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Non-major histocompatibility complex-restricted cytotoxicity of bovine coronavirus-infected target cells mediated by bovine intestinal intraepithelial leukocytes

Dale L. Godson,[1,2] Manuel Campos[1] and Lorne A. Babiuk[1,2]*

[1] *Veterinary Infectious Disease Organization, 124 Veterinary Rd., Saskatoon, Saskatchewan S7N 0W0 and*
[2] *Department of Veterinary Microbiology, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, Saskatchewan S7N 0W0, Canada*

Non-specific cellular mechanisms of defence against intestinal virus infections of cattle were investigated using bovine coronavirus (BCV) as a representative enteric virus. Since BCV infection is limited to the epithelial cells of the intestinal tract, defence mechanisms must be capable of acting at that site to be effective. Therefore, the intraepithelial leukocyte (IEL) population of the intestinal mucosa was chosen for initial study. Treatment of intestinal samples with DTT and EDTA in calcium- and magnesium-free buffers allowed recovery of viable IEL populations appropriate for further functional assessment. Studies of IELs isolated from neonatal calves revealed that non-major histocompatibility complex (MHC)-restricted cytotoxicity of BCV-infected target cells was more prevalent in calves with concurrent virus infection, suggesting *in vivo* activation of the cytotoxic response. Peripheral blood mononuclear cells from the same calves did not mediate cytotoxicity, emphasizing the difference in function of lymphocytes isolated from different anatomical sites. IELs from normal adult animals rarely showed spontaneous non-MHC-restricted cytotoxicity. However, interleukin-2 (IL-2) was a potent activator of IEL cytotoxicity *in vitro*, enhancing the killing of BCV-infected target cells after just 18 h of treatment. Incubation of IELs with interferon-γ and tumour necrosis factor (TNF) did not induce cytotoxic activity, but TNF could augment the levels of IL-2-induced cytotoxicity. Although further analysis of the cytotoxic effector cells present in the intestinal epithelium is required, the present study indicates that the IEL population may play a role in enteric antiviral activity.

Introduction

Many virus diseases result from infection of mucosal surfaces, or from entry of viruses into the body through mucosal surfaces, and, therefore, it is necessary to determine the virus–host interactions that occur at these mucosal sites so that strategies to prevent infection or bolster host defence can be developed. Bovine coronavirus (BCV) was chosen as a representative virus for the study of antiviral defence mechanisms of the bovine intestine because it replicates in the epithelium of the intestinal tract. BCV is a common primary pathogen of cattle and is responsible for a large percentage of neonatal viral diarrhoea cases (Langpap *et al.*, 1979; Snodgrass *et al.*, 1986). In addition, it has been implicated as an aetiological agent of winter dysentery, an enteritis of mature cattle (Van Kruiningen *et al.*, 1987; Durham *et al.*, 1989). Consequently, the study of this virus model appeared to be relevant to the understanding of a basic biological problem, as well as providing insight into means of reducing economic losses from BCV infection.

The study of host resistance to this disease and other enteric infections requires a better understanding of the immune cells present at the site of infection because these are the cells that ultimately will be the mediators of host defence. The gut-associated lymphoid cells are categorized on the basis of location to differentiate between those lymphocytes congregated in the Peyer's patches, the cells dispersed throughout the lamina propria and the lymphocytes between the epithelial cells. Not only are these cells located in different areas of the intestine, but it has been suggested that the lymphocytes present in these different regions are associated with specialized functions. For instance, Peyer's patches contain B and T lymphocytes and antigen-processing cells, which are organized for the uptake of antigen and the initiation of the immune response, whereas the

lamina propria contains many immunoglobulin-secreting cells and regulatory T cells for amplification of the immune response (reviewed in Husband, 1990). By contrast, the intraepithelial lymphocyte (IEL) population is characterized by a high percentage of $CD8^+$ T cells (Ernst *et al.*, 1985). Since the IELs are located at the interface between the host and the environment, it has been proposed that they participate in the first line of defence against enteric pathogens or in the maintenance of mucosal homeostasis by regulating the response to dietary antigens (Brandtzaeg *et al.*, 1989). The IELs are present in all vertebrate species and constitute a significant proportion of the total lymphocyte population, suggesting that they are likely to be an important component of host defence (Fichtelius *et al.*, 1969).

Techniques for the isolation of the IELs of cattle have been developed (Nagi & Babiuk, 1987; Clough & Dean, 1988), and preliminary characterization of this population initiated (Nagi & Babiuk, 1989). However, the function of these cells in the host remains to be resolved. Since natural killer (NK) and lymphokine-activated killer (LAK) cells have been implicated in antiviral defence (Bukowski *et al.*, 1985, 1988), we investigated the ability of IELs to mediate non-major histocompatibility complex (MHC)-restricted cytotoxicity against BCV-infected target cells. The effect of cytokine treatment on cytotoxic effector cells was also studied because activation of the IELs was required to elicit this function.

Methods

Cells and virus. Madin Darby bovine kidney (MDBK) cells obtained originally from the ATCC were used as target cells for infection with BCV. The cells were grown in MEM with Earle's salts supplemented with 10% foetal bovine serum (FBS). The MDBK cell line was periodically tested and found to be free of *Mycoplasma* species. K562 cells, used as target cells for cytotoxicity assays, also were obtained originally from the ATCC. These cells were cultured in RPMI 1640 medium containing 50 μg/ml gentamicin, and 25 mM-HEPES supplemented with 10% FBS. Lymphocyte populations were cultured routinely in RPMI supplemented with 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, 0·25 μg/ml amphotericin B and 50 μM-2-mercaptoethanol.

The Quebec isolate of BCV (Dea *et al.*, 1980) was plaque-purified three times and propagated at an m.o.i. of 0·1 p.f.u./cell in confluent monolayers of MDBK cells (Deregt *et al.*, 1987). The titre of the stock virus was approximately 2×10^6 p.f.u./ml.

Bovine herpesvirus type 1 (BHV-1) strain 108 was produced in cultures of Georgia bovine kidney cells (Rouse & Babiuk, 1975) and prepared for use in challenges as described previously (Bielefeldt Ohmann & Babiuk, 1985).

Isolation of leukocytes. Bovine blood was collected using citrate anticoagulant and peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Paque (Pharmacia) density gradient centrifugation (Campos & Rossi, 1985). Leukocytes from the intestinal epithelium were isolated using a modification of the procedure described by Nagi & Babiuk (1987). Abattoir samples were obtained from 1- to 3-year-old cattle of either sex with no signs of disease in ante-mortem or post-mortem inspection. In addition, some samples were collected from a group of neonatal calves which were removed from their mother at birth and housed in isolation until they were humanely killed at 4 to 7 days of age; samples were collected and processed immediately after death. Some calves were challenged with BHV-1 using an intranasal aerosol (Bielefeldt Ohmann & Babiuk, 1985); approximately 4×10^7 infectious particles were used for this challenge. The calves were treated in accordance with the guidelines for experimental animals established by the Canadian Council on Animal Care.

Intestinal samples from experimental animals or abattoir specimens were transported to the laboratory in ice-cold citrate-buffered saline (CBS) (Lyscom & Brueton, 1982). The mesentery and fat were removed, and the intestinal samples were washed once by stirring in CBS and then everted. After three more washes in CBS, the samples were incubated for 20 min at 37 °C in Hanks' balanced salt solution (HBSS) containing 2% FBS and 5 mM-DTT to remove mucus. After washing to remove DTT, the mucosa was removed from the muscle and serosal layers, cut into small pieces, suspended in calcium- and magnesium-free HBSS with 2 mM-EDTA and 10 mM-HEPES buffer, and placed in a shaking water bath at 37 °C. After a 20 min incubation, the supernatant containing detached epithelial cells was collected. The EDTA extraction was repeated twice, and the supernatants were pooled and placed on ice for 20 min to allow the large debris and mucus to settle. Cell suspensions were then filtered through sterile 60 μm nylon mesh to remove aggregated cells and clumps. After washing three or four times with HBSS, the cells were resuspended in an isotonic solution of Percoll (Pharmacia) in MEM with a density of 1·044 g/ml, and centrifuged at 600 ***g*** for 10 min at room temperature. This step removed some of the dead and epithelial cells, improving the purification of lymphocytes in the subsequent discontinuous density gradient centrifugation (Petit *et al.*, 1985). Cells were resuspended in 1·055 g/ml Percoll and layered on 1·080 g/ml Percoll. HBSS was layered over the cell suspension and then the tubes were centrifuged at 800 ***g*** for 30 min. The cell band at the interface of the 1·055 g/ml and 1·080 g/ml layers was collected and washed. Cell viability was determined by trypan blue exclusion, and morphology from Wright-Giemsa-stained cytocentrifuge preparations.

Monoclonal antibodies (MAbs) and flow cytometry. MAbs used for subset analysis of bovine leukocyte populations were obtained from Veterinary Medical Research and Development Inc. The following MAbs specific for surface antigens of bovine leukocytes were used: B26A4 (CD2), CACT83B (CD4), BAQ82A (CD6), BAQ111A or CACT80C (CD8), B7A1 (WC1-γ/δ T cells), BAQ44A (B cell) and DH59B (monocyte and granulocyte) (Davis *et al.*, 1990).

The leukocyte populations were stained with the MAbs and fluorescein isothiocyanate-conjugated goat anti-mouse IgG, as described (Griebel *et al.*, 1987/1988) and analysed with an EPICS C flow cytometer (Coulter Electronics). Data from 10000 cells were collected and two-parameter analysis of forward angle light scatter versus 90° light scatter was used to gate the population for fluorescence analysis. Using cells stained with second antibody only, a threshold of fluorescent intensity was determined, above which cells were considered positively stained. The percentage of cells with a fluorescence intensity above this threshold minus the percentage obtained with cells stained with an irrelevant MAb was the value recorded.

Cytotoxicity assays. These were performed in triplicate or quadruplicate in round-bottom 96-well plates. K562 target cells were prepared by labelling 10^7 cells with 100 μCi $Na_2{}^{51}CrO_4$ for 2 h in 1 ml RPMI and 10% FBS. Target cells were washed three times in RPMI and 1×10^4 cells were added to each well.

To obtain BCV-infected target cells, confluent monolayers of MDBK cells in 100 mm tissue culture plates were infected with BCV

(m.o.i. of 1) for 1 h. The inoculum was removed and replaced with 5 ml MEM and 10% FBS containing 250 μCi ^{51}Cr. At 24 h post-infection (p.i.), the plates were rinsed once with Versene and then the cells were removed by trypsin treatment. After washing in MEM, the cells were added to the cytotoxicity assay. Mock-infected MDBK cells were labelled and harvested similarly.

Effector cells were added to the assay to give various effector cell:target (E:T) ratios. In some cases, effector cells were cultured in the plate for 18 to 72 h prior to the addition of the target cells. Since the effector cell concentrations within the wells were not readjusted prior to the assay, the results reflect the cytotoxic potential of the cultured population rather than the cytotoxic potential per viable cell. This procedure allowed effector cells to be treated with cytokines during the culture and/or assay period. Recombinant bovine tumour necrosis factor (TNF) and recombinant bovine interferon-γ (IFN-γ) (Ciba-Geigy), as well as recombinant human interleukin-2 (IL-2) (Boehringer Mannheim) were used at various concentrations, alone and in combination, to determine their effect on the cytotoxic capability of lymphocytes. The cytotoxicity assays were incubated for 18 h. Supernatants were harvested using a supernatant collection system (Skatron) and counted in a gamma counter (Model 5500; Beckman Instruments). Spontaneous ^{51}Cr release was determined from the mean of target cell cultures incubated in medium alone, and maximal release from the mean of target cell cultures incubated with 2% Triton X-100. The spontaneous release was always less than 30% of maximal release, and the standard deviation within replicate cultures was usually less than 10% of the mean. The percentage cytotoxicity was calculated using the formula [(experimental c.p.m.) − (spontaneous c.p.m.)/(maximal c.p.m.) − (spontaneous c.p.m.)] × 100.

The percentage enhancement of cytotoxicity due to cytokine treatment was calculated as the difference between the cytotoxicity mediated by IELs treated with cytokine combinations and IELs receiving IL-2 alone, divided by the level of killing by IELs receiving IL-2 alone, multiplied by 100.

The effects of cytokine treatment on cytotoxicity were assessed by analysis of variance with the SOLO 2.0 program (BMDP Statistical Software), and the difference between treatments was determined by Fisher's least significant difference comparison.

Results

Isolation and characterization of the IEL population

Since BCV infection is limited to the epithelial cells of the intestinal and respiratory tracts, assessment of cell-mediated defence mechanisms requires examination of the cells present at the site of infection. Therefore, the leukocytes present in the epithelial layer of the intestinal mucosa were isolated. Examination of histological sections of intestine following EDTA treatment showed that all the epithelium, except for some remnants at the base of the crypts, was removed, but the lamina propria architecture remained intact. Therefore the majority of IELs were isolated with minimum contamination by cells from the lamina propria. Yields of IELs by this isolation procedure ranged from $0{\cdot}4 \times 10^7$ to $5{\cdot}5 \times 10^7$ cells/g of mucosa, with a mean value of $1{\cdot}3 \times 10^7$ cells (*n*, 56 animals). The isolated cells consisted of over 75% lymphocytes with other cell types, such as granulocytes, macrophages, mast cells and epithelial cells, contributing

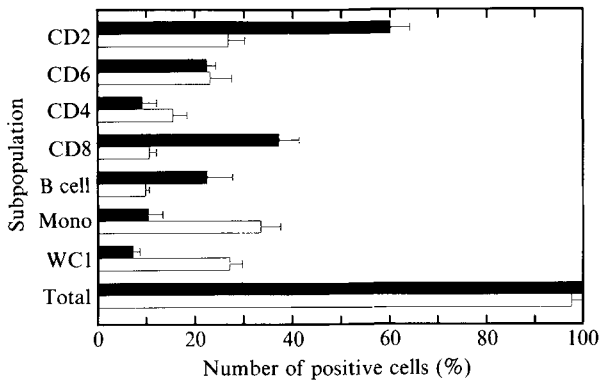

Fig. 1. Leukocyte subpopulations of the intestinal epithelium and blood of neonatal calves. The results are the mean (+S.E.M.) percentage of cells stained positively for selected leukocyte differentiation markers using flow cytometry (*n*, 9). The total value refers to the sum of the percentages of the four major subpopulations, CD2$^+$ T cells, B cells, WC1$^+$ γ/δ T cells and monocytes/granulocytes. ■, IELs; □, PBMCs.

less than 10% each on average. Virtually all of the granulocytes were eosinophils. The isolated IELs proliferated in response to both concanavalin A and IL-2, demonstrating their functional integrity (data not shown). Furthermore, equivalent levels of cytotoxicity against BCV-infected target cells were mediated by untreated PBMCs, and PBMCs treated with DTT and EDTA for periods similar to those in the IEL isolation procedure (data not shown), indicating that the chemical treatments used did not affect cell function.

IELs from neonatal calves were analysed initially because disease due to BCV infection is most commonly seen in calves between 1 and 3 weeks of age. The phenotypic profile of the IEL population was compared to that of PBMCs (Fig. 1). CD2$^+$ T cells were more abundant in the IEL population (60%) than in PBMCs (27%). Within the T cell subset, CD8$^+$ cells were more prevalent in the IEL population, resulting in a CD4/CD8 ratio of 0·24, whereas this ratio was 1·5 in PBMCs. The PBMC population had higher levels of monocytes (34%) and WC1$^+$ cells (27%) than the IEL population (8% and 4% respectively). These results confirmed the need to study the lymphocyte populations at the site of infection because the differences in subpopulation composition may be reflected in differences in function.

Non-MHC-restricted cytotoxicity mediated by IELs of neonatal calves

Having determined that IELs represent a unique population of lymphocytes present at the site of BCV infection, they were tested for their ability to mediate non-MHC-restricted cytotoxicity, one mechanism that may be involved in controlling virus infections. Using an

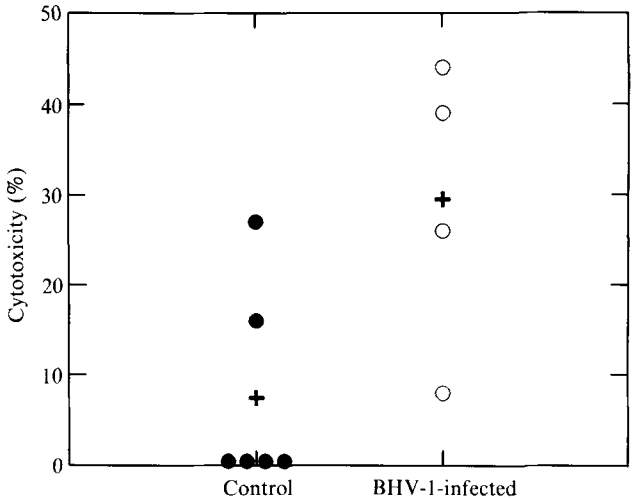

Fig. 2. Non-MHC-restricted cytotoxicity mediated by the IELs of neonatal calves. The level of killing of BCV-infected MDBK target cells (E:T, 100:1) in an 18 h ^{51}Cr release assay for each calf is represented as a data point. The calves were divided into two groups based upon challenge with BHV-1. The mean cytotoxicity for each group is shown by a cross.

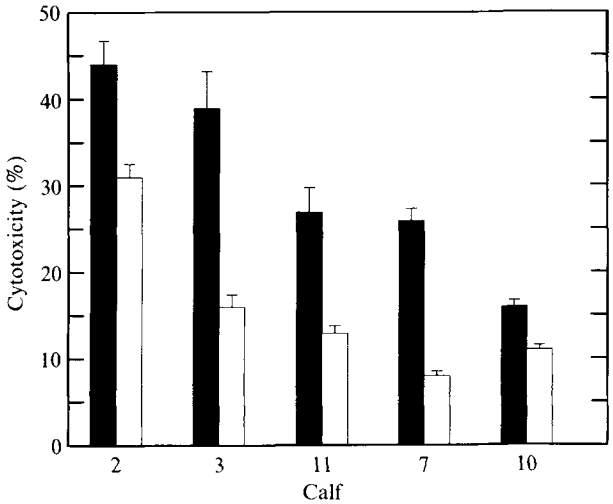

Fig. 3. Preferential killing of BCV-infected MDBK cells. IELs isolated from neonatal calves (3 to 7 days old) were tested for the level of killing (+S.D.) of BCV-infected (solid bars) and uninfected (open bars) MDBK cells (E:T, 100:1).

Table 1. *Cytotoxicity mediated by IELs from a neonatal calf*

	IEL*			PBMC
Target cell	100:1†	50:1	25:1	100:1
BCV-infected MDBK	39‡	22	13	0
Uninfected MDBK	16	15	10	0

* IELs were isolated from a 6-day-old calf which had been infected with BHV-1 4 days previously.

† E:T.

‡ Percentage cytotoxicity.

18 h ^{51}Cr release assay with BCV-infected MDBK cell targets, the IELs from only two of six calves mediated cytotoxicity (Fig. 2). However, when IELs from a group of calves of similar age (3 to 7 days old), which had been challenged with BHV-1 2 to 4 days previously, were tested, cytotoxicity was found in all cases. The differences in cytotoxicity between these two groups ($P = 0{\cdot}055$ in a Mann–Whitney test) indicate that non-MHC-restricted cytotoxicity may be induced during a virus infection. There was a direct correlation between the level of killing and the number of IELs in the assay (Table 1). In contrast, PBMCs from the virus-infected calves did not mediate cytotoxicity even at an E:T ratio of 100:1 (Table 1), which emphasizes the differences in the function of lymphocytes from different anatomical compartments. Since IELs killed BCV-infected MDBK cells to a greater extent than uninfected MDBK cells ($P < 0{\cdot}05$) (Fig. 3), this mechanism of cytotoxicity could possibly play a role in antiviral defence.

In vitro *treatment of IELs with IL-2 stimulates cytotoxic activity*

The observation that IELs isolated from virus-infected calves always exhibited cytotoxicity suggested that virus infection might be responsible for the induction of the killing activity. To determine by which mechanisms IELs could be induced to mediate cytotoxicity, IELs were isolated from abattoir specimens and stimulated with various cytokines *in vitro*. IELs were stimulated first with IL-2 because this cytokine is known to induce cytotoxicity by bovine PBMCs (Campos & Rossi, 1986). Since the percentage cytotoxicity mediated by different animals was variable, the level of cytotoxicity was expressed relative to each individual's maximum level of killing (Fig. 4). In the absence of IL-2 there was little (<20% of maximum) or no cytotoxicity; at doses of 4 to 50 U/ml, responses varied from minimal (4%) to near maximal levels of cytotoxicity. However, in the majority of cases, the maximum level of killing achieved by an animal was reached at a dose of 100 U/ml and further increases in IL-2 concentration did not augment killing. Therefore, a concentration of 100 U/ml of IL-2 in *in vitro* assays was considered optimal for eliciting the cytotoxic potential of all tested populations and was used in all subsequent studies.

To evaluate the relationship between duration of IL-2 treatment and induction of cytotoxicity, IELs were incubated with IL-2 for various periods of time before

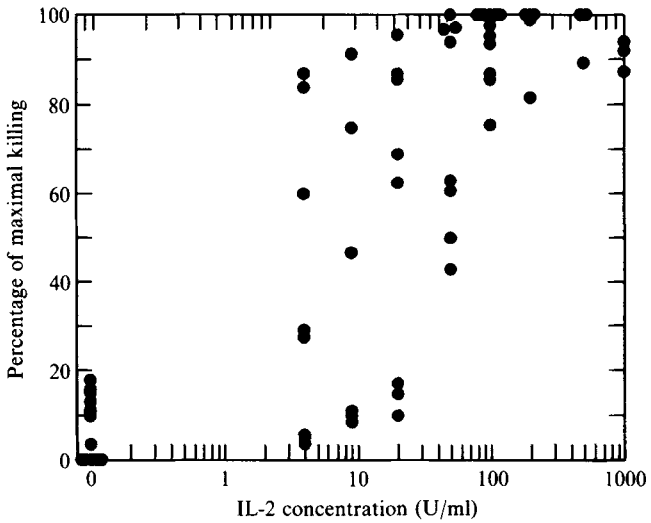

Fig. 4. The effect of IL-2 concentration on the induction of cytotoxicity by IEL. IELs (1×10^6 cells/well) were incubated with the indicated concentration of IL-2 for 36 h. BCV-infected target cells (1×10^4 cells/well) were added for the last 18 h of incubation. The level of cytotoxicity with each treatment was compared to the maximum level of killing by each animal and the percentage of maximal killing was calculated. Each data point at a given concentration is the result from one animal. Not all animals were tested at all concentrations of IL-2.

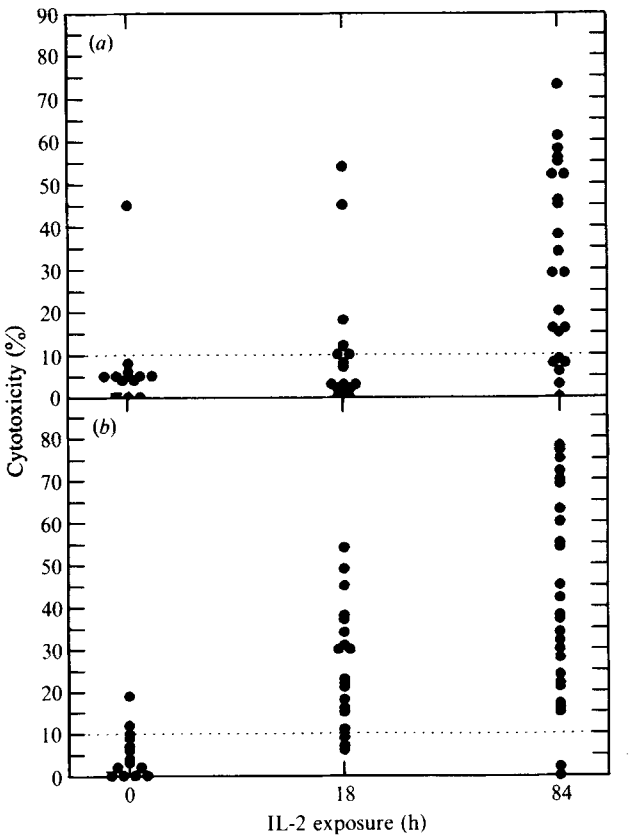

Fig. 5. The development of IL-2-activated killing of K562 (*a*) and BCV-infected MDBK (*b*) target cells by IELs. IELs (1×10^6 cells/well) were treated with IL-2 (100 U/ml) and target cells were added at 0 or 72 h of incubation. Supernatants were harvested 18 h later to determine the level of cytotoxicity. Each data point is the result from one animal for the indicated time of exposure to IL-2. Not all animals were tested at all time points. A threshold of 10% cytotoxicity was used to discriminate between responders and non-responders to IL-2 treatment.

labelled target cells were added. K562 (human NK cell target) cells were also used as targets because they have been shown to be susceptible to bovine cell-mediated cytotoxicity and would thus provide a more consistent target than a virus-infected cell. Using a value of 10% cytotoxicity as the threshold for detection of a significant level of killing, only one of 13 animals tested (8%) was able to mediate cytotoxicity against K562 target cells without IL-2 treatment (Fig. 5). When IL-2 was added to freshly isolated cells for the duration of the cytotoxicity assay (18 h) there was a small change in the level of killing observed; the mean level increased from 7% to 11% cytotoxicity and the proportion of animals responding increased from 8% to 37%. However, when the IL-2 incubation period was increased to 84 h there was a significant ($P < 0.05$) increase in the mean level of killing (32%), and the number of animals responding doubled (75%).

Likewise, when BCV-infected MDBK cells were used as target cells, cytotoxicity was observed only sporadically in the absence of IL-2 treatment; three of 14 animals had cytotoxicity values between 10% and 20% (Fig. 5). In contrast to the K562 cell assays, addition of IL-2 for the duration of the assay resulted in significant activation of the killing ability of IELs. The proportion of animals responding increased from 21% to 84% and the mean level of cytotoxicity increased from 5% to 26%; increasing the length of exposure to 84 h increased the proportion of responders to 93% and increased the mean level of killing to 42%. This IL-2-induced cytotoxic function of IELs was preferential for BCV-infected MDBK cells ($P < 0.05$), killing approximately twice as many of these targets as uninfected MDBK cells (data not shown).

One mechanism for the increased level of cytotoxicity after prolonged IL-2 treatment may have been proliferation of the IELs and hence an increase in the E : T ratio. To investigate this possibility, IELs were harvested following culture with IL-2 for 84 h, purified on Ficoll gradients, counted and added to the target cells at various E : T ratios. The mean recovery of IELs was 44% (S.D., 9·7; *n*, 7) of input. These results indicate that IL-2 treatment did not result in an overall increase in the number of IELs, although it may have stimulated proliferation of a subset of them. However, IL-2 treatment resulted in an increase in the cytotoxic activity per cell, because these cells mediated levels of cytotox-

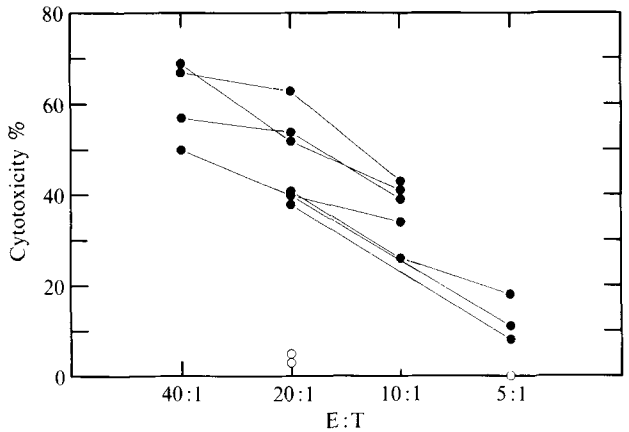

Fig. 6. IELs were harvested following culture with IL-2 (100 U/ml) (●) for 84 h, purified on Ficoll gradients, counted and added to BCV-infected MDBK cells at various E:T ratios. Each line is the result for IELs from one animal. Poor viability of IELs after culture without IL-2 (○) prevented testing of all animals.

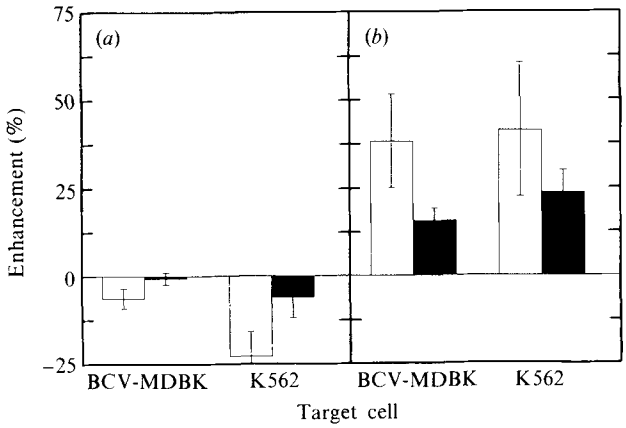

Fig. 7. The effect of IFN-γ (*a*) and TNF (*b*) on IL-2-induced cytotoxicity against BCV-infected MDBK (BCV-MDBK) and K562 target cells. IELs (1×10^6 cells/well), incubated with IL-2 (100 U/ml), also received IFN-γ (100 ng/ml) or TNF (100 ng/ml) during the culture and assay periods (□) (84 h), or the assay period alone (■) (18 h). IELs treated with TNF for the culture period mediated significantly higher ($P < 0.05$) killing of BCV-infected MDBK cells than IELs treated with IL-2 alone. The mean (*n*, 5) percentages of enhancement (+S.E.M.) of cytotoxicity are shown.

icity at a 20:1 E:T ratio (Fig. 6) that were higher than those of freshly isolated IELs at an E:T ratio of 100:1 (Fig. 5).

The effect of other cytokines on IL-2-induced cytotoxicity

Having established that IL-2 was a potent activator of IEL cytotoxic function, two recombinant bovine cytokines, IFN-γ and TNF, were tested in similar experiments. However, in the dose range of 4 to 1000 ng/ml, and up to 72 h of incubation, these cytokines were unable to induce IEL cytotoxicity (data not shown). Therefore, they were tested for their ability to modulate IL-2-induced cytotoxicity. When TNF was present for the entire culture period, it was able to augment IL-2-induced cytotoxicity against BCV-infected MDBK target cells significantly (Fig. 7), causing up to a 40% enhancement of the level of killing. Lower levels of enhancement were seen when TNF was present only during the assay. By contrast, addition of IFN-γ had little effect, with a tendency to decrease the level of killing. When IL-2-activated IELs were purified on Ficoll gradients prior to the addition of TNF, significantly enhanced levels of killing were detected with 10, 100 and 1000 ng/ml of TNF (Fig. 8); there was a dose-dependent increase in enhancement with TNF treatment up to 100 ng/ml. At 1000 ng/ml, the response was more variable, with levels of enhancement for individual animals similar to or less than those with the 100 ng/ml dose, indicating that optimal augmentation was reached at 100 ng/ml. Since the number of IELs was readjusted prior to the assay and TNF was present only during the assay, it is likely that the mechanism of cytokine

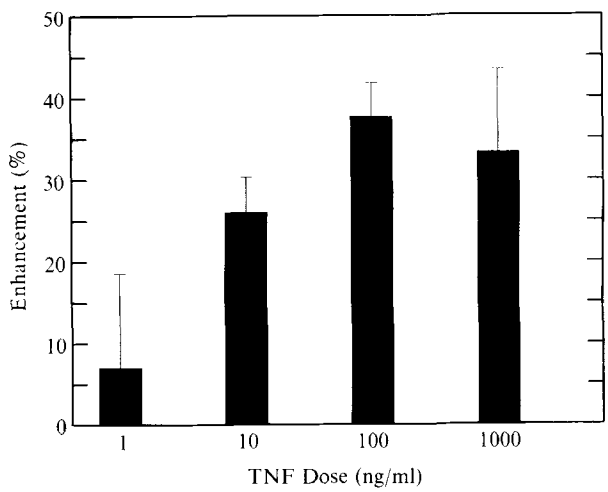

Fig. 8. Dose–response plot of TNF enhancement of IL-2-dependent cytotoxicity. IELs were preactivated with IL-2 for 18 h, harvested, purified on Ficoll gradients and then treated with IL-2 and TNF during the cytotoxicity assay (E:T, 10:1). IELs treated with 10, 100 or 1000 ng/ml of TNF mediated significantly higher killing than IELs treated with IL-2 alone ($P < 0.05$). The mean (*n*, 3) percentage enhancement (+S.E.M.) of cytotoxicity against BCV-infected MDBK cells is shown.

augmentation of killing was not due to enhanced proliferation. The enhancement of IL-2-dependent cytotoxicity by TNF suggests that the regulation of IEL-mediated cytotoxicity may be multifactorial.

Discussion

A distinguishing feature of this study of antiviral defence mechanisms was the assessment of leukocytes present at the natural site of infection, the intestinal epithelium. These IELs, obtained from both neonatal calves and adult cattle, demonstrated the potential to mediate preferential lysis of BCV-infected cells. However, an activation signal seemed to be required for induction of cytotoxic activity because spontaneous IEL-mediated cytotoxicity was detected rarely in normal animals. Cytotoxicity could, however, be detected when IELs were obtained from calves with concurrent virus infection or when IELs were treated with IL-2 *in vitro*. In addition, the level of IL-2-induced cytotoxic activity was enhanced by simultaneous TNF treatment. Thus, cytokines potentially could regulate the degree of non-MHC-restricted cytotoxicity *in vivo*, allowing it to serve as a local antiviral defence mechanism.

The IEL population was chosen for study because the proximity of these leukocytes to the epithelial cells would allow a rapid response to virus infection. Although the use of lymphocytes present at the site of infection makes more biological sense than using those from the blood, the effect of the isolation procedure on cellular function was of initial concern. However, isolated IEL populations were greater than 90% viable as determined by trypan blue exclusion, proliferated in response to mitogen and participated in cytotoxicity, indicating that functional, viable cells could be obtained by this method. Assessment of the function of these cells should, therefore, provide some insight into local defence mechanisms of the intestine.

As well as being strategically positioned to participate as a first line of defence against enteric virus infection, the IELs of calves differed from the PBMC population in both phenotypic profile and cytotoxic function. The IEL population of cattle, similar to those of other species (Ernst *et al.*, 1985; Brandtzaeg *et al.*, 1989), consisted of a majority of T cells, a large proportion of which were $CD8^+$. This phenotypic profile suggests that certain functional subsets of lymphocytes may preferentially migrate into the epithelial compartment. Consequently, this selective recruitment may account for the differences in cytotoxic function observed between IELs and PBMCs in this report and in other species, such as mice (Alberti *et al.*, 1985) and pigs (Cepica & Derbyshire, 1983). Alternatively, some of the unique features of IELs may be secondary to activation *in situ* by stimuli present in the mucosal micro-environment (Goodman & Lefrancois, 1989).

In the absence of *in vitro* activation, IEL-mediated cytotoxicity was observed consistently only if the cells had been isolated from animals with concurrent virus infection. Therefore, we speculated that activation of IEL cytotoxicity could result from the effect of cytokines produced in response to virus infections. Using *in vitro* cytokine treatment of IELs to investigate the mechanism of induction of cytotoxicity, IL-2 was shown to be an effective activator of bovine IELs, increasing both the number of responder animals and the mean level of cytotoxicity. These results corroborate previous studies of non-MHC-restricted cytotoxicity in cattle, which showed that spontaneous cytotoxic activity is low (Campos *et al.*, 1982; Jensen & Schultz, 1990), and that IL-2 is a potent *in vitro* inducer of cell-mediated cytotoxicity (Campos & Rossi, 1986; Cook & Splitter, 1988). In the human and mouse, non-MHC-restricted cytotoxicity may be mediated by NK, natural cytotoxic (NC) or LAK cells. These effector cells can be differentiated on the basis of their ability to kill prototype target cells (e.g. K562 cells for human NK cells) and their response to cytokines (reviewed in Cook & Splitter, 1989*a*). In cattle, the characterization of cytotoxic effector cells has been problematic as there is no prototype NK target cell against which all effector cell populations can be tested. Previous studies, using xenogenic tumour or virus-infected target cells have shown that susceptibility to lysis is variable and influenced by both cell type and infecting virus (Campos *et al.*, 1982; Bielefeldt Ohmann *et al.*, 1985; Cook & Splitter, 1989*b*). Also, both adherent and non-adherent effector cells were capable of mediating cytotoxicity in these studies, suggesting a heterogeneous effector cell population. Therefore the cytotoxic activity identified in this study can be described as non-MHC-restricted and IL-2-inducible, but cannot be characterized as being mediated by NK, NC or LAK cells with the same precision as in the human or mouse systems.

Bovine IFN-γ or TNF were also evaluated for their influence on IEL function, but were unable to activate cytotoxicity directly. Although IFN-γ treatment is usually associated with augmentation of NK cell function (reviewed in Trinchieri, 1989), the cytotoxic response of bovine cells treated with IFN-γ has not been consistent. Bovine PBMCs treated with IFN-γ do not kill BHV-1-infected cells (Campos *et al.*, 1989), but do lyse K562 or YAC-1 target cells (Jensen & Schultz, 1990). Since IFN was present in the cytotoxicity assay, one explanation for the lack of increased cytotoxicity in our experiments may have been IFN-induced target cell resistance to lysis (Welsh *et al.*, 1981). Alternatively, there may be a difference between IEL and PBMC populations in their response to IFN-γ. Human neonatal PBMCs are an example of a cell population which can be activated by IL-2 but not IFN-γ (Ueno *et al.*, 1985). Similarly, human lamina propria lymphocytes from the intestinal mucosa show increased NK cell activity in

response to IL-2 but not IFN-α treatment (Hogan *et al.*, 1985). Thus, the regulation of mucosal cytotoxic cells may differ from that of their systemic counterparts.

Although neither IFN-γ or TNF was able to induce cytotoxicity, the addition of TNF resulted in enhancement of IL-2-induced cytotoxicity. This enhancing effect of TNF may function *in vivo* to potentiate cytotoxic activation in the presence of small amounts of endogenous IL-2 or other activating factors. For example, in humans TNF has been shown to enhance IL-2 receptor expression, allowing lymphocytes to respond to lower concentrations of IL-2 (Chouaib *et al.*, 1988; Owen-Schaub *et al.*, 1988). These types of interaction would facilitate the regulation of non-MHC-restricted cytotoxicity and allow it to be induced earlier in the course of infection. Mechanisms for the regulation of non-MHC-restricted cytotoxicity would also serve to enhance antiviral defence while minimizing host damage.

The non-MHC-restricted cytotoxic response may be an important facet of antiviral defence because conventional cytotoxic T lymphocyte or antibody-dependent cell-mediated cytotoxicity effector cells can destroy infected host cells only after viral antigens are expressed on the cell surface. The activation of IELs at the site of infection to kill BCV-infected cells and, to a certain extent, uninfected cells could provide a defence against the local spread of virus by eliminating cells that are in the 'eclipse' phase of virus replication (Klein *et al.*, 1985). This mechanism might be particularly appropriate in BCV infection because the gut epithelium, which has a high regenerative capacity, can quickly repair small defects in the epithelium surface. Also, the immature crypt cells which replace the villous cells are refractory to BCV infection (Saif, 1990). Thus, despite the loss of a few uninfected cells, a non-specific cytotoxic response early in the course of infection could serve to limit virus spread and maintain the health of the intestinal tract as a whole.

Previously, non-MHC-restricted cytotoxicity mediated by IELs has been implicated in defence against enteric coronavirus infection in pigs (Cepica & Derbyshire, 1984) and mice (Carman *et al.*, 1986), and thus has the potential to play a similar role in cattle. However, if the absence of spontaneous *in vitro* cytotoxicity is an accurate reflection of the *in vivo* situation, then IELs must be activated quickly if they are to play a role in early protection against virus infections. This activation is apparent in the neonatal calves at 2 to 4 days p.i. Induction of cytotoxicity *in vitro* has been demonstrated only using IL-2. However, whether sufficient IL-2 is produced early in the primary immune response to activate cytotoxicity *in vivo* is not known. Thus, there may be other cytokines, or novel methods for production of IL-2, that are involved in the early activation of the cytotoxic response. Alternatively, the importance of IL-2 in the activation of cytotoxicity may explain the severity of clinical signs seen in primary as opposed to secondary BCV infection. Studies are presently in progress to assess the role of other cytokines in the activation of cytotoxicity, and to examine the kinetics of IL-2 production in response to virus challenge. These studies are being undertaken in conjunction with investigations of the ability of BCV to stimulate IEL-mediated cytotoxicity.

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