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Cultivation of Bluetongue virus-specific ovine T cells and their cross-reactivity with different serotype viruses

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SUMMARY

Bluetongue (BT) virus-specific ovine T-cell lines were prepared from BT virus-infected sheep by three cycles of alternate stimulation and resting culture *in vitro*. In antigen-specific proliferation assays and/or cytotoxicity assays, most of these T-cell lines responded not only to homologous serotype virus but also heterologous serotype viruses. This cross-reactivity did not correlate with the relatedness of serotypes as defined by cross-neutralizing antibodies. One cell line, 58-014, has grown continuously for more than 7 months without loss of antigen specificity. However, most of the cell lines lost their antigen specificity 2–4 months after cultivation. Certain BT virus-specific T-cell lines were able to reduce BT virus replication in autologous skin fibroblast cell culture.

INTRODUCTION

Bluetongue is an arthropod-borne virus disease of sheep and cattle, which is characterized in sheep by facial oedema, coronitis and death. The neutralizing antibody against BT virus is serotype specific, and there are at present 24 known BT virus serotypes defined by neutralizing test. Group-specific antibodies for BT virus, which are demonstrated by complement-fixation (CF) and agar gel diffusion tests, cross-react with other orbiviruses such as epizootic haemorrhagic disease virus, Eubenangee and Palyan sero-group viruses (Della-Porta, Parsonson & McPhee, 1985). The role of these antibodies in protection is unclear, since attempts to demonstrate the existence of antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-facilitated antibody-dependent cell-mediated cytotoxicity in both cattle and sheep, using a variety of systems, has consistently failed (Jeggo, Wardley & Taylor, 1983). On the other hand, passive serum transfer studies have shown that antibody is important in protection, but this is type specific and does not correlate with levels of neutralizing antibody (Jeggo, Wardley & Taylor, 1984a). T cells have also been implicated in immune protection against BT. Thus, Stott *et al.* (1979) showed that an inactivated vaccine preparation conferred protection in the absence of neutralizing antibodies but in the presence of a cellular immune response. In mice, BT virus has been shown to induce BT virus-specific cross-reactive cytotoxic T lymphocytes (CTL) (Jeggo & Wardley, 1982). Furthermore, in sheep BT virus has been shown to induce anti-BT virus CTL and, using adoptive transfer techniques in monozygotic sheep, these cells were shown to partially protect animals from BT virus challenge (Jeggo, Wardley & Brownlie, 1984b, 1985). T cells therefore appear to play an important part in protection against BT virus infection. To clarify the role of T cells in protection further, and as a first step towards producing vaccines specifically designed to induce such cells, we have established BT virus-specific T-cell lines from BT virus-infected sheep, and examined their cross-reactivity against different BT virus serotypes.

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MATERIALS AND METHODS

Virus

Bluetongue (BT) virus types were obtained and prepared in baby hamster kidney (BHK) cells as described previously (Jeggo & Wardley, 1982). Titration of virus was carried out in 96-well plates using BHK cells, and the titres are expressed as tissue culture infective doses (TCID50). In some experiments, BT virus was prepared in primary sheep skin cell culture. The method of Mertens, Burroughs & Anderson (1987) was used for BT virus purification.

Immunization, infection and preparation of lymphocytes

Cross-bred Dorset Horn or Southdown sheep were inoculated intradermally with approximately 10^6 TCID50 of live BT type 1 or type 3 virus (both isolated from South Africa). Two of these sheep (PZ57, PZ58) were inoculated with purified and UV-inactivated BT type 1 virus (100 μg, with an equal volume of 3% aluminium hydroxide) intramuscularly 4 weeks before infection. All animals were held during the experiments in insect-proof and BT virus-secure accommodation at the Pirbright Laboratory. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood by Ficoll–Paque (Phar-

macia, Uppsala, Sweden) density gradient centrifugation (400 *g*, 1 hr at 18°). They were subsequently washed four times with Hanks' balanced salt solution and resuspended in culture medium.

Sheep skin fibroblast cultures

Ten millimetre skin punch biopsies obtained from sheep were cut into small sections and treated with trypsin-EDTA (Sigma, St Louis, MO) solution. After trypsinization, cells were cultured in minimal essential medium (MEM) containing 10% tryptase phosphate broth (TPB), 10% heat-inactivated fetal calf serum (FCS; Imperial Lab., Andover, Hants), 5 ng/ml of epidermal cell growth factor (EGF; Sigma Chemical Co., St Louis, MO), 1 mM/ml of sodium pyruvate and antibiotics (skin cell medium). These cells were maintained in culture and passaged every 5–7 days as they reached confluency.

Generation of BT virus-specific T-cell lines

A modified method of Kimoto & Fathman (1980) was used for the generation of BT virus-specific T-cell lines. Briefly, PBMC (5×10^6/ml) from BT virus-infected and/or recovered animals were cultured with different concentrations of UV-inactivated homologous serotype virus or BT virus-infected autologous skin fibroblast cells (12–16 hr post-infection and fixed with 0·01% glutaraldehyde) in RPMI-1640 medium (Gibco Limited, Paisley, Renfrewshire) supplemented with 10% heat-inactivated lamb serum (Gibco), 1 mM/ml of sodium pyruvate and antibiotics for 5–7 days in a 24-well multiplate or in a 15-ml conical tube. After this first stimulation culture, the cells were harvested, pooled and the viable cells collected by gradient centrifugation (Ficoll–Paque, 400 *g* 20 min), washed twice and then 2×10^6/ml of cells were cultured with the same number of gamma-irradiated (1700 rads) autologous PBMC without antigen (resting culture). After a further 10 days, live cells were separated by gradient centrifugation, washed and then restimulated with BT virus antigen in the presence of irradiated autologous PBMC as antigen-presenting cells (APC) for 5–7 days (the second stimulation culture). Either after the first resting culture or the second stimulation culture, cells were cultured in RPMI medium supplemented with 10 U/ml of human recombinant interleukin-2 (HrIL-2, kindly supplied by Cetus Corp., Emeryville, CA) and 10% heat-inactivated FCS. After the three cycles of alternate stimulation and resting culture, the cells were cultured with IL-2-conditioned medium [RPMI-1640 medium supplemented with 10% FCS, 15 U/ml HrIL-2, 10% concanavalin A (Con A)-stimulated bovine PBMC supernatant, 10% Con A-stimulated ovine PBMC supernatant and 200 μg/ml of alpha-methyl-D-mannoside].

Antigen and irradiated autologous PBMC were added to these cultures once weekly. Immunoglobulin (Ig)-bearing cells in the cultures were determined using a fluorescence microscope and fluorescein isothiocyanate rabbit anti-sheep immunoglobulins (Dakopatts, Glostrup, Denmark). Surface Ig (sIg)-positive cells were removed by panning on rabbit anti-sheep Ig (Dakopatts)-coated plates (Djilali & Parodi, 1987) when the cell lines were found to contain more than 10% of sIg-positive cells. Preliminary cell surface marker analysis was carried out using indirect immunofluorescence techniques with an anti-sheep pan T-cell monoclonal antibody ST-1 (Beya *et al.*, 1986). Some cell lines were also stained with monoclonal antibodies ST-8 (CD8; Miyasaka & Trnka, 1986), SBU-T1 (CD5; Mackay *et al.*, 1985), SBU-T4 (CD4) and SBU-T8 (CD8; Maddox, Mackay & Brandon, 1985).

Antigen-specific proliferation assay

PBMC (2×10^5/well) or cultured lymphocytes (1×10^4/well and $5–10 \times 10^4$/well of gamma-irradiated autologous PBMC) were cultured with varying concentrations of UV-inactivated BT viruses or with medium alone in 96-well round-bottomed microplates. UV-inactivated BHK cell-adapted African swine fever virus (ASFV, attenuated Uganda strain; kind gift of Dr E. Anderson, I. A. H., Pirbright) was used as a negative control antigen. Cells were cultured for 96 hr at 37° before the addition of 0·2 μCi of tritiated thymidine ([^{3}H]TdR) to each well. After a further 16-hr culture period, cells were harvested onto glass-fibre filters and [^{3}H]TdR incorporation was determined by liquid scintillation spectrometry (MR300; Kontron Ltd, Watford, Herts). Experiments were performed in triplicate or quadruplicate.

^{51}Cr-release assay

Autologous skin fibroblasts were used as target cells. These were cultured at 37° overnight with BT virus then trypsinized and incubated for 90 min with $Na_2{}^{51}CrO_4$ at 37°, and washed three times. To reduce spontaneous ^{51}Cr release, labelled target cells were incubated at 37° for an additional 1 hr then, after washing, the cell concentration was adjusted to 1×10^5/ml in RPMI-1640. One hundred microlitres of a two-fold serial dilution of PBMC or cultured T cells were incubated in the wells of a 96-well round-bottomed microplate with 100 μl of target cells. Plates were incubated at 37° for 6 hr, after which half of the contents of each well (100 μl) was removed and the amount of ^{51}Cr present in the supernatant determined using a gamma-counter (1282 Compugamma; LKB-Wallac, Turku, Finland). The percentage specific release of ^{51}Cr was calculated as follows:

$$\% = \frac{\text{c.p.m. experimental release} - \text{c.p.m. spontaneous release}}{\text{c.p.m. maximum release} - \text{c.p.m. spontaneous release}} \times 100.$$

Maximum release was obtained by exposing target cells to 2% Triton X-100.

Induction and assessment of IL-2 production by BT virus-stimulated PBMC

PBMC from BT virus-infected sheep were stimulated for 3 days with UV-inactivated homologous serotype virus, and the supernatants were tested for the presence of IL-2 activity by assessing their ability to induce proliferation of an IL-2-dependent bovine T-cell line, TC-6 (H. Takamatsu, Collen, Tucker and Denyer, manuscript in preparation).

Effect of lymphocytes on BTV replication in autologous skin cell cultures

Skin fibroblasts were cultured in 24-well plates, and 500 or 1000 TCID50/well of virus inoculated into each well. After 1 hr incubation at 37°, cells were washed and cultured with skin cell medium for 12–16 hr. The culture fluid was then removed from each well and replaced by 1 ml of fresh medium or the same volume of a lymphocyte suspension. When CPE was observed in control cultures, the culture fluid was harvested from each well, centrifuged and the virus titre in this fluid estimated using BHK cells as described previously (Jeggo & Wardley, 1982).

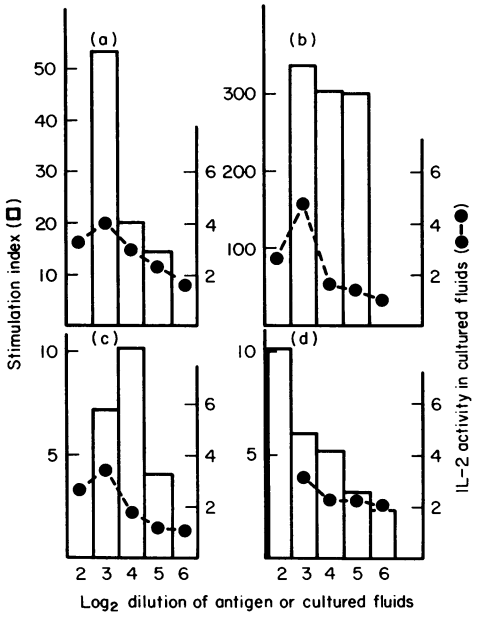

Figure 1. Proliferative responses of ovine PBMC and stimulation of IL-2 (or IL-2-like lymphokine) production by BT virus. PBMC from BT virus-infected sheep were stimulated with UV-inactivated homologous serotype virus (40 μg/ml) for 5 days. Results are shown as stimulation index. Sheep PZ57 (a), PZ58 (b) and PZ59 (c) were infected with BT virus type 1, and PU96 (d) was infected BT virus type 3. PBMC from BT virus-infected sheep were stimulated for 3 days with UV-inactivated homologous serotype virus and the supernatants were tested for the presence of IL-2 activity by an IL-2-dependent bovine T-cell line. IL-2 activity (●) is shown as proliferation of bovine T-cell line.

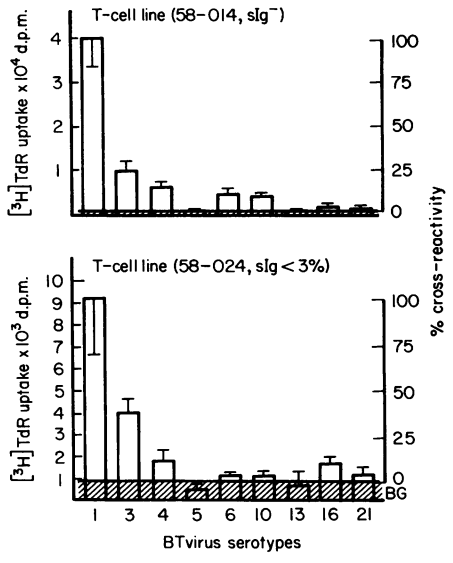

Figure 2. Antigen-specific proliferation of T-cell lines generated from BT virus serotype 1-infected sheep. Homologous and certain heterologous virus serotypes induce proliferation of BT virus-specific T cells. Figure 2 also shows a percentage cross-reactivity against different serotype viruses. Percentage cross-reactivity was calculated as follows:

$$\frac{\text{d.p.m. with heterologous virus} - \text{d.p.m. without virus}}{\text{d.p.m. with homologous virus} - \text{d.p.m. without virus}} \times 100.$$

RESULTS

BT virus antigen-specific proliferation of PBMC was observed from about 7 days post-infection, with the maximum stimulation being reached at Days 13–17 post-infection. Subsequently BT virus-specific proliferation decreased gradually. A dose-dependent, antigen-specific proliferation was observed (Fig. 1). Results shown in Fig. 1 also indicate that supernatant from BT virus-infected ovine PBMC stimulated for 3 days with UV-inactivated virus contains IL-2 or IL-2-like activity. Antigen-specific proliferation was also observed when BT virus-infected autologous skin cells were used as the stimulating antigen, although the magnitude of stimulation was lower (the stimulation index was usually between 3 to 4) than that obtained using BT virus particles (data not shown).

Since BT virus-specific proliferation could be demonstrated, large numbers of PBMC from BT virus-infected sheep were stimulated for 5–7 days *in vitro*. After three cycles of alternate stimulation and resting, the antigen specificity of cultures was determined using the antigen-specific proliferation assay. From this, several BT virus-specific cell lines were obtained. Preliminary surface marker analysis demonstrated the major population of cells in each cell line, except 61D110, to have a T-cell phenotype as defined by positive staining with ST-1 monoclonal antibody. About 51% of 96S116 and the major proportion of 97D120 also stained with ST-8. More than 90% of the 58-014 cell line stained both with SBU-T1 and ST-1 and 11–24% of them were very strongly positive, 65–70% of the cells positively stained with SBU-T4 and less than 7% of them were SBU-T8 positive.

These cell lines responded well to stimulation by both crude and purified BT virus, but did not respond to ASFV (data not shown). The cells were most effectively stimulated by the homologous virus serotype, although several heterologous virus serotypes also had the ability to stimulate these T-cell lines (Fig. 2). The cross-reactivity of these cells to different BT virus serotypes is summarized in Tables 1 and 2. This cross-reactivity against different serotypes was slightly different for each T-cell line and the culture period used for assay. T-cell lines generated from BT virus type 1-infected sheep responded well to stimulation by BT virus types 1, 3 and 4 but poorly to stimulation by BT virus serotypes 2, 5, 6, 9 and 13. The response of T-cell line 58-014 to stimulation by BT virus types 2, 9 and 15 was less than that to types 3, 4 and 10, even when these viruses were used at 10 times the concentration (optimum concentration) normally used in these assays (Table 1). In general, T-cell lines generated from BT virus type 3-infected sheep showed higher cross-reactivity than lines obtained from BT virus type 1-infected sheep. T-cell lines obtained from BT virus type 3-infected sheep showed a lower stimulation by BT virus types 13, 16 and 21 than stimulation by types 1, 3, 4 and 6 (Table 2). Similar cross-reactivity was observed when PBMC were used for assay (Tables 1 and 2).

Some T cell lines (96S116, 97D120, 97D217) not only showed antigen-specific proliferation but also cytotoxic activity against BT virus-infected autologous skin fibroblast cells (Fig. 3). The cytotoxicity of these T-cell lines was higher than that of the PBMC or even 4-day stimulated PBMC. T-cell line 97D120, derived from a BT virus type 3-infected sheep (PU97) was highly cytotoxic for BT virus-infected autologous skin cells,

Table 1. Cross-reactivity between BT virus serotypes evaluated by antigen-specific proliferation assay with PBMC and T-cell lines derived from BTV type 1-infected sheep*

BTV serotypes	% cross-reactivity: 58 PBMC	T-cell line: 58-014	59-014	58-024	58-014
1	100	100	100	100	100
2	—†	—	—	—	13·4 (45·7)‡
3	11·6	22·8	68·7	36·4	61·9
4	36·3	14·5	102·8	11·8	54·7
5	0·0	0·7	−7·2	−4·5	—
6	14·8	10·4	−9·9	3·5	—
9	—	—	—	—	0·2 (20·4)‡
10	19·6	9·0	—	3·3	46·8
13	−1·9	34·4	—	−1·6	—
15	—	—	—	—	16·5 (18·4)‡
16	−15·5	3·0	6·5	10·5	—
21	10·3	2·2	88·3	4·5	—

* Cross-neutralizing antibody was not observed in the sera from these animals. PBMC or T cells lines were stimulated with UV-inactivated 5×10^3 TCID50 virus.

† Not tested.

‡ Stimulated with 5×10^4 TCID50 virus.

% Percentage cross-reactivity =

$$\frac{\text{d.p.m. with heterologous serotype virus} - \text{d.p.m. without virus}}{\text{d.p.m. with homologous serotype virus} - \text{d.p.m. without virus}} \times 100.$$

Table 2. Cross-reactivity between BT virus serotypes evaluated by antigen-specific proliferation assay with PBMC and T-cell lines derived from BTV type 3-infected sheep

BTV sero-types	% cross-reactivity: T-cell lines: 61D110	96S116	96D612	96-314	96PBMC	97D120	97D217
1	88	124	—*	79	116	39	52
3	100	100	100	100	100	100	100
4	101	108	—	76	87	28	4
5	—	—	97	—	—	—	—
6	73	103	128	—	—	—	—
10	—	—	—	106	106	25	12
13	53	89	69	—	—	—	—
16	47	85	—	96	101	8	0
21	60	86	89	—	—	—	—

* Not tested.

Cells were stimulated with UV-inactivated 1×10^2 TCID50 BT virus.

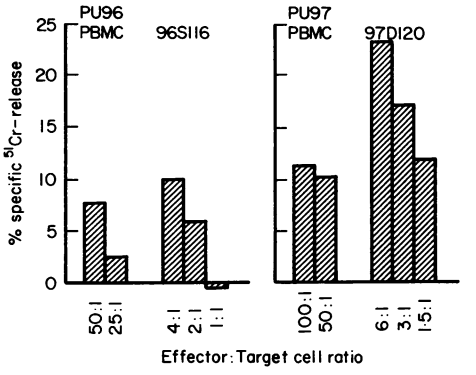

Figure 3. Cytotoxicity of short-term stimulated PBMC and T-cell lines to homologous serotype BT virus-infected autologous cultured skin fibroblast cells. PBMC were stimulated 4 days in *vitro* with homologous serotype BT virus, then used for cytotoxicity assay. T-cell lines more effectively killed BT virus-infected autologous skin fibroblast cells than short-term stimulated PBMC.

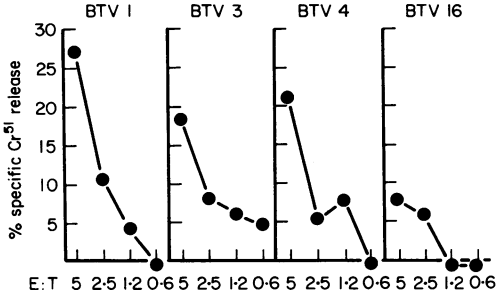

Figure 4. Cytotoxic T-cell line 97D120 generated from BT virus type 3-infected sheep kills autologous skin cells infected with different virus serotypes. E : T, effector (T cells) to target (skin cells) ratio. Similar levels of lysis were observed against autologous skin cells infected with BTV types 1, 3 and 4, whilst a little cytolysis occurred against type 16-infected skin cells.

although this cell line did not kill BT virus-infected skin cells obtained from sheep PU61 or PU96. This cytotoxic T-cell line was used to evaluate cross-reactivity using skin cells infected with different virus serotypes (Fig. 4). Similar levels of lysis were observed against autologous skin cells infected with BT virus types 1, 3 and 4, whilst little cytolysis occurred against type 16-infected skin cells.

The effect of T-cell lines with CTL activity on BT virus replication was estimated by co-cultivation with BT virus-infected autologous skin cells. Usually BT virus replication and cytopathogenic effect (CPE) occurrence in skin fibroblasts were slower (5–7 days) than in BHK cells (2–4 days). When T cells were added to the cultures during the early infection period (within 24 hr), desquamation of skin cells from the culture plate was observed within 20 hr after co-cultivation with these T-cell lines and almost all infected skin cells were removed from the culture wells before the appearance of CPE. The titres of BT virus in supernatants from these cultures were 1–2 logs lower than those from untreated infected skin cell cultures. This reduction in virus titre correlated with the number of T cells added. IL-2 medium on its own did not reduce BT virus

replication in skin fibroblast cells. Similar results were observed when skin cells were co-cultured with PBMC or short-term cultured lymphocytes. However, in some of these cases a reduction in the titre of virus was observed without complete rejection of the skin cells.

The T-cell lines generated from sheep PZ58 were usually stable. One cell line (58-014) was continuously grown for more than 7 months while retaining its antigen specificity, and sublines distinguished by their antigen specificity were established from this cell line (H. Takamatsu, Mertens, Burroghs and M. H. Jeggo, manuscript in preparation). However, most of the T-cell lines lost their antigen specificity and often failed to grow from between 1 and 3 months after the three cycles of alternate stimulation and resting culture.

DISCUSSION

In this paper we have demonstrated that it is possible to generate BT virus antigen-specific ovine T-cell lines that can be maintained for several months *in vitro*. Their reactivity with BT antigens is shown to be specific and several of the cell lines obtained were shown to have a cytotoxic effect on BT virus-infected autologous fibroblastic cells. Since this cytotoxicity was directed only against BT virus-infected autologous skin cells, it is concluded that these cells are neither natural killer cells (NK) nor lymphokine-activated killer cells (LAK). This observation confirms a previous study indicating that BT virus-specific ovine cytotoxic T lymphocytes are MHC restricted (Jeggo *et al.*, 1985).

In this study only three of the cell lines obtained showed clear cytotoxic activity (96S116, 97D120 and 97D217). This may in part be due to the use of inactivated BT virus for *in vitro* stimulation of T cells. This approach was chosen as we have previously observed that ovine lymphoblastic cells support BT virus replication *in vitro* (H. Takamatsu, unpublished data).

The BT virus group has been classified into serotypes using neutralizing antibodies (Howell, 1960), and it has been shown that virus protein 2 (Huismans, Van der Walt & Erasmus, 1985) is responsible for the induction of these neutralizing antibodies. Protection has been shown to be serotype specific, that is, animals infected with one BT virus serotype are protected against re-infection by the same serotype but not by others. Furthermore it has been clearly shown that sheep experimentally infected with one BT virus serotype produce neutralizing antibodies to only that serotype and the sera does not cross-neutralize other BT virus serotypes. However, serial inoculation with two BT virus types will result in sera containing neutralizing cross-reactive antibodies to several serotypes, and these animals have been shown to be protected against more than the two immunizing serotypes of virus (Jeggo, Gumm & Taylor, 1983). This broad cross-protection could be due to the generation of a range of cross-reactive neutralizing antibodies. However, the phenomenon could also be due to the induction of a cross-protective cell-mediated immune response to different BT virus protein(s). Anti-BT virus CTL have been described both in mice (Jeggo & Wardley, 1982) and sheep (Jeggo *et al.*, 1984a, 1985) and these are capable of heterotypic lysis (Jeggo & Wardley, 1982). Furthermore, *in vitro* culture of PBMC with BT virus has been shown to induce cross-reactive stimulation by a number of different serotypes of the virus (Ghalib, Schore & Osburn, 1985; Ghalib *et al.*, 1985). The results of this study further support the concept. Cross-reactivity was observed not only with PBMC but also with long-term cultured cells of both the CTL type and the non-cytotoxic type cell lines.

Not surprisingly, these cell lines were most effectively stimulated by the virus type used to generate them, but they also gave varying responses to other serotypes. This cross-reactivity did not correlate with the relatedness of serotypes as defined by cross-neutralization studies with antibody (Howell, 1970). This early work showed that, although the BT virus group could be classified into serotypes, within this classification groups with a degree of relatedness could be shown. Studies with T-cell responses indicate that again within the BT virus group a relatedness occurs but that it is dissimilar to that observed using antibodies. This could therefore indicate that different viral proteins are responsible for the induction of cellular immune responses compared to antibody responses. The viral protein(s) specificity of these T-cell lines is still unknown. Identification of BT virus-specific T-cell epitopes would be greatly facilitated by the construction of T-cell clones from these lines.

The importance of cytotoxic cells in the immune response is further shown by the ability of these cell lines to reduce BT virus replication *in vitro*. Although the role of lymphokines secreted by contaminating helper T cells was not examined, the ability of these cells to decrease or prevent virus replication in skin fibroblastic cells could be important *in vivo*. Indeed skin cells are those first encountered by infecting virus and the prevention of virus replication at this site could be crucial in halting an infection.

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