</tr>
<tr>
<td>1 x inactivated virus in FCA</td>
<td>0  0  0</td>
</tr>
<tr>
<td>2 x inactivated virus in FCA</td>
<td>0  0  0</td>
</tr>
</tbody>
</table>

* C3H mice inoculated with 0.5 ml intraperitoneally either once and tested seven days later or twice, the two inoculations separated by two weeks and then tested seven days later. Mean results from three separate experiments. Two mice per immunisation procedure. SD less than 7 per cent
† Effector cells — C3H spleen cells. Target cells L cells infected 24 hours before assay with BT4 and labelled with $51$Cr.
‡ Seven hour release assay; 100:1 effector to target cell ratio
§ BT4 TCID50 $10^6$
FICA Freund's incomplete adjuvant
FCA Freund's complete adjuvant

**TABLE 2: Induction of CTLs in C3H mice following inoculation* with live and glutaraldehyde inactivated bluetongue virus infected cells**

<table>
<thead>
<tr>
<th>Immunisation procedure</th>
<th>Target cell lysis expressed as % of value obtained with live virus</th>
</tr>
</thead>
<tbody>
<tr>
<td>live virus</td>
<td>100  1  99</td>
</tr>
<tr>
<td>inactivated virus</td>
<td>97   7  90</td>
</tr>
<tr>
<td>1 x inactivated virus</td>
<td>28   6  22</td>
</tr>
<tr>
<td>2 x inactivated virus</td>
<td>59   9  50</td>
</tr>
<tr>
<td>1 x inactivated + FICA</td>
<td>5    4  1</td>
</tr>
<tr>
<td>2 x inactivated + FICA</td>
<td>0    0  0</td>
</tr>
<tr>
<td>1 x inactivated + FCA</td>
<td>24   14-7 9-3</td>
</tr>
<tr>
<td>2 x inactivated + FCA</td>
<td>35-7 13-9 18-2</td>
</tr>
</tbody>
</table>

* As in Table 1
§ Inactivated by 0-15 per cent glutaraldehyde 35 minutes at $4^\circ C$
effective. Inactivated virus without adjuvant was least inducing a high level antibody response while inoculations of live virus were most effective in and inactivated virus, is shown in Fig 1. Clearly, two specific ELISA tests, following administration of live virus preparations, using the group specific ELISA test, (Pooled sera from two mice per preparation) x 1 — single intraperitoneal injection of 0.5 ml preparation. x 2 — double intraperitoneal injection of 0.5 ml of preparation. Sera collected 10 days after final injection. Live — BT type 4 10^7 TCID50/ml. Inactivated — BT type 4 inactivated by 0.3 BPL for 30 minutes at 37°C and overnight at 4°C. FCA inactivated — inactivated as above and emulsified with an equal volume of Freund’s complete adjuvant. FCA inactivated — inactivated as above and emulsified with an equal volume of Freund’s incomplete adjuvant

![FIG 1: Measurement of the serum antibodies produced in C57BL/6 mice following the intraperitoneal injection of various bluetongue virus preparations, using the group specific ELISA test. (Pooled sera from two mice per preparation) x 1 — single intraperitoneal injection of 0.5 ml of preparation. x 2 — double intraperitoneal injection of 0.5 ml of preparation. Sera collected 10 days after final injection. Live — BT type 4 10^7 TCID50/ml. Inactivated — BT type 4 inactivated by 0.3 BPL for 30 minutes at 37°C and overnight at 4°C. FCA inactivated — inactivated as above and emulsified with an equal volume of Freund’s complete adjuvant. FCA inactivated — inactivated as above and emulsified with an equal volume of Freund’s incomplete adjuvant]

(Table 3). However, as was observed with the primary stimulation, the level of lysis was much lower than that caused by live virus preparations.

The antibody response as measured by the group-specific ELISA test, following administration of live and inactivated virus, is shown in Fig 1. Clearly, two inoculations of live virus were most effective in inducing a high level antibody response while inactivated virus without adjuvant was least effective.

**Discussion**

The concept that recovery from many viruses is a function of a cell mediated response is now well established and, although many different T cell subsets have been recognised, it appears that cytotoxic T cells may act by destroying virus-infected cells and preventing virus spread (Zinkernagel and Welsh 1972, Blandon 1974). The evidence that cell mediated immunity plays a role in BTV infections is increasing (Stott et al 1979, Jeggo and Wardley 1982) and we have also noticed that in sequential challenge experiments using different BTV types sheep have shown resistance to challenge in the absence of neutralising antibody (Jeggo, unpublished data). Although a role for CTL remains to be proven in the sheep, work in other animals has shown the importance of these cells (Webster and Askonas 1980) and we have also recently measured significant development of BTV induced CTLs in sheep undergoing BTV infections (Jeggo and Wardley, unpublished observation). It thus seems probable that CTLs are important in BTV recovery and protection, and hence their induction can be assumed to be a desirable characteristic of any bluetongue vaccine preparation.

Our results indicate that on primary assays none of the inactivated virus preparations produced a CTL response (Table 1), even though the preparations were immunogenic as shown by their induction of antibody (Fig 1). On the other hand, virus infected fixed cell preparations induced a primary response which was improved by a second inoculation and such preparations may, as has been shown with other viruses (Powell 1975), provide an effective vaccine. Further work needs to be done to improve these preparations and to substantiate this claim. From a
theoretical viewpoint, however, the presentation of virus antigen fixed in a cell membrane has a number of distinct advantages because of the way in which CTLs recognise and kill virus infected cells (Zinkernagel and Welsh 1976, Davidson 1977). It may also be that virus induced products which would be present in fixed cell preparations and displayed at cell surfaces, but are not present in purified virus preparation, play some role in CTL induction.

The secondary stimulation assays may also supply an explanation for the protection seen by Stott et al (1979) using inactivated vaccines, in that although such preparations produce no primary CTL response, a low level of memory cells is induced (Table 3). Thus, on challenge, these cells could quickly proliferate to limit virus multiplication and clinical signs.

Evidence that the presentation of virus or induced antigens are responsible for the induction of an antibody or CTL response highlights the need for improved purified BTV preparations so that the proteins responsible for these immune effects can be identified and used to produce improved vaccines.

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References
ZINKERNAGEL, R. M. & WELSH, R. M. (1972) Journal of Immunology 17, 1495-1501
The Induction of Murine Cytotoxic T Lymphocytes by Bluetongue Virus

By

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With 1 Figure

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Summary

After inoculation with live bluetongue virus, mice produced cytotoxic T lymphocytes (CTL) which showed virus and H-2 restriction. Inactivated preparations failed to induce CTLs. On secondary in vitro stimulation, specifically sensitised memory cells also produced high numbers of CTLs. The need for replicating virus to induce primary CTLs, evidence for partial type specificity and the role which cell-mediated immunity might play in the early stages of a bluetongue virus infection are discussed.

Introduction

Bluetongue virus is an orbivirus affecting sheep, cattle, goats and wild ruminants. Many aspects of the pathogenesis and immune response of infected animals to this virus are not fully understood (16) and both inactivated and attenuated vaccines have been advocated to help control the disease. In South Africa attenuated vaccines have enjoyed widespread use in the control of the disease. However, the possibility of reversion to virulence, abortion in pregnant ewes, reassortment among bluetongue (BT) virus genomes in multivalent preparations and possible persistence of vaccine virus in cattle make the use of live attenuated vaccines in bluetongue endemic areas questionable and precludes their use in non-endemic areas. It is therefore essential to evaluate the ability of various inactivated vaccine preparations and vaccine protocols.

Workers have recently suggested that a cell-mediated immune (CMI) response is involved in protection from BT virus re-infection and that sheep produce such a response on administration of an inactivated vaccine (28). Of the T cell subsets involved in CMI responses (1, 3), cytotoxic T cells have the ability to destroy virus-infected cells early in infection and so prevent viral spread (32). CTL induction by bluetongue virus was studied in a mouse model with special reference to the ability of live and inactivated preparations to produce CTLs in vivo or on secondary stimulation in vitro.
Materials and Methods

Viruses

Bluetongue virus types four, three and two (BT4, BT3, BT2) were originally obtained from the Veterinary Research Institute, Onderstepoort. BHK grown virus stocks were prepared by three passages in suckling mouse brain followed by six passages (BT4 and BT2) or seven passages (BT3) in BHK cells; BT4 mouse brain virus stocks were obtained by a further three passages in suckling mouse brains. Titrations of virus were carried out in roller tubes using BHK cells. Stock viruses had titres of 10^{6.8} TCID_{50}/ml. The mouse brain BT4 stock had a titre of 10^{8.5} TCID_{50}/ml. Pseudorabies virus was kindly supplied by the Central Veterinary Laboratory, Weybridge, and was grown in pig kidney cells (IB-RS-2).

All stock viruses were held at −70°C prior to use.

Virus Inactivation

Virus was inactivated with 0.3 per cent beta-propiolactone (BPL) buffered with 0.1 M Tris (22). The mixture was held at 37°C for half an hour and overnight at 4°C before use. Heat inactivation was carried out by holding the virus at 56°C for 60 minutes. Residual infectivity was detected by titration in BHK cells.

Virus Isolation

Tissues from infected mice were examined for virus using the following method. Heart and a small section of spleen were removed immediately after killing the mice and stored at −70°C. Before titration, the heart and spleen were ground with sterile sand and the mixture suspended in phosphate buffered saline containing 0.2 per cent bovine albumin (PBS/BSA) to give a 10 per cent (w/v) suspension. After sonication the samples were centrifuged for 5 minutes at 3000 rpm and the resulting supernatant was diluted in 10-fold dilution steps in PBS/BSA. Dilutions were then titrated in monolayer roller tube cultures of BHK cells, using 5 tubes per dilution.

Mice

C3H (H-2k) mice were supplied by the Laboratory Animal Centre, Carshalton; the Balb C (H-2d) mice were reared at this Institute.

Six to eight week old mice were immunized by a single intraperitoneal injection of 0.5 ml of a stock virus or inactivated virus and at various times afterwards were killed by cervical dislocation and the spleens removed aseptically.

Cells

L929 (H-2k) and 3T3 B (H-2d) cells were obtained from Flow Laboratories and maintained on Eagle’s medium containing 10 per cent ox serum together with penicillin (110 units/ml) and streptomycin (10 mg/ml).

Mouse spleen cell suspensions were prepared using the technique described by Lawman et al. (17) and finally resuspended in RPMI 1640 medium containing 10 per cent foetal calf serum, 2 mM HEPES buffer and antibiotics at the above levels (RPMI-HEPES).

T cell enriched populations were obtained from mouse spleen cell suspensions by the nylon wool adherence technique of Julius et al. (13) or by treating suspensions with rabbit anti-mouse IgG serum plus normal rabbit serum to provide a source of complement. T cell depleted populations were obtained by treating cells with anti-thymocyte sera (Miles Laboratories) and rabbit complement.

Experimental Design

Confluent monolayers of L929 or 3T3 B cells in 90 mm plastic Petri dishes were simultaneously incubated with 100 μCi of Na_2^{[51]Cr}O_4 (Radiochemical Centre, Amersham) and 10^{8.8} TCID_{50} of BHK grown BT virus type 4 or 3 for 1 hour at 37°C. Cells were washed twice in Eagle’s medium, re-fed with fresh medium and left for 24 hours, a time at which a radioimmunoassay to detect cell surface virus antigen gave
maximum results. They were then removed from dishes with trypsin and versene, washed three times in Eagle’s medium and adjusted in RPMI-HEPES to give $10^5$ cells/ml. Now designated as target cells, $10^4$ were added in $100 \mu l$ volumes to the wells of flat-bottomed microtitre plates. Effector cells were obtained from the spleens of mice that had been infected with BT virus and were suspended in RPMI-HEPES and added to microtitre plates in $100 \mu l$ volumes. All assays were carried out in six-well replicates using various effector-to-target ratios. Plates were incubated at $37^\circ C$ in a humidified incubator containing 5 per cent CO$_2$ in air for 7 hours and then centrifuged at $200 \times g$ for 1 minute before harvesting. Half of the contents of each well were taken and used to estimate the release of $[^{51}Cr]$, using a gamma counter (MR 1032, Kontron Ltd.).

The percentage specific release of $[^{51}Cr]$ was calculated as follows: (Effector cell/target cell release minus target cell alone release) divided by (total releasable chromium minus target cell alone release) multiplied by 100. Total releasable chromium was obtained by exposing target cells to 2.0 per cent Triton X-100.

For in vitro culture, spleens were removed from mice inoculated at least 14 days previously with inactivated or live stock BT4 virus. Fifteen ml of a spleen cell suspension adjusted to contain $1 \times 10^7$/ml in RPMI-HEPES plus $5 \times 10^{-5}$ M 2-mercaptoethanol were incubated in plastic flasks (Falcon flask number 3024F) with 1 ml of live or heat-inactivated BT4 for 8 days at $37^\circ C$ in a humidified atmosphere containing 5 per cent CO$_2$. Prior to the cytotoxicity assay the cells were harvested, the viable cells counted, approximately 30 per cent of cultured cells, washed twice in Eagle’s medium and resuspended in RPMI-HEPES.

**Results**

**Induction of Cytotoxic T Lymphocytes**

**Primary Induction**

Infection of C3H mice with BT4 gave rise to the development of cytotoxic spleen cells which caused the maximum lysis (28 per cent) of target L cells between 7 and 8 days post infection and dropped to undetectable levels by 18—20 days after infection (Fig. 1). There was a fall in the extent of lysis as the effector-
to-target ratio was reduced from 100:1 to 25:1 (Table 1). Uninfected cells were also lysed by spleen cells harvested between 2 and 7 days after infection but the level of lysis was always very much lower than that seen in infected cells (Fig. 1). Although after 7 days, lysis of uninfected cells was normally less than 2.5 per cent on some occasions higher levels did occur (Table 1) the reason for this variability is not known.

Similar numbers of CTLs were produced whether mice were infected with virus from mouse brain or from BHK cells and so virus grown in BHK cells was used routinely. Unlike previous workers (20), we found (Table 3) that BHK or mouse-adapted virus could be recovered from the heart or spleen of mice following intraperitoneal inoculation. The titres of virus reached a peak between 3 and 4 days after infection, the time of which CTL activity appeared (Fig. 1); by 8 days virus was no longer recovered. However, in spite of this replication, the development of CTLs was shown to depend on the immunising dose of virus given (Table 2).

Mice inoculated with virus inactivated with heat or BPL did not produce CTLs (Table 2), so live virus is needed for the primary induction of CTLs by a single inoculation.

Table 1. Primary induction of anti-bluetongue CTL using various effectora to targetb cell ratios

<table>
<thead>
<tr>
<th>Effector: Target cell ratio</th>
<th>% specific lysisc</th>
<th>Infected targets</th>
<th>Non-infected targets</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>100:1</td>
<td>43.6</td>
<td>13.3</td>
<td>30.3</td>
<td></td>
</tr>
<tr>
<td>50:1</td>
<td>28</td>
<td>8.3</td>
<td>19.7</td>
<td></td>
</tr>
<tr>
<td>25:1</td>
<td>16</td>
<td>7.4</td>
<td>8.6</td>
<td></td>
</tr>
</tbody>
</table>

a C3H spleen cells immunized 7 days previously with 0.5 x 10^6 TCID_{50}/ml BT4
b L cells + BT4
c 7 hour assay time. Minimum of 2 mice per experiment. Lysis expressed as a mean of the individual mouse results

Table 2. Primary induction of anti-bluetongue virus CTL using various effector cell populations

<table>
<thead>
<tr>
<th>Effector cell population</th>
<th>Stimulant</th>
<th>% specific lysisa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immune C3H spleen cells</td>
<td>10^4.8 BTV 4 mouse brainb</td>
<td>35.3 1.1</td>
</tr>
<tr>
<td>10^6.8 BTV 4 BHK</td>
<td>35.7 0.7</td>
<td></td>
</tr>
<tr>
<td>10^2.0 BTV 4 BHK</td>
<td>9.6 2.1</td>
<td></td>
</tr>
<tr>
<td>10^1.0 BTV 4 BHK</td>
<td>7.0 1.6</td>
<td></td>
</tr>
<tr>
<td>10^6.8 BTV BPL inactivated</td>
<td>0.0 0.7</td>
<td></td>
</tr>
<tr>
<td>10^6.8 BTV 56°C 60 minutes</td>
<td>0.0 0.0</td>
<td></td>
</tr>
</tbody>
</table>

a Mean of 6 assays; assay time 7 hours. Effector to target cell ratio of 100:1. Standard error less than 6 per cent.
b Mice inoculated 7 days prior to assay with 0.5 ml of virus. Minimum of 2 mice per experiment. Lysis expressed as a mean of the individual mouse results.
Secondary Induction

Spleen cells from mice infected at least 14 days previously with live virus developed high levels of cytotoxicity upon secondary stimulation \textit{in vitro} with either live or inactivated BT4 virus (Table 4). At an effector-to-target cell ratio of 25:1, cells stimulated by live virus showed a specific cytotoxicity of 64.8 per cent, whereas the previous use \textit{in vivo} of similar numbers of stimulation effector cells at this level had never produced lytic levels above 10 per cent (Table 2). Even though the numbers of effector cells in primary assays (Table 2) were increased four-fold such high levels of cytotoxicity were not found.

Table 3. \textit{Level of virus in tissues following inoculation\(^a\) of C\(_2\)H mice with bluetongue virus type 4}

<table>
<thead>
<tr>
<th>Days post infection</th>
<th>BT4 BHK(^b)</th>
<th>BT4 Mouse brain(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Heart(^d)</td>
<td>Spleen(^e)</td>
</tr>
<tr>
<td></td>
<td>10(^{2.1})</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>10(^{2.5})</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>10(^{3.3})</td>
<td>10(^{2.4})</td>
</tr>
<tr>
<td>5</td>
<td>10(^{1.8})</td>
<td>10(^{1.3})</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
<td>Trace</td>
</tr>
<tr>
<td>9</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>11</td>
<td>N.T.</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^a\) 0.5 ml of virus injected intra-peritoneally. 2 mice per protocol
\(^b\) Bluetongue virus type four passaged 6 times in baby Hamster kidney cell line prior to inoculation
\(^c\) Bluetongue virus type four passaged 3 times in suckling mice prior to inoculation
\(^d\) Whole heart ground up with sterile sand and diluted out using log dilutions in phosphate buffered saline (PBS) + 0.2 per cent bovine albumin
\(^e\) Spleen cell suspension (containing approx. 1 x 10\(^6\) spleen cells), diluted out using log dilutions in PBS + 0.2 per cent bovine albumin
\(^f\) Virus titration: Expressed as the TCID\(_{50}\)/ml using 5 tubes per dilutions
0 No virus detected
Trace 1/5 tubes using undiluted heart/spleen
N.T. Not tested

Table 4. \textit{Secondary induction of cytotoxic T lymphocytes by \textit{in vitro} stimulation of primed spleen cells\(^a\)}

<table>
<thead>
<tr>
<th>Effector cells(^a)</th>
<th>Target cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{In vivo}</td>
<td>Infected targets(^c)</td>
</tr>
<tr>
<td>\textit{In vitro}</td>
<td></td>
</tr>
<tr>
<td>Stimulant</td>
<td></td>
</tr>
<tr>
<td>BT4 None</td>
<td>1.2</td>
</tr>
<tr>
<td>BT4 Inactivated virus(^d)</td>
<td>37.9</td>
</tr>
<tr>
<td>BT4 Live virus(^e)</td>
<td>64.8</td>
</tr>
<tr>
<td>Inactivated BT4</td>
<td>25.7</td>
</tr>
</tbody>
</table>

\(^a\) Spleen cells removed from mice infected 14 days with BPL inactivated or live virus (10\(^{6.8}\) TCID\(_{50}\)/ml) pooled preparations incubated \textit{in vitro} for 5 days prior to assay
\(^b\) Carried out after 7 hours incubation of effector and target cells, 6 well duplicates.
\(^c\) Standard error less than 7 per cent
\(^d\) L cells + BT4 25:1 effector to target cell ratios
\(^e\) BT4 inactivated for 1 hour at 56°C, added to an \textit{in vitro} spleen cell culture 5 days prior to assay

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Spleen cells from mice inoculated with BPL-inactivated BT4 and secondarily stimulated \textit{in vitro} with live BT4 also produced CTL's (Table 4). Hence, although inactivated preparations do not produce primary CTL induction, they do produce specific memory cells.

**T Cell Dependence**

To substantiate the fact that the lysis observed in primary assays is mediated by T cells, procedures aimed at enriching T cell numbers in spleen cell suspensions were carried out. The passage of spleen cell suspensions over nylon wool columns led to an increase in lysis from 25.3 to 35.3 per cent (Table 5). Treatment of spleen cell suspensions with anti-thymocyte serum and complement completely prevented lysis of infected target cells (Table 5).

\textbf{Table 5. Effects of various treatments on anti-bluetongue CTL populations}\(^a\)

<table>
<thead>
<tr>
<th>Effector cell population</th>
<th>L cells + BTV</th>
<th>L cells + BTV pseudorabies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-immune</td>
<td>0</td>
<td>0.7</td>
</tr>
<tr>
<td>Immune Balb c spleen</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Immune C(_3)H spleen cells</td>
<td>25.7</td>
<td>0.4</td>
</tr>
<tr>
<td>Nylon wool non-adherent</td>
<td>35.9</td>
<td>0.6</td>
</tr>
<tr>
<td>Antithymocyte 1.2 serum + complement</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Anti IgG + complement</td>
<td>37.4</td>
<td>0.2</td>
</tr>
<tr>
<td>Complement</td>
<td>26.9</td>
<td>0.2</td>
</tr>
</tbody>
</table>

\(^a\) C\(_3\)H mice spleen cells. Immunized 7 days with 0.5 \(\times 10^6\) TCID\(_{50}\)/ml, BT4, BHK

\(^b\) Means of six assays. Assay time 7 hours. Effector to target ratio 100:1 standard error less than 4 per cent. Minimum of 2 mice per experiment. Lysis expressed as a mean of the individual mouse results.

Following the use of anti-IgG serum, an increase in lysis occurred from 25.7 to 37.4 per cent (Table 5). This increase may reflect the removal of humoral mediated suppression of CMI (12, 15) and an increase in the percentage of CTLs in the spleen cell suspensions after treatment with anti-IgG serum.

Although similar experiments were not carried out on secondarily stimulated CTLs it is also assumed that these cells are cytotoxic T cells and not \textit{in vitro} generated NK cells. Apart from direct evidence in other systems that this is the case (34) mouse NK cell reactivity has been shown to be relatively labile in culture and hence after 5 days would not be expected to contribute to the observed lysis (11, 21, 29).

**Viral and Target Cell Specificity**

To demonstrate that the phenomenon of H-2 restriction occurred in this system, Balb C and C\(_3\)H immune spleen cells were used as effector cells against non-histocompatible targets and no lysis occurred (Table 5).

To demonstrate virus specificity, L929 or 373 B cells were infected with pseudorabies virus and again no lysis occurred (Table 5).
Hence, the characteristic viral and target cell specificity noted in other murine T cell mediated anti-viral cytotoxicities (6, 33, 10, 17) were also seen in the BT virus assay.

However, L cells infected with BT3 or 3T3B cells infected with BT2 were susceptible to lysis by histocompatible BT4 immunized mouse spleen cells, although the level of lytic activity was reduced by approximately 65 per cent (Table 6) in comparison to BT4 infected targets.

Table 6. Lysis by anti-bluetongue type 4 CTL against various target cells

<table>
<thead>
<tr>
<th>Effector cell population*</th>
<th>Target cell infection</th>
<th>Target cell type</th>
<th>% specific lysisb &lt;br&gt;(Infected targets minus uninfected control cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immune C3H</td>
<td>BT4</td>
<td>L929</td>
<td>27.0</td>
</tr>
<tr>
<td>Immune C3H</td>
<td>BT3</td>
<td>L929</td>
<td>9.6</td>
</tr>
<tr>
<td>Immune Balb c</td>
<td>BT4</td>
<td>3T3B</td>
<td>14.9</td>
</tr>
<tr>
<td>Immune Balb c</td>
<td>BT2</td>
<td>3T3B</td>
<td>5.1</td>
</tr>
</tbody>
</table>

* Mice spleen cells inoculated 7 days previously with 0.5 ml of BT4 intra-peritoneally. Minimum of 3 mice per experiment

b 7 hour assay time: 100:1 effector to target cell ratio. Minimum of 2 mice per experiment. Lysis expressed as a mean of the individual mouse results

Discussion

The characteristics of the cytotoxicity response of mice to BT4 virus infection provides evidence that it is T cell mediated and conforms to the pattern of virus and H-2 restriction described for many other murine systems (6, 33, 10, 17).

At two to five days after infection, spleen cell suspension caused significant lysis of non-infected target cells (Fig. 1). The early appearance of this activity, the fact that it was only observed in spleen cell suspensions from virus-infected mice and that it was also observed against non-infected targets argues that we are observing natural killer cell activity (25, 31). BT virus is known to be highly effective at producing interferon (24)—a powerful augmenter of NK cell activity (30, 9, 31). This may be important in control of BT virus infection in mice prior to the development of specifically sensitized CTLs. The maximum lysis observed by the CTLs was considerably lower than that described for some other systems (23) and it is possible that some regulating event occurs in vivo, as has been argued with CTL induction in mice infected with herpes simplex virus (17). As has been found with murine cytomegalovirus (23, 18), the level of the lytic response to BT virus was also dose-dependent.

We were unable, however, to demonstrate primary CTL induction with inactivated preparations (Table 1). This is important at two levels. Firstly, there are considerable differences in the ability of various live or inactivated viruses to produce a CMI response (5, 7, 14). Thus, live and inactivated vaccinia (10) and Sendai virus (27) preparations are equally effective in promoting a CMI response, whereas some authors (5, 17) have shown that inactivated herpes and influenza virus fail to elicit CTL production. It has been argued (10) that some inactivated viruses do not fuse into the membrane of stimulator cells to produce the necessary
H-2 interaction for CTL production. Recent work with herpes simplex virus (26) has investigated this facet of CTL production and emphasized the importance of antigen triggering of helper cells to produce a factor which acts on precursor cells.

Secondly, it has been shown in sheep, using adjuvanted inactivated BT vaccines (28), that such preparations can mediate protection against infection, apparently by stimulating a CMI response, as measured by a lymphocyte transformation test. In mice, however, we have been unable to demonstrate primary CTL production by inactivated virus (Table 1). However, as has been found with herpes simplex virus (17), BT virus inactivated virus did induce memory T cell production which on in vitro culture gave large numbers of CTLs (Table 4). If this situation could be shown with BT virus in sheep, then rapid CTL production upon challenge could confer protection and be the mechanism by which inactivated vaccines protect sheep against live virus challenge.

Although the protective effect of both the humoral and CMI components in BT virus infections still await evaluation, serum neutralizing antibody is produced only in response to type antigen (4). Also Luedke and Jochim (19) have shown that in many animals there is a poor correlation between serum neutralizing antibody and protection which argues for a protective CMI component in the immune response. Our data in both C3H and Balb C mice, however, indicate that a single BT virus inoculation results in the production of CTL's which can lyse heterologously infected targets. Such cross-reactive T cells have been described in other virus systems (8, 34). If such a response is a general phenomenon in the BT virus group and the CTLs produced can be shown to have a protective effect, then single-type attenuated virus vaccines could provide a degree of heterotypic immunity.

References


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Clinical and serological response of sheep to serial challenge with different bluetongue virus types

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A group of British sheep was infected with bluetongue virus 5 (BTV5) and subsequently challenged with the same virus type. Protection from this challenge and a homotypic BTV neutralising antibody response were observed. A second group of sheep was infected serially with three different BTV types. Animals previously exposed to BTV4 and BTV3 were found to be resistant to challenge by BTV6. Animals infected with BTV4 and challenged with BTV3 were shown to produce a transient heterotypic neutralising antibody response to a number of types. Although the level of this heterotypic response diminished with time, after challenge with BTV6 these animals developed a similar broad heterotypic response. The nature of this response and its implications in terms of observed protection merit consideration in future vaccine design and evaluation of field survey work.

The causative agent of bluetongue, an orbivirus, has been classified into 20 serological types based on an in vitro virus neutralisation test (Howell 1960, Boulanger and Frank 1975). Protection from disease in areas in which a number of bluetongue virus (BTV) types exist depends on the use of polyvalent virus vaccines, but the degree of protection afforded by these vaccines is ill defined (Howell 1979).

The ability of serial infection with two or more BTV types to evoke a broad spectrum antibody response and heterotypic immunity could have wide implications in terms of both polyvalent vaccine design and analysis of field sera results to predict virus types present in an area. The work described below examines the effect of serial challenge in terms of clinical manifestation of disease and the humoral response in a group of British sheep.

Materials and methods

Viruses

BTV 3, 5 and 6 were obtained from the Veterinary Research Institute, Onderstepoort; BTV3 had undergone one passage in embryonated eggs and five passages in BHK21 cells (E1/BHK5) and BTV5 and 6 were in passage E2/BHK4. BTV4 was obtained as an isolate from the 1969 Cyprus bluetongue outbreak and was designated A SOT1 (E1/BHK4).

Each BTV was plaque purified three times in baby hamster kidney (BHK) cells using the agar gel double sandwich suspension technique (Cooper 1961). Following plaque purification, virus type specificity was verified in a virus neutralisation test using type-specific guinea pig sera.

Experimental animals

Male crossbred Dorset Horn sheep obtained at six to eight months old were used. In the first experiment a group of four sheep was infected with BTV5 and subsequently challenged with homologous virus. In the second experiment, a group of eight sheep was serially inoculated with BTV4, 3 and 6. After one exposure, the subsequent inoculation was begun only when the previously established viraemia was no longer detected. At the time of the second and third inoculations, groups of sheep susceptible to BTV were infected with the appropriate virus to act as inoculation controls.

Animals were infected by inoculating 1 ml containing approximately 10^7 median tissue culture infective doses (TCID50) of BTV intradermally in the left ear. The animals were clinically examined daily, temperatures being recorded at the same time each day for up to three weeks after inoculation. Daily serum samples were collected together with heparinised blood (5 iu heparin per ml final concentration) for virus isolation. All animals were held in insect-proof and BTV-secure accommodation during the experiments.

Virus isolation procedures

Heparinised whole blood was centrifuged and the packed cells washed three times in an approximately
equal volume of isotonic saline solution. These washed cells were sonicated at 110 watts for 15 seconds (MSE Soniprep 150) before titration for the presence of BTV in roller tubes. Blood was diluted in 10-fold steps in phosphate buffered saline (PBS) containing 0.2 per cent bovine serum albumin. Each of five roller tubes containing a 24 hour old monolayer of BHK cells was inoculated with 0.2 ml of the appropriate blood dilution. Adsorption of virus was allowed for 30 minutes at 37°C before refeeding with Glasgow modified Eagle's medium containing 5 per cent newborn calf serum, 10 per cent tryptose phosphate broth and antibiotics at the usual concentrations. The tubes were rolled at 37°C. The following day all cultures were washed twice with PBS and refed with the above medium. The tubes were examined daily for BTV cytopathic effect for up to eight days and fluid changed every second day. Viraemia levels were expressed as log_{10} TCID50/ml of original packed cells. From each experiment, a virus isolated from the blood of an infected sheep was typed using the virus neutralisation test.

Serological procedures

Agar gel tests for BTV group-specific precipitating antibodies (Jochim and Chow 1969) were conducted on microscope slides, using antigen derived by ammonium sulphate precipitation of infected cell cultures (Eisa et al 1982). Virus neutralisation tests were carried out in a microneutralisation system with an initial serum dilution of 1:10 against 100 TCID50 of virus per well. It was necessary to double the number of cells added to test wells when examining sera from animals receiving three different BTV types, owing to the cytotoxic effect of these sera against BTV cells (Parker et al 1977).

Results

Inoculation and challenge of sheep with BTV5

Four sheep inoculated with BTV5 showed an incubation period of five days followed by a pyrexia lasting five days (Fig 1); the mean peak temperature for this group of animals was 40.3°C attained on day 6 after inoculation. No other clinical abnormalities were observed. Virus was detectable in the blood as early as day 2 in all four sheep and rose to peak levels between days 6 and 8. Viraemia levels declined slowly but virus continued to be recovered from the blood until day 23 in two of the four animals. After 43 days, these sheep were challenged with the homologous virus type; no pyrexia was seen for the following 21 days and BTV could not be detected in the blood of these animals.

All four animals developed BTV group-specific precipitating antibodies on day 10 and retained this response for the rest of the experiment. The presence of neutralising antibody to BTV5 was first detected on day 10 in three of the four sheep and on day 11 in the fourth animal. The level of neutralising antibody rose to reach maximum titres of around 1:120 by day 15. No significant increase occurred following challenge at day 43. Sera from these animals were also examined for the presence of antibodies to the remaining 19 BTV types and no neutralising antibodies were detected in any sera collected during the periods before and after challenge.

Inoculation of eight sheep with BTV4 followed by sequential exposure with types 3 and 6

A group of eight sheep was inoculated with BTV4. The patterns of temperature response and blood virus levels were similar to those described above for BTV5. Mean peak temperature and viraemia levels were recorded on day 7 and the viraemia lasted 24 days in six of the eight animals and 26 days in the remaining two animals. No other clinical abnormalities were noted. All eight animals produced precipitating antibodies on day 11 and these were maintained throughout the remainder of the experiment. The development of neutralising antibodies to BTV4 can be seen in Fig 2. Antibodies were first detected on day 10 and mean levels remained at around 1:40 until the second virus inoculation. Examination for the presence of antibodies to the other 19 BTV types in these animals gave negative results.

Sixty-six days later the eight principal sheep were inoculated with BTV3. The pattern of temperature response and viraemias obtained was similar to that following the BTV4 inoculation. No distinction could be made between the pyrexia and viraemias established in sheep previously inoculated with BTV4 and a group of control sheep receiving BTV3 only.
Bluetongue in sheep

The four control sheep showed a neutralising antibody response to type 3. The principals also developed a type 3 neutralising antibody response (Fig 2) together with an increase in the previously established type 4 neutralising antibody level (Fig 2). Significantly, antibodies to BTV6 were detectable between the ninth and 74th days after this second virus infection, although sheep had not as yet been exposed to this virus type. Other aspects of this non-specific antibody response are described below.

Finally, on experimental day 176, the principals were inoculated with BTV6. Although inoculation controls demonstrated the typical response to a single BTV serotype, the eight principal sheep showed neither pyrexia nor viraemia after this inoculation. The pattern of development of antibodies to BTV6 in the principal sheep was dissimilar to that in the control animals (Table 1). In the latter group, as had been observed with the previous BTV inoculations, specific neutralising antibodies were first recorded around day 10 after inoculation, while in seven of the eight principals, levels above 1:20 were detectable by as early as day 6 following exposure to BTV6; by day 12, levels were similar in both groups.

| TABLE 1: Development of neutralising antibodies to BTV6 after inoculation of BTV6 |
|----------------------------------|------|------|------|------|------|------|------|------|
|                                  | 0    | 4    | 6    | 8    | 10   | 12   |      |      |
| Principal sheep*                | —    | —    | —    | 1:25 | 1:23 | 1:68 | —    | —    |
| Control sheep†                  | —    | —    | —    | —    | 1:30 | 2:13 | —    | —    |

* Previously exposed to BTV 4 and 3  
† Previously unexposed to BTV  
‡ Neutralising antibody titre expressed as geometric mean of reciprocal log10 VN50  
§ Neutralising antibody titre <1:15

Heterotypic antibody responses

Sera collected during this series of inoculations were examined for neutralising antibodies to all designated types of bluetongue virus. The presence of non-specific neutralising substances in the sera of some animals (Klontz et al 1962) makes it difficult to state clearly the lowest neutralising antibody titre considered positive evidence of the animal having experienced BTV. However, in these experiments, the lowest dilution at which sera were tested was a 1:20 final dilution and, in this laboratory, sera with antibody levels of 1:15 or less are considered negative. Figures for antibody levels were obtained by calculating group geometric means, giving a value of zero to negative (less than 1:15) animals. This occasionally resulted in group means being below the lowest dilution at which individual animals were tested.

The extent of the heterotypic antibody response following the sequential exposure of BTV4 immune sheep to BTV 3 and 6 can be seen in Figs 3, 4 and 5.

Following infection with BTV 4 and 3 sera were examined for the presence of neutralising antibodies to all 20 BTV types. The cross reactivity of the sera produced was estimated by summing the number of virus types neutralised by a particular serum at levels equalling or exceeding 1:20, 1:30 or 1:40 (Fig 3). At low serum dilutions most sera neutralised a number of virus types to which these experimental animals had not been exposed. This effect was not detectable before the inoculation of BTV and was not therefore due to non-specific activity in the sera. At these low serum dilutions, a greater number of virus types were neutralised than at higher dilutions and the extent of cross reactivity of sera from individual animals...
increased with time up until day 24. The control animals which had received type 3 only were tested at a single dilution of 1:20; the response remained homologous except for a short period around day 24 after inoculation.

Group levels of neutralising antibodies to each of the 20 BTV types in the 77 day period following the inoculation of BTV3 into sheep previously infected with BTV4, can be seen in Fig 4. Before the challenge with BTV3, only antibodies to BTV4 were detected but seven days after this first challenge, group levels of 1:15 or higher were recorded to BTV 2, 8 and 20 as well as types 4 and 3 (Fig 4). The heterologous character of the response increased further but was transient and, by day 77, titres of 1:15 or higher were recorded only against BTV 3, 4, 17 and 20.

In the four control animals receiving BTV3 only, the response was predominantly homotypic (Fig 4),
FIG 5: Histograms depicting the level of serum neutralising antibodies to BTV types induced by the inoculation of BTV6 into eight sheep which had previously been inoculated with BTV4 and challenged with BTV3. All titres calculated from geometric mean of reciprocal log_{10} VN50 values and expressed as a neutralising antibody titre. Sera with titres less than 1:15 scored as zero. Antibody levels in the control animals receiving BTV6 only are shown in hatching although antibodies at low levels, ie, group means of 1:15, were recorded against BTV 6 and 17 on day 19, and against BTV 4, 6 and 20 on day 25. Only antibodies to BTV3 were recorded at levels above 1:15.

The levels of type-specific neutralising antibody during the 42 days following inoculation of BTV6 into sheep previously exposed to BTV 4 and 3 can be seen in Fig 5. Again, the response was broadly heterotypic but at a slightly higher level and more types were detected at titres of 1:15 and above. A small heterotypic response occurred in the BTV6 control group, with neutralising antibodies to BTV 3, 6, 11 and 14 being detected but, BTV6 apart, the titres of neutralising antibody were extremely low.

Sera from all these experiments were also examined for the presence of neutralising antibodies to epizootic haemorrhagic disease of deer virus (New Jersey strain; EHDV1) and Ibaraki virus, with negative results.

Discussion

Throughout this series of experiments, the only clinical manifestation following the inoculation of BTV was that of pyrexia and no experimental animals developed characteristic bluetongue lesions (Bowen 1971) or became noticeably ill. The authors have consistently noted that a variety of apparently virulent field isolates fail to produce characteristic signs of disease in laboratory sheep and, in the present experiment, all isolates used were relatively unadapted by laboratory passage. This experience is shared with other laboratories (Luedke and Jochim 1968). Thus, the effect of BTV on these animals was only measurable in terms of pyrexia, viraemia and evaluation of the immune response.

The homologous challenge of animals previously infected with BTV5 did not give rise to pyrexia or a detectable viraemia. Thus, it appears that previous exposure to BTV5 had protected these animals. The antibody response following homologous challenge remained monotypic. Similar protection and a monotypic antibody response have been shown in homologous virus challenge with BTV2, 3 and 4 (M. H. Jeggo and I. D. Gumm, unpublished observations). On the other hand, the outcome of heterotypic challenge was different in that previous exposure to BTV4 did not protect sheep against BTV3 and both...
The possible permutations of types in immunisation were recorded in that serum than at a titre of 1:20, neutralising antibodies to far more BTV types were present at a particular BTV type at a titre of 1:40. This could give rise to erroneous interpretations when screening sera at single dilutions for the presence of neutralising antibodies to BTV.

Following challenge with a third BTV type, a similar broad heterotypic response occurred. It should be noted, however, that antibodies to this serotype (BTV6) had been detectable for about 30 days during the previous phase of the experiment (Fig 2). The development of antibodies to this third challenge type occurred more rapidly than in control animals (Table 1), although by 12 days after inoculation the titre reached in the principal sheep was no higher than that observed in control animals. This, then, can be termed an accelerated immune response (Roitt 1980) and demonstrated the likely existence of memory cells to BTV6. These cells would probably have been evoked when antibodies to BTV6 were stimulated following the second administration of BTV — which would argue the true existence of antibodies to BTV6 and not an in vitro test aberration. It must be noted that, after inoculation of BTV6, an accelerated antibody response to other previously evoked BTV types did not occur and the reason for this is not clear.

The usefulness of this broad heterotypic antibody response must rest on the role neutralising antibodies play in protection and recovery from a BTV challenge. In these experiments, administration of BTV4 and 3 protected these animals from challenge from BTV6. However, whether this protection was mediated by neutralising antibody is not so easily determined. The role of neutralising antibody in many other infectious diseases has been well documented but in virus diseases the position is not clear (Rouse and Babiuk 1978, Askonas et al 1981). With bluetongue, protection has been demonstrated in the absence of detectable neutralising antibody but in the presence of a cell-mediated immune response (Stott et al 1979). Recent work in mice has shown that BTV can evoke cytotoxic T lymphocytes (CTL) and that these CTLs cross react with a number of BTV types (Jeggo and Wardley 1982a, b). Furthermore, CTLs have been shown in some virus diseases to have a protective role (Webster and Askonas 1980).

So, although an antibody response was induced before challenge with BTV6 and an accelerated humoral response was seen after the inoculation of BTV6, the role of neutralising antibody in the observed protection cannot be assessed clearly and full evaluation will require the use of passive antibody transfer techniques.

Neitz (1948) reported a series of classic studies demonstrating the existence of antigenic plurality among bluetongue viruses and the majority of the virus strains used in his experiments were later allotted to serotypes by Howell (1960). Neitz (1948) clearly showed that each of a variety of virus types produced a solid immunity to challenge with the same virus type but, when challenged with a different virus type, two different features could be observed. First, a second serotype could establish a clinical infection in a sheep that had recovered from a first serotype. Second, the extent of this heterologous infection was much less severe than in fully susceptible animals. Although there was no alteration to either the length of the incubation period or height of the ensuing pyrexia in previously exposed sheep, the course of the clinical disease was shortened and the severity of the mouth and foot lesions decreased. Such observations were similar using numerous combinations of strains and Neitz (1948) formulated a concept of common or basic immunity between strains. In the present study, it was possible to confirm the fact that sheep resist homologous challenge but not to confirm the observation of basic immunity. No difference could be detected in the reaction of British sheep that had experienced BTV4 when these and unexposed controls were subsequently infected with BTV3.

Neitz (1948) also carried out experiments in which BTV3, 4 and 8 were inoculated into sheep in...
sequence and in different combinations. Here he found no increase in the basic immunity after the first inoculation and sheep were just as susceptible after infection with two virus types as with one type. From this he concluded that polyvalent immunity could only be produced by inoculation of a number of different virus strains, a concept that has been fundamental to the use of live vaccine strains ever since. This study indicates a completely different conclusion in that, within the limitations of the test system, it has shown that prior exposure to two virus types abrogates the viraemia and pyrexia following administration of a third BTV type.

More recently, Thomas and Trainer (1971), Della-Porta et al. (1981) and Gorman et al. (1981) have reported cross reactivity between virus isolates. Studies of the virus proteins giving rise to type specificity have been restricted by problems associated with virus purification (Huismans and Howell 1973). However, recent work by Huismans and Erasmus (1981) and Gumm and Newman (1982) has shown that the outer capsid polypeptide P2 of BTV is the main serotype-determining antigen, whereas the core polypeptide P7 is responsible for group specificity. By the use of these purified proteins it may now be possible to define more clearly the serological relationships within the BTV group. It may be that the administration of type-specific proteins instead of whole virus in the sequential manner described in the above challenge experiments would result in a type-specific antibody response. However, the use of such viral subunits as vaccines would only be of use in areas in which one or two BTV types are to be expected, as a broad heterotypic response is highly desirable in endemic areas with many BTV types.

In conclusion, it would seem that the ability of the serial inoculation of two or more bluetongue virus types to evoke a broad spectrum antibody response merits serious consideration in areas where protection from a number of BTV types is considered.

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Host Response to Bluetongue Virus

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Introduction

Bluetongue (BT) is a virus disease of sheep, goats and cattle characterized by edema of the face and coronitis, particularly in sheep. There are at present some 22 known bluetongue virus (BTV) types, classified by means of an in vitro neutralization test. Because of its insect vector, the only feasible method of control in endemic areas is vaccination, the success of which relies on the inclusion of the endemic BTV types in the vaccine. In areas where many types are present, this results in multivalent vaccines linked to complex vaccination schedules and the degree of protection afforded is often poor.

To improve current vaccine design, a detailed understanding of the host's immune response to the inoculation of BTV is required. The work briefly reviews the current information regarding immunity to BTV infection and details the results of an investigation into both the humoral and cellular response of cattle, sheep, and mice to challenge with different BTV types.

Clinical Response to BTV Infection

The inoculation of BTV into British breeds of sheep and cattle is characterized by a very mild clinical response. Pyrexia, coronitis and hyperaemia of mucous membranes can occur in sheep, although cattle fail to show any response. Inoculation of field isolates in British sheep can, however, on occasions cause severe clinical signs with high mortality (Taylor, personal observation). Both species develop detectable viraemias. BTV can first be recovered two or three days after subcutaneous inoculation of the virus. Peak
Immune Response to BTV Infection

The humoral response to BTV is summarized in Fig. 1. BTV type specific neutralizing antibody is first detected in sheep around 10–12 days post-inoculation (p.i.) and in cattle around 10–20 days p.i. The response following the inoculation of a single BTV type is monotypic and titers remain high in both species for at least six months (Herniman, personal communication).

The cellular immune response following the inoculation of BTV is less clearly defined. Lymphocyte blastogenesis has been shown to occur following the administration of AEI inactivated BTV and in the absence of a neutralizing antibody response. BTV has further been shown to induce murine cytotoxic T lymphocytes (CTL) and these have been found to be highly cross-reactive. Maximum CTL activity occurred around day 7 p.i. of BTV and was detectable for about three weeks. Preliminary studies in sheep demonstrates that ovine CTLs do exist, that they peak around day 14 p.i. and are detectable for some 2½ weeks following virus inoculation (Fig. 1). The cross-reactive nature of ovine CTLs and the effect of serial inoculation of BTV on the character of their response has not been investigated.
Table 1. Development of Antibody-Dependent Cell-Mediated Cytoxicity Against IBR and BTV Infected Cells as Measured by a Chromium Release Assay

<table>
<thead>
<tr>
<th>Effector cells</th>
<th>Target cells plus infecting virus</th>
<th>Assay time (hrs)</th>
<th>Serum</th>
<th>Serum titre</th>
<th>% specific lysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine—Udder</td>
<td>GBK</td>
<td>7</td>
<td>Mouse Anti</td>
<td>1/50</td>
<td>2.3</td>
</tr>
<tr>
<td>Bovine—Udder</td>
<td>GBK + IBR</td>
<td>7</td>
<td>IBR</td>
<td>1/50</td>
<td>62</td>
</tr>
<tr>
<td>Bovine—Udder</td>
<td>GBK + IBR</td>
<td>7</td>
<td>IBR</td>
<td>1/500</td>
<td>14</td>
</tr>
<tr>
<td>Bovine PBL</td>
<td>GBK + IBR</td>
<td>7</td>
<td>IBR</td>
<td>1/50</td>
<td>60</td>
</tr>
<tr>
<td>Bovine Udder</td>
<td>GBK + BT4</td>
<td>7</td>
<td>Mouse Anti BT4</td>
<td>Neat, 1/50, 1/5000</td>
<td>0</td>
</tr>
<tr>
<td>Macrophages</td>
<td>GBK + BT4</td>
<td>7</td>
<td>Mouse Anti BT4</td>
<td>Neat, 1/50, 1/5000</td>
<td>0</td>
</tr>
<tr>
<td>Macrophages</td>
<td>LFK + BT4</td>
<td>7</td>
<td>Sheep Anti BT4</td>
<td>Neat, 1/50, 1/5000</td>
<td>0</td>
</tr>
<tr>
<td>Bovine PBL</td>
<td>LFK + BT4</td>
<td>7</td>
<td>Sheep Anti BT4</td>
<td>Neat, 1/50, 1/5000</td>
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</tr>
<tr>
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<td>LFK + BT3</td>
<td>7</td>
<td>Sheep Anti BT3</td>
<td>Neat, 1/50, 1/5000</td>
<td>0</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>LFK + BT3</td>
<td>7</td>
<td>Sheep Anti BT3</td>
<td>Neat, 1/50, 1/5000</td>
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</tr>
<tr>
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<td>LFK + BT3</td>
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<td>Sheep Anti BT3</td>
<td>Neat, 1/50, 1/5000</td>
<td>3</td>
</tr>
<tr>
<td>Neutrophils</td>
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<td>Sheep Anti BT3</td>
<td>Neat, 1/50, 1/5000</td>
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<tr>
<td>Bovine Udder</td>
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<td>36</td>
<td>Sheep Anti BT3</td>
<td>Neat, 1/50, 1/5000</td>
<td>1.7</td>
</tr>
</tbody>
</table>

Antibody-dependent cell-mediated cytotoxicity assay carried out as described previously using target cells infected with infectious bovine rhinotracheitis (IBR) and bluetongue virus (BTV). Target cells used were Georgia bovine kidney cells (GBK), primarily lamb fetal kidney cells (LFK) and primary lamb testis cells (LT). Effector cell populations were obtained as described previously. The effector to target cell ratio was 100:1 and % specific lysis was obtained from a minimum of 6-well duplicates.

Attempts to demonstrate the existence of BTV antibody-dependent killer cells through assay of antibody-dependent cell-mediated cytotoxicity (ADDC) and complement facilitated ADCC in both cattle and sheep, using a variety of systems, has consistently failed (Table 1). This would suggest that, if BTV neutralizing antibody is involved in protection and recovery, it operates by direct neutralization of extra-cellular virus.

In order to investigate the immune response further, a group of cattle was sequentially inoculated with BTV types 4, 3, and 6.
Table 2. Levels of Viraemia Detected Following the Inoculation of Bluetongue Virus (BTV) Type 6<sup>a</sup> Animals Previously Exposed to BTV Type 4 and 3

<table>
<thead>
<tr>
<th>Animal number</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days post inoculation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
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<td>—</td>
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<td>—</td>
<td>1.6</td>
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<td>—</td>
</tr>
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<td>5</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>2.2</td>
<td>2.8</td>
<td>—</td>
</tr>
<tr>
<td>6</td>
<td>—</td>
<td>—</td>
<td>0.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.2</td>
<td>2.4</td>
<td>—</td>
</tr>
<tr>
<td>7</td>
<td>—</td>
<td>—</td>
<td>2.2</td>
<td>3.8</td>
<td>3.2</td>
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</tr>
<tr>
<td>8</td>
<td>—</td>
<td>—</td>
<td>2.4</td>
<td>4.2</td>
<td>4.0</td>
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</tr>
<tr>
<td>10</td>
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<td>3.6</td>
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<td>—</td>
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<td>—</td>
<td>—</td>
<td>0.4</td>
<td>0.4</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>30</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
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</tr>
</tbody>
</table>

<sup>a</sup>Animals inoculated intradermally with 10<sup>6</sup> TCID<sub>50</sub>/ml of BTV type 6.

<sup>b</sup>Animals 1, 2, 3 and 4: Cattle previously exposed to BTV types 4 and 3. Numbers 5 and 6 control animals not previously exposed to BTV.

<sup>c</sup>Virus isolation as described previously. Titers expressed as TCID/ml of washed blood.

Clinical Response Following Sequential Challenge

The inoculation of BTV type 4 followed four months later by BTV type 3 resulted on both occasions in a typical viraemia of the usual duration. However, after the inoculation of the third BTV, type 6, virus could be detected in only one of the four animals (Table 2). Viraemia was established in control animals receiving BTV type 6 only.

Immune Response Following Sequential Challenge

Following the inoculation of BTV type 4, neutralizing antibodies could be detected to BTV type 4 only. The inoculation of a second BTV type, in this case type 3, evoked antibodies not only to BTV type 3 but to a large number of BTV types (Fig. 2). This broad heterotypic response peaked around three weeks following the inoculation of this second virus type. However, by day 63 p.i. neutralizing antibody titres of 1/10 or greater were only detectable against BTV types 3, 4, and 17. Control animals receiving only BTV type 3 showed a monotypic neutralizing antibody response (Fig. 2).

The development of neutralizing antibodies to the three inoculated viruses can be seen in Fig. 3. Significantly, antibodies to BTV type 6 occurred following the administration of BTV type 3 and prior to the inoculation of BTV type 6.
Figure 2. Neutralizing antibody response in cattle following serial inoculation of two bluetongue virus types. Histograms showing level of neutralizing antibodies to bluetongue virus types at various times after challenge with bluetongue virus type 3 in a group of 4 cattle previously exposed to bluetongue virus type 4. Virus neutralization tests were carried out in a microneutralization system using BHK cells and a starting serum dilution 1/10 against 100 TCID_{50} of virus per well. Titers calculated as group geometric means of individual reciprocal log_{10} VN_{50} values and expressed as the neutralizing antibody titer. Sera with titers < 1/15 scored as zero. Antibody levels in the control animals receiving bluetongue virus type 3 only are shown in hatching.
This transient production of antibodies to BTV type 6 may have resulted in the production of BTV type 6 memory cells, where rapid antibody production on stimulation with type 6 could have been responsible for abrogating the viraemia following inoculation of BTV type 6 in three of the four animals (Table 2). Similar results have been obtained in serial challenge of sheep with BTV. The observation that the administration of two BTV types gives protection against the development of viraemia following the inoculation of a third BTV type correlates with the development of a broad heterotypic neutralizing antibody response and the possible induction of cross-reactive CTLs. This indicates that it should be possible to produce vaccine cocktails consisting of a small number of BTV types but giving broad heterotypic cover. However, before a clear assessment can be made of the types to include in this modified vaccine, it will be necessary to examine the relative functional importance of the humoral and cellular immune response in protection and recovery. This will require not only the use of antibody and cellular adoptive “transfer” techniques but a clearer understanding of those viral components which give rise to the immune response. At present it is known that the outer capsid protein,
Host Response to Bluetongue Virus

designated P2, gives rise to type-specific antibodies but further analysis of the relative immunogenic importance of BT viral proteins must await virus purification.

Conclusion

The ability of a limited number of BTV types to induce a broad heterotypic neutralizing antibody response and a single BTV type to induce cross-reactive CTLs should be carefully considered in future BTV vaccine design.

References