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Generation of cross-reactive cytotoxic T lymphocytes following immunization of mice with various bluetongue virus types

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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has been shown to induce a cell-mediated response in mice following the administration of live virus (Jeggo & Wardley, unpublished observations).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

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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 (E_3BHK_7), BT3 (E_1BHK_5), BT6 (E_3BHK_4), BT10 (E_1BHK_7), BT16 (E_2BHK_6). 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 (BHK_3) was obtained from the National Institute of Animal Health, Tokyo, Japan, Epizootic Haemorrhagic Disease, New Jersey strain (BHK_5) was supplied by K. Herniman (A.V.R.I.) and Corriparta (BHK_6) 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 ^{51}Cr 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% CO_2 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 ^{51}Cr was calculated as follows: % specific lysis (SL) = [(effector cell/target cell release − target cell alone release)/ (total releasable ^{51}Cr − target cell alone release)] × 100. Total releasable ^{51}Cr was calculated from release in the presence of 1% Triton × 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 ^{51}Cr 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

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

Effector cell types	Target cell types						Mean (%) Lysis across six targets	Uninfected
	One	Three	Four	Six	Ten	Sixteen		
One	33‡	23	24	31	47	8	27·6	2
Three	14	9	8	11	13	0	9·2	2
Four	19	15	20	7	28	2	15·1	1
Six	31	19	17	25	42	7	23·5	2
Ten	29	20	21	20	42	4	22·6	0
Sixteen	8	2	5	3	14	0	5·3	2
Control§	1	5	5	3	1	0		—

* 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 5×10^6 $TCID_{50}$ 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/effector 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 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†

In vitro stimulant of memory cells	Target cells‡
1 ml BT4 ($10^{6·8}$ $TCID_{50}$/ml)	44·3§
1 ml BT4 ($10^{8}$$TCID_{50}$/ml)	43·3
1 ml BT10 ($10^{6}$$TCID_{50}$/ml)	44·4
1 ml BT3 ($10^{6}$$TCID_{50}$/ml)	17·9

* 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 $10^{6·8}$ $TCID_{50}$ 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

In vitro stimulant of memory cells*	Target cells		
	BT4	BT3	BT10
10^6TCID$_{50}$/ml BT4	36·7†	15	53
None	8·8	6	16

* C3H mice spleen cells. Mice inoculated 3 weeks earlier with $10^{6\cdot8}$TCID$_{50}$ BT4 given intraperitoneally.

† Percentage lysis using ^{51}Cr 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 vivo*

Effector cells* second *in vivo* inoculation	Target cells†			
	BT4	BT10	BT16	Uninfected
BT4	9‡	8	8	2
BT10	8	2	5	2·5
BT16	23	19	24·5	0
Pseudorabies virus	7·5	9	3·5	0

* C3H mice spleen cells. Mice inoculated 2 weeks before second *in vivo* inoculation with $10^{6\cdot8}$TCID$_{50}$ BT4 given intraperitoneally. Second inoculation approximately 10^6TCID$_{50}$ of virus intraperitoneally.

† L929 cells infected with various BT virus types.

‡ Percentage specific lysis following 7 hr ^{51}Cr 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 ^{51}Cr 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.

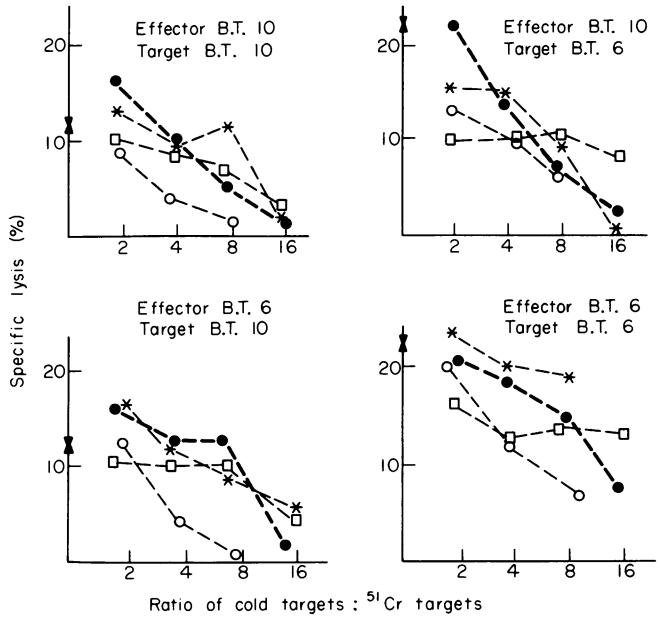

Figure 1. Inhibition of immune spleen cell effectors (100:1) in a 7 hr assay using different ratios of cold, unlabelled competitor cells. The competitors were normal L cells, or L cells infected with Bluetongue types 10, 6 or 16. Both cold and labelled L cells were added to the effector cells at the same time. Cold targets BT 10 (○—○); 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

	Target cells†				
Effector cells*	BT1	BT16	Ibaraki	EHD‡	Corriparta
BT1	24§	3	0	0	0
BT16	11	5	0	0	0

* C3H mice spleen cells. Mice immunized 7 days previously with $10^{6\cdot8}$TCID$_{50}$ BT virus type 1 or 16.

† L929 cells infected with appropriate virus. Approximately 5×10^6TCID$_{50}$. 7 Hr ^{51}Cr 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).

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