Immunology 1984 **52** 403

A study of the role of cell-mediated immunity in bluetongue virus infection in sheep, using cellular adoptive transfer techniques

M. H. JEGGO, R. C. WARDLEY & J. BROWNLIE* *Animal Virus Research Institute, Pirbright, Woking, Surrey, and *Institute for Research on Animal Diseases, Compton, Berkshire*

Accepted for publication 3 February 1984

Summary. The transfer of thoracic duct lymphocytes from sheep inoculated 14 days, but not 7 days previously with bluetongue virus into their monozygotic twin resulted in some protection from challenge with bluetongue virus. T cell enrichment of the 14 day thoracic duct lymphocyte population resulted in a similar effect, indicating the T cell basis of the observed protection.

Animals recovered from infection with bluetongue virus type 3 and which received thoracic duct lymphocytes from an identical twin recently infected with the same bluetongue virus type were protected from challenge with bluetongue type 4.

These observations suggest that T lymphocytes play an important role in protection against bluetongue virus.

INTRODUCTION

Bluetongue virus (BTV), a double-stranded RNA virus belonging to the family Reoviridae and group orbivirus causes disease in sheep, cattle and goats characterized by facial oedema and coronitis.

A single inoculation of mice with one BTV serotype induces formation of cytotoxic T cells capable of lysing syngeneic targets infected with other BTV types (Jeggo & Wardley, 1982). Such plurality of the humoral response is only seen, however, after two or more sequential inoculations of BTV (Jeggo, Gumm & Taylor, 1983). These observations suggest that some present vaccines, based on the serotypic definition of BTV types (Howell, 1960) and involving the inoculation of up to 15 BTV types, may be unnecessarily complicated. Nevertheless, before simplifying vaccines, it is first necessary to assess the relative importance of the humoral and cellular responses in initial protection and later recovery from disease.

Antibody transfer has given a clearer understanding of the role of humoral immunity in the prevention of re-infection by BTV (Jeggo, Wardley & Taylor, 1983). However similar approaches applied to cellular components have been limited by the requirement for syngeneic lymphocytes. To examine in more detail the role of cell-mediated immunity in BTV infections we have used monozygotic animals in experiments involving the transfer of thoracic duct lymphocytes.

Correspondence: Dr R. C. Wardley, Animal Virus Research Institute, Pirbright, Woking, Surrey.

MATERIALS AND METHODS

Animals

Monozygotic sheep, approximately 1 year old, were kindly given by the Institute of Animal Physiology, Babraham.

All animals were held during the experiments in insect proof and BTV secure accommodation at the Animal Virus Research Institute, Pirbright.

Viruses

BTV3 was obtained from the Veterinary Research Institute, Onderstepoort and had been passaged twice in embryonated eggs and three times in baby hamster kidney (BHK) cells. BTV4 was isolated from an outbreak of bluetongue in Cyprus in 1969 and had been passaged once in eggs and four times in BHK cells. Both BTV types were plaque purified three times in BHK cells and their type specificity was verified in a micro-neutralization test (Jeggo *et al.*, 1983).

Virus inoculation and isolation

Animals were inoculated with 10^6 $TCID_{50}$ of BTV intradermally. Virus isolation was carried out using heparinized blood and BHK monolayers as indicator cells (Jeggo *et al.*, 1983). Viraemia levels were expressed as $\log_{10}TCID_{50}$/ml of original packed cells which constituted approximately half the total volume of blood sample. In each experiment virus isolates were retyped using the virus neutralization test.

Neutralizing antibody detection

Tests for neutralizing antibodies to BTV types were carried out using the micro-neutralization system as described previously (Jeggo *et al.*, 1983). Virus and positive and negative serum controls were included with each batch of sera tested.

Cellular adoptive transfer techniques

Thoracic duct cannulation. Donor monozygotic animals were inoculated with 10^6 $TCID_{50}$/ml of BTV and thoracic duct cannulation was carried out 7 or 14 days later.

Pooled collections of TDLs from each animal were washed three times in RPMI-1640 medium containing 10% foetal calf serum, 2 mM HEPES buffer and antibiotics (penicillin 110 IU/ml; streptomycin 0·2 mg/ml) (RPMI-HEPES). Cell viability was checked using trypan blue exclusion and found to be approximately 98%. TDLs and fluid were also examined for the presence of BTV and BTV antibodies.

Cell characterization

TDLs were characterized 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 ovine T cell identification (Higgins, 1981). In each case 5×10^5 cells/ml in 1 ml was mixed with an equal amount of a 1/10 dilution of the conjugate in phosphate-buffered saline (PBS). The mixture was incubated for 1 hr at 4° 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.

T cell enrichment procedures

Anti-sheep immunoglobulin. Normal sheep serum was precipitated using sodium sulphate (18% final concentration) (Hudson & Hay, 1976) and the resulting precipitate redissolved in a small amount of PBS and dialysed against PBS overnight at 4°. This solution was adjusted to contain 2 mg/ml of immunoglobulin and emulsified with an equal volume of Freund's incomplete adjuvant. Rabbits were inoculated once weekly for 4 weeks at multiple sites with 1 ml amounts of the emulsified antigen. The rabbits were bled out 1 week later, the serum pooled, precipitated with sodium sulphate, and finally adjusted to contain 1 mg/ml of immunoglobulin. Fourteen millilitres of this rabbit anti-sheep IgG was added to each 140 mm plastic petri-dish (No. 305v, Sterilin Ltd., U.K.) and incubated for 18 hr at 4°. Immediately prior to use of the plates the anti-IgG was drawn off and the plates washed three times in 15 ml of PBS.

TDLs were adjusted to contain $1{\cdot}5 \times 10^7$ cells/ml in bicarbonate free culture medium containing 5% foetal calf serum. Twenty-one millilitres of TDL suspension was added to each treated petri dish and incubated for 30 min at room temperature on a level table. After this time the plates were gently agitated and incubated for an additional 30 min. 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.

Cell transfer

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

RESULTS

Adoptive transfer of TDLs, 14 days after inoculation of the donor with BTV; homologous virus challenge

The inoculation of BTV3 into the three donor sheep induced a pyrexia and viraemia typical of a BTV

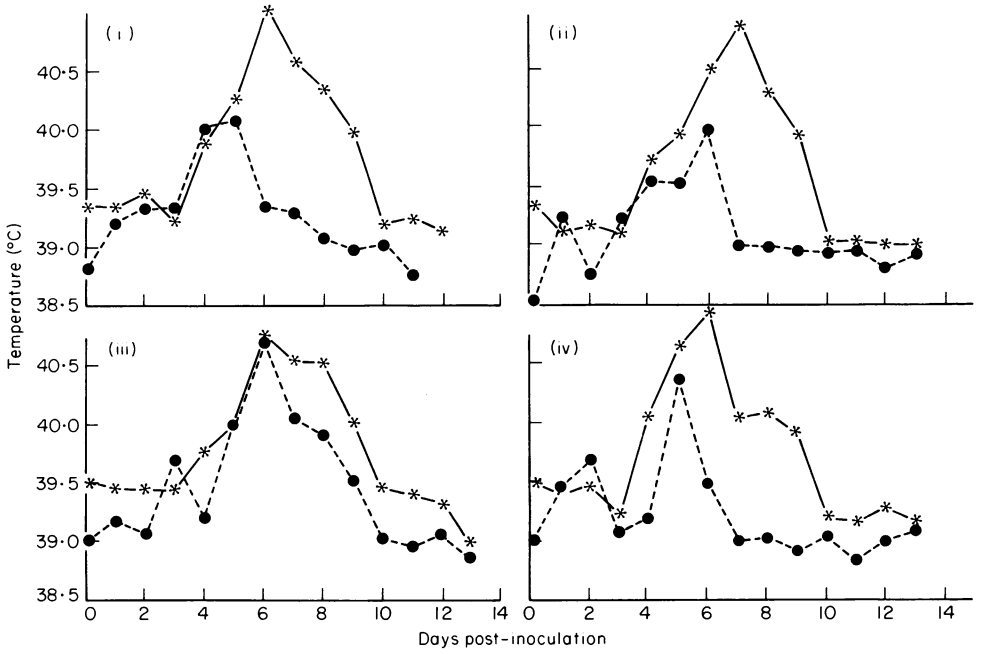

Figure 1. Elevation of temperature following the inoculation of BTV into monozygotic animals. Thoracic duct lymphocyte transfer at 14 days p.i. (*——*) Donor(s); (● - - - ●) recipient(s). Rectal temperature in °C. (i) Mean of individual animals results; (ii), (iii) and (iv) individual animal results.

infection (Jeggo *et al.*, 1983) (Figs 1, 2). Peak temperature and viraemia levels were obtained on day 7 post-inoculation (p.i.) and virus persisted in the blood for at least 20 days (Fig. 2).

The amount and composition of the thoracic duct fluid collected at day 14 p.i. can be seen in Table 1. Although it did not contain BTV, the thoracic duct lymph contained low levels (1/20) of neutralizing antibody to BTV3.

The transfer of cells and challenge of the recipient animals resulted in the temperature and viraemia response shown in Figs 1 and 2. The peak temperature response was obtained 1 day earlier and was below that of the donor animals. Peak viraemia in the recipients was detected between 3 and 4 days p.i., compared to 7 days in the donors, and then fell rapidly, so that by 7 days p.i. levels were 2–5 logs 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 p.i., the viraemia was at considerably lower levels than that in the donor animals (Fig. 2).

In the control animal receiving TDLs from an uninoculated donor the response was typical of that shown in fully susceptible animals with the height and duration of the viraemia and pyrexia being indistinguishable from that following the inoculation of BTV3 into the other donor animals (data not shown). Examination of the sera of both donor and recipient animals for neutralizing antibodies to BTV3 gave the results shown in Fig. 3. In the donor animals, neutralizing antibody was first detected around day 10 and rose rapidly to reach plateau levels of around 1/1000 by 13 days p.i. In the recipients, antibodies to BTV3 were first detected around day 5, reached 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 TDLs from BTV inoculated donors. The recipient receiving TDLs from an uninfected animal developed antibodies in a similar manner to that of the donor animals (data not shown). Neutralizing antibodies to the other 21 BTV types were not detected in the sera of any of these animals.

These results indicate that 14 day TDLs from a BTV-immune animal can reduce both the pyrexia and viraemia normally associated with BTV infections in sheep. The earlier appearance of neutralizing antibody in the recipient animals indicates that either this

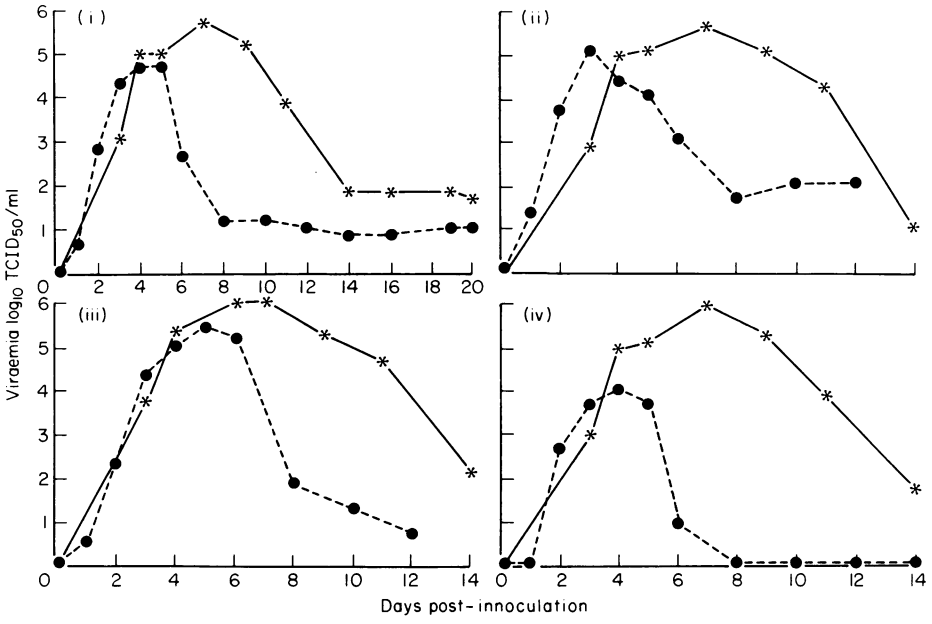

Figure 2. Viraemia in monozygotic animals following the inoculation of BTV. Thoracic duct lymphocyte transfer at 14 days p.i. (*——*) Donor(s); (●– – –●) recipient(s). Viraemia $TCID_{50}$/ml. (i) Mean of individual animal results; (ii), (iii) and (iv) individual animal results.

Table 1. Nature of thoracic duct fluid collected following cannulation 14 days after the inoculation of BTV into sheep

Animal No.	Amount collected (cm^3)	Time taken (hr)	Cell count ($\times 10^9$)	Amount transferred (cm^3)*	% of B cells†	% of T cells‡
1	500	4	0·85	20	36	26
2	1300	16	6	20	42	37
3	1300	8	4	20	46	17
4	1000	16	4·6	20	31	22

* Inoculated intravenously into recipient monozygotic twin.
† Identified using fluoroscein-conjugated rabbit anti-sheep IgG.
‡ Identified using fluoroscein-conjugated peanut agglutinin.

antibody was formed by B cells transferred in the TDL population (approximately 40% of the TDLs were B cells) (Table 1) or that immunologically specific T helper cells more rapidly primed the B cell response of the recipient and hence antibody was formed more quickly.

Adoptive transfer of TDLs, 7 days after the inoculation of the donor with BTV; homologous virus challenge

The temperature, viraemias and neutralizing antibody response in both donor and recipient following the inoculation of BTV3 can be seen in Fig. 4. The donor animal showed a pyrexia and viraemia typical of that found following the inoculation of BTV into fully susceptible animals. Unlike the 14 day thoracic lymph this fluid contained $10^{2\cdot4}$ $TCID_{50}$/ml of BTV and $10^{1\cdot4}$ $TCID_{50}$/ml of BTV was still present in the washed cells. Hence approximately $10^{2\cdot4}$ $TCID_{50}$ of virus was transferred along with the thoracic duct lymphocytes. No neutralizing antibody however was demonstrated in this fluid.

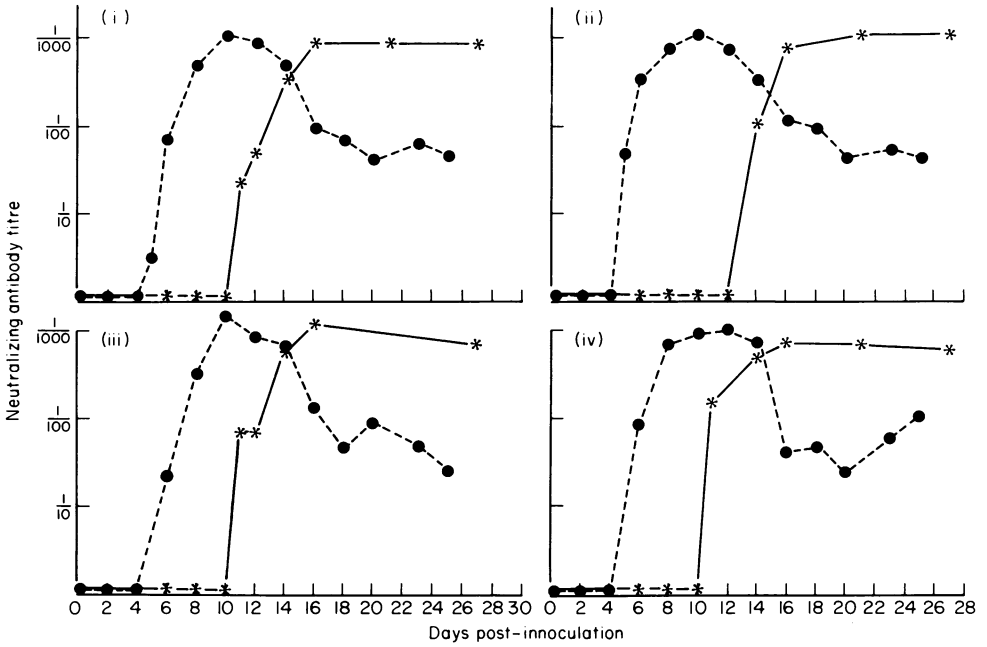

Figure 3. Neutralizing antibody to BTV3 in monozygotic animals following the inoculation of this BTV type. Thoracic duct lymphocyte transfer at day 14 p.i. (*——*) Donor(s); (● - - - ●) recipient(s). (i) Mean of individual animal results; (ii), (iii) and (iv) individual animal results.

The pyrexia obtained following the inoculation of BTV3 into the recipient was similar in pattern to that observed in the 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, and reflects the intravenous inoculation of virus associated with the TDLs compared to the intradermal challenge of all other animals. Examination of sera from both donor and recipient for the presence of neutralizing antibodies to BTV3 revealed similar titres to those following the inoculation of BTV into fully susceptible animals.

These results indicate that TDLs collected 7 days after the inoculation of BTV are not effective in curtailing the course of a BTV infection.

Adoptive transfer of T cell-enriched population of TDLs, 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 (Fig. 5). The use of anti-sheep IgG-coated plates removed 93% of the B cells; their percentage fell from 34% to 2·3%. It is highly likely that the remaining 48·7% of unlabelled cells are not all 'null' cells as the T cell marker used (peanut agglutinin) is thought not to label all T cell subsets (Dumont & Nardelli, 1979).

The inoculation of these enriched T cells into the recipient animal and challenge 12 hr later with BTV3 resulted in the viraemic and pyrexic response shown in Fig. 5. Although the pyrexia is similar to that of the donor, a 90% reduction in the viraemia was obtained and virus could be detected in the blood of the recipient for 13 days p.i. only, whereas in the donor virus was isolated for 28 days.

In contrast to the result obtained with the transfer of the total TDL population, the reduction in B cells led to the development of neutralizing antibody occurring at a similar time in both donor and recipient (data not shown), indicating that the mechanism responsible for this protection was not antibody-mediated.

Adoptive transfer of TDLs, 14 days after the inoculation of BTV into the donor; heterologous virus challenge

The inoculation of BTV3 into the donor animal and

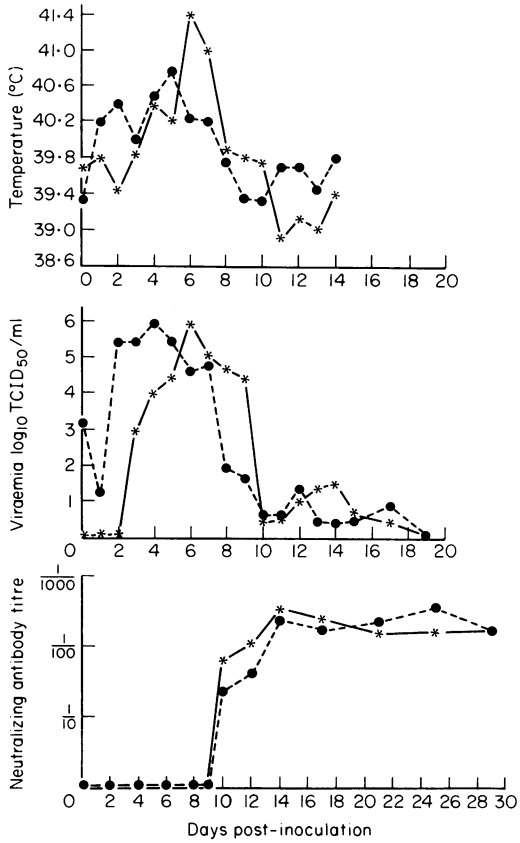

Figure 4. Temperature, viraemia and neutralizing antibody response in two monozygotic animals following the inoculation of BTV. Thoracic duct lymphocyte transfer at 7 days p.i. (*——*) Donor; (●– – –●) recipient. Rectal temperature °C. Viraemia $TCID_{50}$/ml.

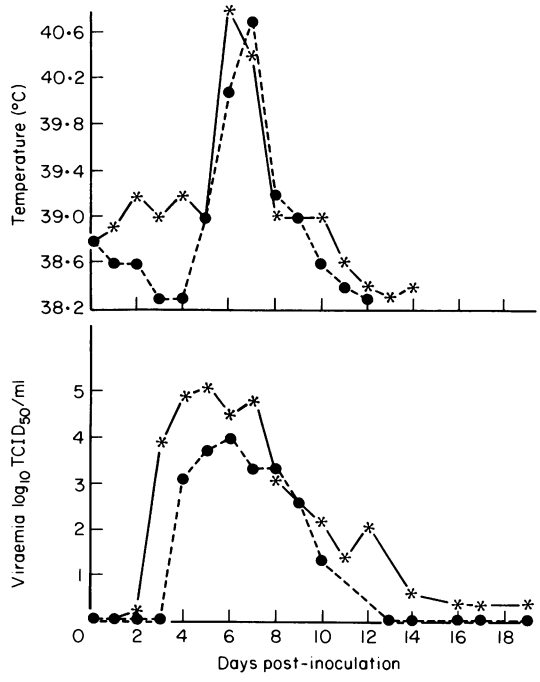

Figure 5. Development of temperature and viraemia response following the inoculation of BTV in monozygotic twins; TDLs 'panned' prior to transfer at 14 days p.i. (*——*) Donor; (●– – –●) recipient. Rectal temperature °C. Viraemia $TCID_{50}$/ml.

BTV4 into the control animal (which had previously been inoculated with BTV3) resulted in the typical pyrexia and viraemia (Fig. 6) seen in other animals.

In the recipient, however, which had been inoculated with BTV3 previously and received TDLs from the donor inoculated with BTV3, the inoculation of BTV4 induced neither pyrexia nor viraemia (Fig. 6).

Both the recipient and control animal had antibodies to BTV3 prior to the inoculation of BTV4 and following BTV4 inoculation both formed neutralizing antibodies to this type. However, the antibody response in the donor animal following an homologous challenge remained monotypic (data not shown).

Thus the transfer of immune TDLs 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 response although no evidence of a viraemia was obtained.

Both recipient and control animals had antibodies to BTV3 prior to challenge with BTV4 but the clinical signs in the control animal show that antibodies alone 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 14 day TDL population and the presence of non-neutralizing heterotypic antibody.

DISCUSSION

These results indicate that the inoculation of BTV into sheep activates a population of TDLs which when transferred to another animal at 14 days is capable of reducing the pyrexia and viraemia normally associated

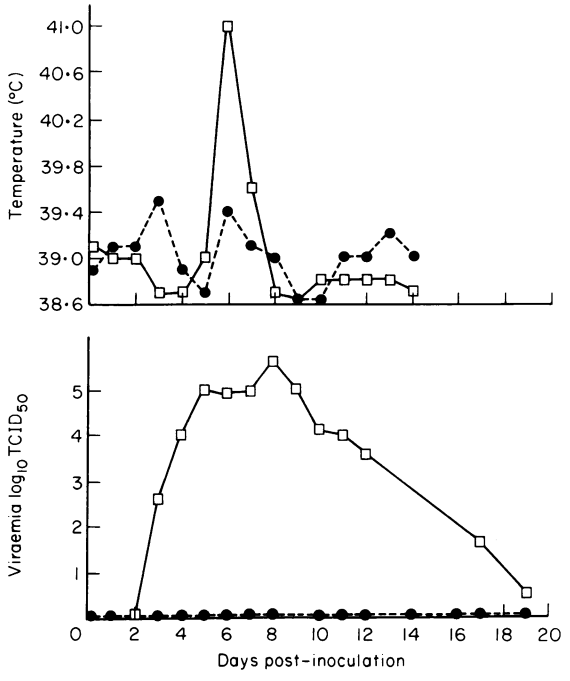

Figure 6. Development of temperature and viraemia response in monozygotic animals following the inoculation of BTV; heterologous virus challenge. (●– – –●) Recipient; (□——□) control. Rectal temperature °C. Viraemia $TCID_{50}$/ml. Both recipient and control animal inoculated with BTV3 6 weeks previously. Recipient received TDLs from BTV3-immune donor prior to challenge of both recipient and control with BTV4. Time expressed as days p.i. with BTV4.

with a BTV infection. The transfer of the total cell population results in an early antibody response, presumably because of the BTV-immune B cells it contains. It could be argued that the observed protection was therefore antibody-mediated. However, the removal of B cells delayed the antibody response without affecting the degree of protection observed. This would indicate that this protection is mediated by T cells through a mechanism which does not rely on antibody production.

Moreover, in the presence of heterotypic antibody, these TDLs 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 prior to challenge of TDLs induced to another type prevents the expected viraemia and pyrexia.

In this context, our previous work with mice has identified a cross-reactive cytolytic T cell (Jeggo & Wardley, 1982) and in sheep has shown maximal CTL development 14 days after BTV infection (Jeggo *et al.*, 1983). This indicates that it is this subset which is important in the protection observed in a TDL recipient. The resistance of BTV to the effects of antiviral substances like interferon (Jeggo, 1983) and the fact that replicating virus can be found in macrophages throughout the course of BTV infections (Lawman, 1979) adds further weight to the argument that, in fully susceptible homologously challenged sheep, these immune T cells are acting through their cytolytic effect. However, in the sheep which had recovered from BTV and which had received TDLs, the possibility also exists that cross-reactive helper T cell subsets were seeded into a bed of cross-reactive anti-BTV CTL memory cells which, by inducing a more rapid and higher level of cytotoxicity, resulted in the complete protection to heterologous challenge. These observations have parallels in studies carried out with influenza virus in mice (Wells & Ennis, 1981) and man (Zweerink *et al.*, 1977). Several reports involving the transfer of CTLs (Yap & Ada, 1978) or a cloned line of anti-influenza CTLs (Lu & Askonas, 1980) have shown that they are able to protect mice against the effects of influenza infection.

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 CTLs may also contribute to the protective mechanism. This could be either by increasing the susceptibility of infected cells to the action of CTLs, augmenting NK cell activity, or by interfering with the assembly of infectious virus (Sethi, Omata & Schneweis, 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 CTLs and helper cells it appears to prevent virus replication completely. The mechanism of this synergism is not clear, although with herpes simplex virus, CTLs and a non-neutralizing monoclonal 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 characterized, or that perhaps it improves antigen recognition and processing so that the recipients response is more vigorous and then the combination of antibody and transferred cells is sufficient to prevent virus replication.

The cross-reactive nature of the short-term CTL response, in contrast to the long lived monotypic antibody protection seen after primary infection (Jeggo *et al.*, 1983), would suggest that in the field, where sheep undergo seasonal BTV challenge, the short-term cross-reactive CTL may be the major mechanism which gives protection from heterologous challenge within a season, whereas the long-lived antibody response would provide protection against homotypic challenge between seasons. This hypothesis would explain the observation that during BTV outbreaks in a flock it is uncommon to isolate more than one BTV type from an individual sheep despite evidence that more than one BTV type is circulating in that area. In such a situation, the short-term cross-reactive CTLs produced following infection with one type would be expected to limit viral replication of any other type. As animals become infected with different BTV types this would be expected to augment both the cross-reactive CTL and antibody response.

Such factors are obviously of prime importance when considering vaccination policies. 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 cross-reactive humoral and cellular immune response which would preclude the use of inactivated preparations (Jeggo & Wardley, 1982). However, if homotypic protection is desired, then vaccines capable of inducing high levels of protective antibody should be used.

REFERENCES

DUMONT F. & NARDELLI J. (1979) Peanut agglutinin (PNA)-binding properties of murine thymocyte subpopulations. *Immunology*, **37**, 217.

HIGGINS D.A. (1981) Markers for T and B lymphocytes and their application to animals. *Vet. Bull.* **51**, 925.

HOWELL P.G. (1960) A preliminary antigenic classification of strains of bluetongue virus. *Onderstepoort J. Vet. Res.* **28**, 357.

HUDSON L. & HAY F.C. (1976) *Practical Immunology*, 1st edn, p. 11. Blackwell Scientific Publications, Oxford.

JEGGO M.H. (1983) The immune response to bluetongue virus infection. *PhD Thesis*, Surrey University.

JEGGO M.H., GUMM L.D. & TAYLOR W.P. (1983) Clinical and serological response of sheep to serial challenge with different BTV types. *Res. Vet. Sci.* **24**, 205.

JEGGO M.H. & WARDLEY R.C. (1982) Generation of cross-reactive cytotoxic T lymphocytes following immunisation of mice with various bluetongue virus types. *Immunology*, **45**, 629.

JEGGO M.H., WARDLEY R.C. & TAYLOR W.P. (1983) Host response to BTV. In: *Double-Stranded RNA Viruses, Oct. 1982, Virgin Islands* (eds R. W. Compans and D. H. L. Bishop), p. 351. Elsevier Biomedical, Amsterdam.

LAWMAN M. (1979) Observations on the pathogenesis of bluetongue virus infection in the sheep. *PhD Thesis*, University of Surrey.

LU L.Y. & ASKONAS B.A. (1980) Cross-reactivity for different type A influenza viruses of a cloned T killer cell line. *Nature (Lond.)*, **288**, 164–165.

MCMICHAEL A.J., ASKONAS B.A., WEBSTER R.G. & LAVER W.G. (1982) Vaccination against influenza: B cell or T cell immunity? *Immunol. Today*, **3**, 231–257.

SETHI K.K., OMATA Y. & SCHNEWEIS K.E. (1983) Protection of mice from fatal herpes simplex virus type 1 infection by adoptive transfer of cloned virus specific and H-2 restricted cytotoxic T lymphocytes. *J. gen. Virol.* **64**, 443.

WELLS M.A. & ENNIS F.A. (1981) Recovery from a viral respiratory infection. II. Passive transfer of immune spleen cells to mice with influenza pneumonia. *J. Immunol.* **126**, 1042.

YAP H.L. & ADA G.L. (1978) The recovery of mice from influenza virus infection: adaptive transfer of immunity with immune T lymphocytes. *Scand. J. Immunol.* **7**, 339.

ZWEERINK H.J., ASKONAS B.A., MILLICAN D., COURTEIDGE S.A. & SKEHEL J.J. (1977) Cytotoxic T cells to type A influenza virus: Viral haemagglutinin induces A strain specificity while infected cells confer cross-reactive cytotoxicity. *Eur. J. Immunol.* **7**, 630.