# SUPPLEMENT

# Verotoxin 1 from *Escherichia coli* Affects $Gb_3$/CD77$^+$ Bovine Lymphocytes Independent of Interleukin-2, Tumor Necrosis Factor-α, and Interferon-α

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Verotoxin (VT)-induced immunomodulation has been implicated in the ability of VT-producing *Escherichia coli* (VTEC) to cause persistent infections in cattle. VT1, also referred to as Shiga toxin 1, is a potent cytotoxin that modulates cytokine secretions and functions. This prompted the current investigation to examine whether the inhibiting effect of VT1 on bovine lymphocytes correlates with the expression of the cellular VT1 receptor $Gb_3$/CD77 or is mediated instead via perturbation of cytokine secretion. Using blood mononuclear cells stimulated by mitogens as a model, VT1 significantly blocked lymphoblast transformation and proliferation in the BoCD8$^+$ T cell and BoCD21$^+$ B cell population. In contrast, VT1 dramatically reduced the number of viable $Gb_3$/CD77$^+$ blast cells within all subpopulations identified (BoCD2$^+$, BoCD4$^+$, BoCD8$^+$, WC1$^+$ [i.e., γδ T cells] BoCD21$^+$, and BoCD25$^+$). Similar effects of VT1 were observed when the culture medium was supplemented with selected cytokines: tumor necrosis factor-α-sensitizing endothelial cells against VT1, interferon-α (IFN-α) as bovine IFN-α receptors are partially homologous to the B-subunit of VT1, and interleukin-2 that is critical for lymphocyte proliferation *in vitro*. The addition of these cytokines was neither able to mimic nor to overcome the effects of VT1. Therefore, it is concluded that VT1 directly acts on bovine lymphocytes rather than inducing a cytokine-mediated effect. VT1 considerably affects all main bovine lymphocyte subpopulations, implicating that the immune system is a predominant target for VT1 in cattle.** Exp Biol Med 228:377–386, 2003

**Key words:** verotoxins; immunity; cattle; Shiga toxin; *Escherichia coli*; food safety

Killing the host immune cells by exotoxins (1) represents a mechanism of pathogenic bacteria to survive inside their host. However, evasive strategies also include those that counteract cytokine action by either blocking production of particular cytokines, mimicking cytokines and/or cytokine receptors, and inhibiting cytokine release or action (2). Analogous to the established classes of virulence factors such as adhesins, invasins, aggressins, and impedins, Henderson *et al.* (3) suggested the term "modulin" to describe this class of molecules. Although classified as aggressins, many bacterial exotoxins, originally defined by cytopathic effects, may possess additional modulating activities. The capacity of exotoxins (3) to elicit synthesis and secretion of pro- and anti-inflammatory cytokines may be as important as their direct toxic effects in pathogenesis. Verotoxins (VT; also referred to as Shiga toxins) are commonly known as potent cytotoxins that efficiently truncate protein synthesis and cause subsequent death of susceptible cells (4). VT-producing *Escherichia coli* (VTEC) are foodborne pathogens causing dramatic illnesses such as the hemolytic uremic syndrome (HUS) in humans (5). During pathogenesis of HUS, kidney failure predominantly results from VT cytotoxic effects on endothelial cells (6). VTs also significantly interfere with cytokine secretion and function

C.M. was supported by a predoctoral fellowship of the Hessische Graduiertenförderung.
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1535-3702/03/2284-0377$15.00

within tissues (7). VT-induced liberation of tumor necrosis factor-α (TNF-α) and interleukin-1 from macrophages (8) renders endothelial cells susceptible to VT by upregulating the VT-receptor $Gb_3$/CD77 (9). In addition, the receptor-binding B-subunit of VT1 and the Type 1 interferon receptor (IFNAR) share a $Gb_3$/CD77-binding domain and one of the physiological functions of $Gb_3$/CD77 is to facilitate the binding of α2 interferon to the IFNAR (10). VT1 and IFNAR efficiently compete for $Gb_3$/CD77 binding and VT1 pretreatment of cells resulted in a diminished IFN-α sensitivity (10, 11).

Ruminants show a high prevalence of asymptomatic VTEC infections and represent the biological reservoir of these pathogens (12, 13). The work of Cornick *et al.* (14) suggested that VT are profoundly involved in the ability of VTEC to cause persistent infections in ruminants. This suggestion is underlined by the findings of our group and others that VT are able to block proliferation of bovine lymphocytes *in vitro* (15) and *in vivo* (16). Therefore, a VT-induced immunomodulation represents a highly attractive hypothesis to explain how VTEC facilitate persistence of infection. However, the exact mechanisms are poorly understood. VT1 efficiently induced apoptosis in a bovine lymphoid cell line (15), and only inhibited proliferative responses to mitogens in primary cultures of bovine lymphocytes without inducing cell death (15, 17). The latter effect was putatively due to a blockage of cellular activation particularly in cells of the $BoCD8^+$ T cell and $BoCD21^+$ B cell subpopulations (15). Although bovine lymphocytes are able to express $Gb_3$/CD77 *in vivo* and *in vitro*, $Gb_3$/CD77 expression itself relies on activation of the cells (18). It should be noted that $Gb_3$/CD77 expression is not limited to $BoCD8^+$ and $BoCD21^+$ cells as it has a very broad cellular distribution, including $BoCD4^+$ T cells proliferation of which is marginally affected by VT1. It is not known whether VT1 can act as a leukotoxin to affect $Gb_3/CD77^+$ bovine lymphocytes. VT1 may function as a modulin by interfering with cytokines that subsequently cause negative effects such as inhibition of $BoCD8^+$ and $BoCD21^+$ cell proliferation (15). The objective of this study was to investigate whether the inhibition of bovine lymphocytes by VT1 is linked to the activation-dependent $Gb_3$/CD77 expression by lymphocytes and whether a selected number of cytokines are involved in the immunomodulation caused by VT1 in cattle.

## Materials and Methods

**Experimental Animals.** Blood samples were collected from 25 healthy, lactating cows (Holstein × German black pied) from the dairy herd of the Teaching and Research Farm of our institution. All cows were healthy throughout the study.

**Toxin Purification.** VT1 was purified from the bovine VTEC1 strain 2403 $H^-$ (19) by a procedure that was described previously (15). Briefly, bacteria were grown at 37°C for 12 hr in minimal essential medium, harvested, and sonicated. The supernatant was applied to a column containing Cibacron blue 3G-A linked to agarose beads (HiTrap blue, Pharmacia, Freiburg, Germany). The bound material was eluted with a gradient from 0 to 1 *M* NaCl in 10 m*M* sodium phosphate buffer (pH 7.4). Fractions with the highest verotoxicity (determined by using the Vero [African Green Monkey kidney] cell toxicity assay) were pooled. For subsequent immunoaffinity chromatography, protein A/G agarose (Schleicher & Schuell, Dassel, Germany) was loaded with mouse anti-VT1 B-subunit (anti-VTB1) monoclonal antibodies (mAb clone 13C4) that became noncovalently linked to the gel matrix (20). The partially purified and dialyzed toxin was applied to this column and bound material was eluted with a pH gradient from 3.5 to 2.15 in 0.75 *M* NaCl. The fractions with the highest verotoxicity were pooled and dialyzed against 0.15 *M* NaCl overnight. Finally, toxin preparations were passed through Detoxi-Gel columns (Pierce, Old-Beijerland, The Netherlands) to remove endotoxin contaminants.

**Cytotoxicity Assay.** The cytotoxic activity of VT1 preparation was determined on Vero cells (ATCC CRL 1587) as described by Gentry and Dalrymple (21) with minor modifications. Briefly, 50 μl of 10-fold dilution series of toxin preparations were generated with 0.15 *M* NaCl in microtiter plates (Nunc, Wiesbaden, Germany) in triplicate. Fifty microliters of 0.15 *M* NaCl served as a negative control, whereas 50 μl of 1% SDS in 0.15 *M* NaCl were used as a positive control. A total of 50 μl of cell culture medium (RPMI 1640 supplemented with 10% fetal calf serum, 2 m*M* glutamine, 100 units of penicillin, and 100 μg of streptomycin per milliliter) then were added to each well. In neutralization studies, medium was additionally supplemented with purified anti-VTB1 (mAb 13C4) leading to a final concentration of 1.5 μg of immunoglobulin per milliliter. Initial experiments in our laboratory revealed that this concentration of antibody is sufficient to completely neutralize the biological activity of at least 200 $CD_{50}$/ml of VT1 (for definition see below) used in this study. After incubation (at room temperature for 30 min) 50 μl of Vero cell suspension ($8 \times 10^5$ cells/ml of culture medium) were applied to each well, and the plates were incubated at 37°C for 96 hr under a 5% $CO_2$ environment. Cellular metabolic activity was assessed by 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl tetrazolium bromide (MTT) reduction assay. Cytotoxic dose 50% ($CD_{50}$) was calculated from dose-response curves geometrically as the reciprocal of the toxin dilution causing 50% reduction in cellular metabolic activity.

**Peripheral Blood Mononuclear Cell (PBMC) Preparation and Stimulation.** Bovine PBMC were prepared according to the method of Bøyum (22) by using Ficoll-Paque (Pharmacia) with minor modifications (15). The cells were resuspended at $5 \times 10^6$ cells/ml in a modified cell culture medium (RPMI 1640 supplemented with 10% fetal calf serum and 3 μ*M* 2-mercaptoethanol). The cell suspension was subsequently added to 96-well flat-bottomed microtiter plates (50 μl per well). The plates were prepared in a manner similar to that for the cytotoxicity

assay. In the PBMC cultures, the medium also was supplemented with concanavalin A (ConA), phytohemagglutinin P (PHA-P), pokeweed mitogen (PWM), or lipopolysaccharide from *E. coli* O111:B4 (LPS; Sigma, Taufkirchen, Germany) at final concentrations of 5, 5, 10, and 25 μg/ml, respectively. In some studies, the medium was supplemented with VT1 (leading to a final concentration of 200 $CD_{50}$/ml as determined in the Vero cell assay) in the absence or presence of monoclonal anti-VTB1 (a final concentration of 1.5 μg/ml) and varying concentrations of selected cytokines. Cytokines were rboIL-2 (supplied by R.A. Collins, IAH, Compton, UK), rboTNF-α (supplied by R.F. Steiger, CIBA-GEIGY, Basel, Switzerland), and rhuIFN-α(2b) purchased from TEBU (Frankfurt, Germany). Plates were incubated at 37°C for 1 to 8 days under 5% $CO_2$.

**MTT Reduction Assay.** Cellular metabolic activity was assayed by measuring the reduction of MTT (Sigma, Taufkirchen, Germany) as described previously (15). Twenty-five microliters of MTT solution (5 mg/ml in phosphate-buffered saline [PBS]) were added per well. Upon incubation at 37°C for 4 hr, the reaction was stopped by adding 100 μl of 10% sodium dodecyl sulfate in distilled water. After an overnight incubation, the optical density (OD) was read on a Titertek Multiscan MCC/340 ELISA plate reader (Flow, Meckenheim, Germany) by using a test wavelength of 540 nm and a reference wavelength of 690 nm. The percentage of cellular metabolic activity was calculated as: (OD sample − OD positive control)/(OD negative control − OD positive control) × 100.

**Immunophenotyping and Flow Cytometry Analysis.** After stimulation, PBMC were thoroughly resuspended and transferred to V-shaped microtiter plates (Greiner, Frickenhausen, Germany) at the end of the cultivation period and were pelleted by centrifugation (150*g* at 4°C for 7 min) as described by Menge *et al.* (23). The pellets were resuspended in 50 μl of cell culture medium as a negative control or with supernatant of hybridoma cell lines (IL-A 43 for BoCD2, IL-A11 for BoCD4, IL-A105 for BoCD8, IL-A65 for BoCD21, IL-A111 for BoCD25, and IL-A 29 for WC1). The cells were incubated on ice for 20 min, pelleted, resuspended with 25 μl of rat IgM (1 mg/ml, 1:50 in PBS; Camon, Wiesbaden, Germany) as a negative control or with anti-human CD77 antibody (1:10 in PBS; Beckman-Coulter, Krefeld, Germany) and incubated for an additional 20 min. The cells then were washed once and resuspended with 50 μl of anti-mouse PE-conjugate (Sigma) diluted 1:100 in PBS. After 20 min, the cells were pelleted and resuspended in 50 μl of anti-rat IgM FITC-conjugate (Dianova, Hamburg, Germany) 1:200 diluted in PBS and containing 2 μg/ml propidium iodide (Sigma). After another 20 min on ice, the cells were washed twice and analyzed with an EPICS ELITE Analyser (Beckman-Coulter, Krefeld, Germany). A total of 5000 events were acquired from each sample. Data analysis was performed by using the ELITE 4.01 software provided by the manufacturer. Electronic gates were set according to the negative control included in each test series defining less than 2% of the cells as positive. Populations of enlarged lymphoblast cells and untransformed nonblast cells were defined according to its light scatter characteristics as described (18) and were analyzed separately.

**Statistical Analysis.** Data were analyzed statistically by paired *t* test and the Wilcoxon signed rank test

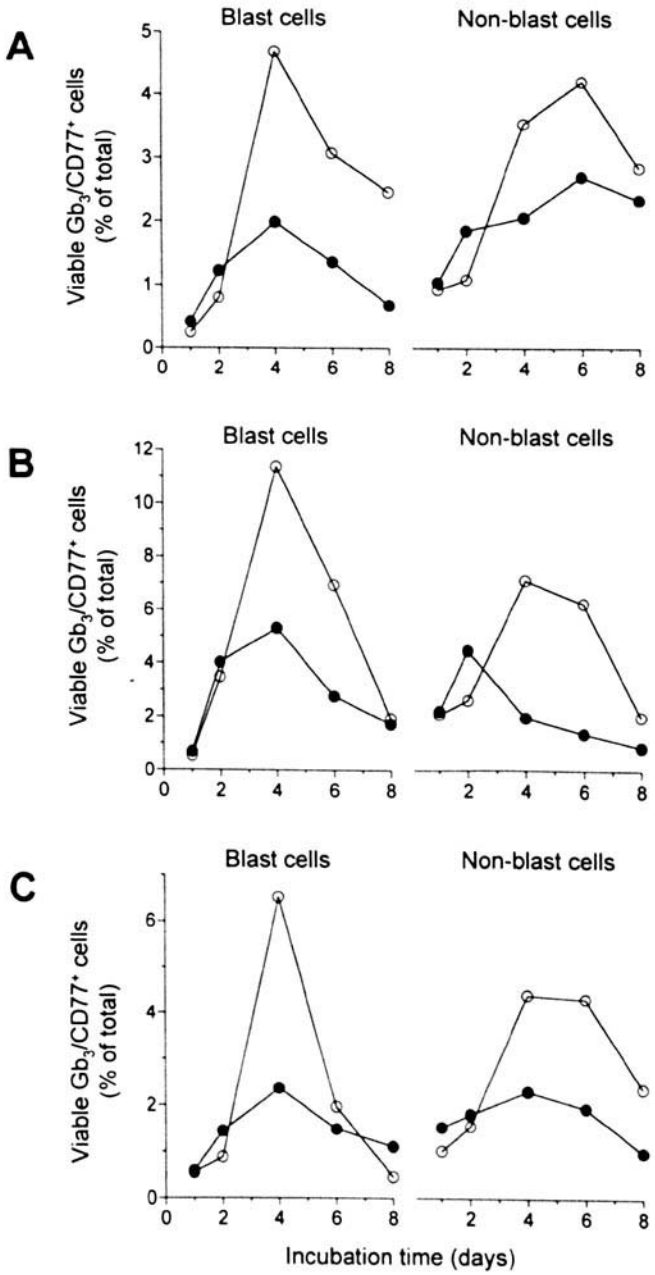


**Figure 1.** Effect of VT1 on $Gb_3$/CD77 expression by bovine PBMC. Cells were incubated with VT1 (200 $CD_{50}$/ml, quantified on Vero [African Green Monkey kidney] cells) at 37°C. Culture medium was free of mitogen (A) or was supplemented with 5 μg/ml PHA-P (B) or 25 μg/ml LPS (C). Observed effects were assigned to VT1 by comparison of the results obtained in the absence (●) or presence of 1.5 μg/ml anti-VTB1 mAb 13C4 (○). Expression of $Gb_3$/CD77 was quantified by flow cytometry at the indicated time points. Data analysis was performed by calculating the percentage of viable $Gb_3$/CD77$^+$ cells belonging to the blast cell or nonblast cell population of single determinations of one cow.

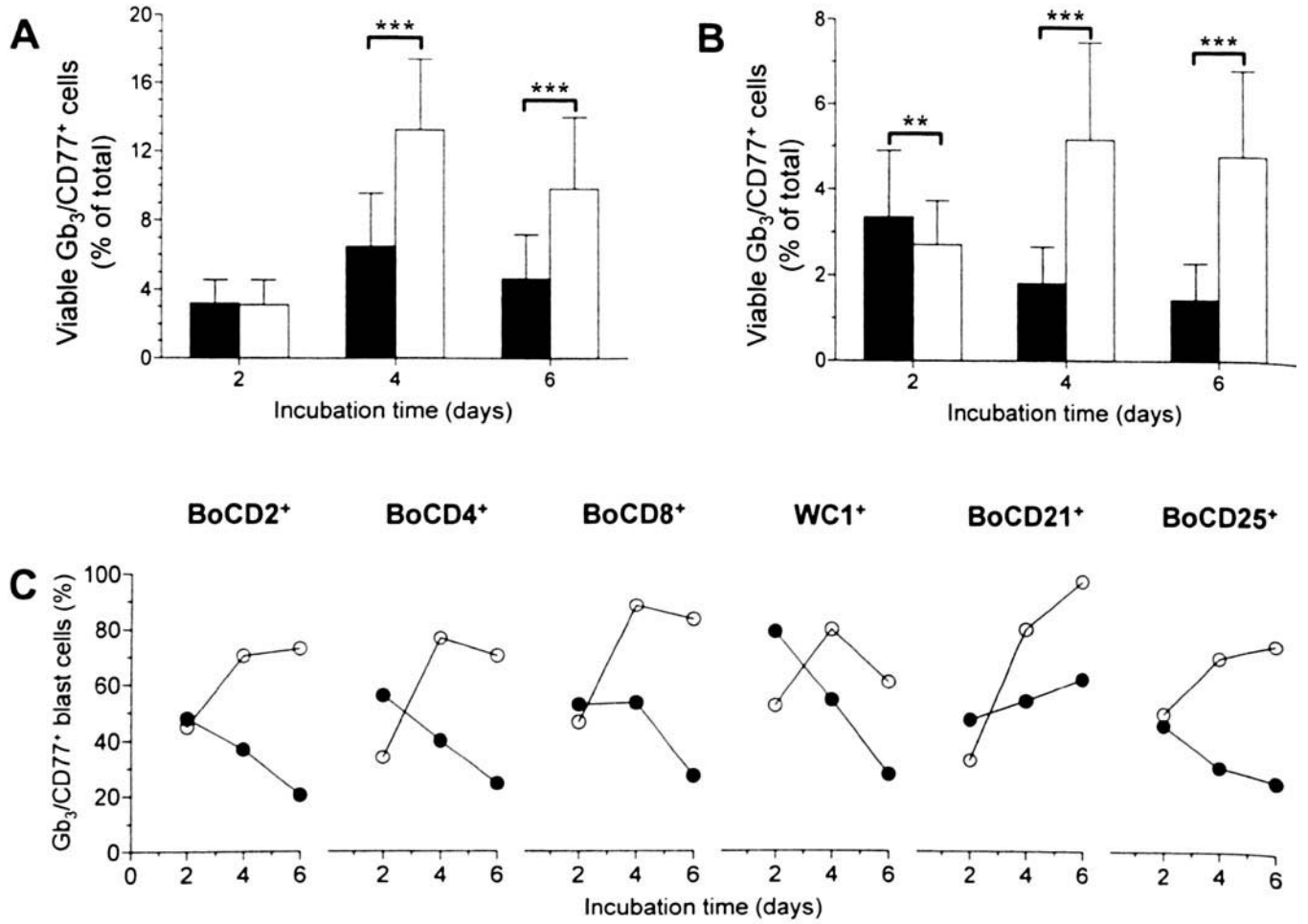


**Figure 2.** Effect of VT1 on $Gb_3$/CD77 expression by bovine PBMC subpopulations. Cells were incubated with VT1 (200 $CD_{50}$/ml, quantified on Vero cells) at 37°C. Culture medium was supplemented with 5 μg/ml PHA-P. Observed effects were assigned to VT1 by comparison of the results obtained in the absence (●) or presence of 1.5 μg/ml anti-VTB1 mAb 13C4 (○). Expression of $Gb_3$/CD77 was quantified at the time points indicated by flow cytometry. Effect of VT1 on $Gb_3$/CD77 expression within the blast cell (A) and nonblast cell (B) population is shown by calculating the percentage of viable, $Gb_3$/CD77$^+$ cells belonging to the blast cell and nonblast cell population, respectively. Bars represent mean ± SD of 20 cows. Horizontal brackets enclose bars that are different ($P \leq 0.01$ [**]; $P \leq 0.001$ [***]). (C) Effect of VT1 on $Gb_3$/CD77 expression by PBMC subpopulations of one cow was depicted by calculating the percentage of $Gb_3$/CD77$^+$ blast cells belonging to the indicated subpopulations (single determinations; WC1, antigen solely expressed by bovine γδ T cells).

using SigmaStat 2.0 software (1992; SPSS Inc., Chicago, IL). Significant differences were separated at $P \leq 0.001$ [***], $P \leq 0.01$ [**], and $P \leq 0.05$ [*].

## Results

**Effect of VT1 on $Gb_3$/CD77$^+$ Bovine PBMC.** Using PBMC cultures as a model, the percentage of $Gb_3$/CD77$^+$-enlarged lymphoblast cells as well as $Gb_3$/CD77$^+$ nonblast cells increased constantly within 1 to 2 days after the initiation of culture and peaked at Day 4 in the absence or the presence of the mitogens PHA-P and LPS, respectively (Fig. 1). The presence of nanogram concentrations of VT1 (200 $CD_{50}$/ml as determined on Vero cells; one verotoxic dose 50% was previously calculated to be equivalent to 0.4–0.8 pg/ml of purified VT) (24) dramatically reduced the number of viable $Gb_3$/CD77$^+$ cells in the blast cell and nonblast cell population over time (Fig. 1). The effect of VT1 became obvious only after 4 days of incubation, implicating that $Gb_3$/CD77 expression on Days 1 and 2 is a

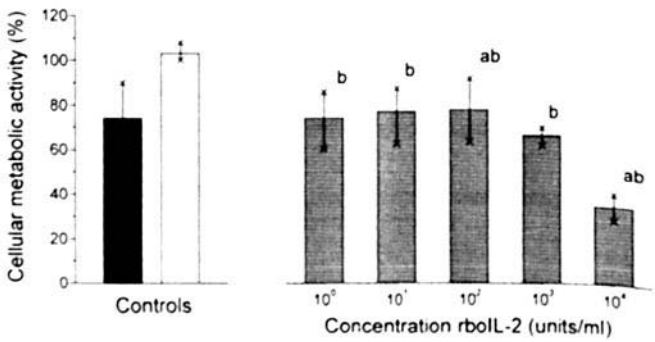


**Figure 3.** Effect of rbolL-2 and VT1 on the cellular metabolic activity of bovine PBMC. Cells were either incubated with VT1 (200 $CD_{50}$/ml, quantified on Vero cells) with (open bar) or without (black bar) anti-VTB1 mAb 13C4 (1.5 μg/ml) or with VT1 together with indicated concentrations of rbolL-2 (striped bars) at 37°C. Culture medium was supplemented with 5 μg/ml PHA-P. Cellular metabolic activity was determined by MTT reduction assay after 96 hr of incubation. Data represent mean, minimum, and maximum of nine determinations with cells from three cows. Differences ($P \leq 0.05$) to VT1 (a) and VT1 + anti-VTB1 (b) were detected.

prerequisite for the effect of VT1 to occur. Analysis of PHA-P-stimulated PBMC of a significant number ($n = 20$) of cows (Fig. 2, A and B) indicated that VT1 did not completely eliminate $Gb_3/CD77^+$ cells. Even in the presence of VT1, viable $Gb_3/CD77^+$ cells were detectable within the cultures over the entire cultivation period, although initial experiments had shown that the concentration of VT1 applied was sufficient to induce maximum effects (data not shown). Surprisingly, at an early phase of cultivation the number of viable $Gb_3/CD77^+$ nonblast cells in VT1-treated cultures significantly exceeded the number of positive cells in cultures treated with VT1 plus anti-VTB1 mAb (Fig. 2B). Irrespective of susceptibility of different PBMC subpopulations to the inhibitory effects of VT1, with $BoCD8^+$ T cells and $BoCD21^+$ B cells being the most sensitive cells, a marked reduction of $Gb_3/CD77^+$ blast cells in the presence of VT1 was detectable for all lymphocyte subsets investigated (Fig. 2C).

**Interference of Selected Cytokines and the Proliferation Inhibiting Effect of VT1.** Checking the hypothesis that VT1 might have impaired a paracrine IL-2 release in the cultures, addition of recombinant bovine interleukin-2 (rboIL-2; 1–10,000 units/ml) did not overcome the reduction of the cells' metabolic activity caused by VT1 (Fig. 3). At the lowest rboIL-2 concentration used (1 unit/ml), the metabolic activity was as much reduced as could be seen with VT1 alone. This reduction was even amplified at increasing rboIL-2 concentrations. Likewise, rboIL-2 neither influenced inhibitory effects of VT1 on $BoCD8^+$ and $BoCD21^+$ cells nor the toxin-induced reduction of $Gb_3/CD77^+$ blast cells at a any significant level (Fig. 4; $P > 0.05$ for VT1 + rboIL-2 versus VT1).

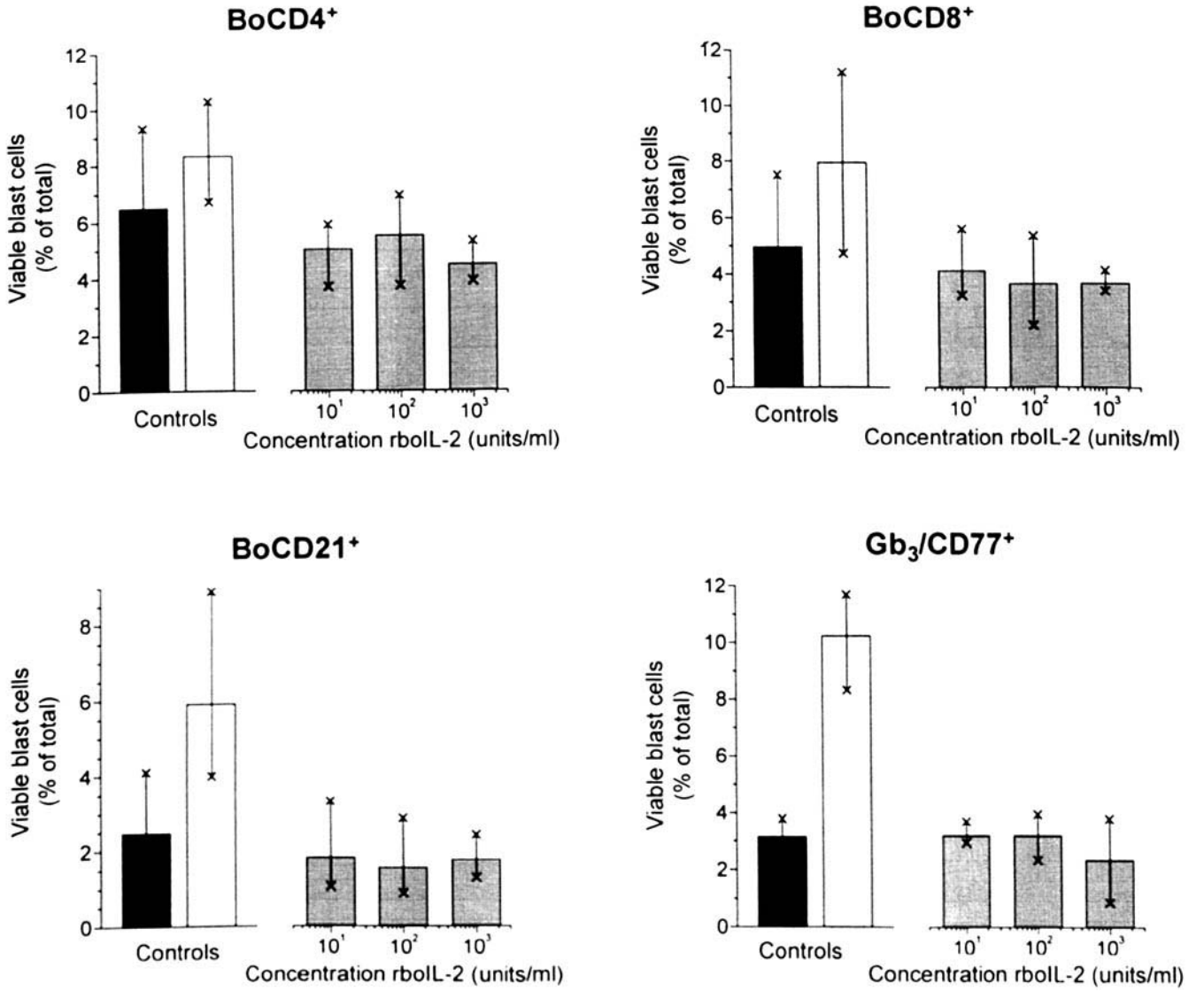


**Figure 4.** Effect of rboIL-2 and VT1 on transformation and proliferation of PBMC subpopulations. Cells were either incubated with VT1 (200 $CD_{50}$/ml, quantified on Vero cells) with (open bars) or without (black bars) anti-VTB1 mAb 13C4 (1.5 μg/ml) or with VT1 together with indicated concentrations of rboIL-2 (striped bars) at 37°C. Culture medium was supplemented with 5 μg/ml PHA-P. Lymphocyte subpopulations were identified by immunophenotyping after 96 hr of incubation, quantified by flow cytometry, and the percentage of viable blast cells belonging to the indicated subpopulation was calculated. Bars represent the mean, minimum, and maximum of determinations from three cows.

The effects of VT1 on bovine lymphocytes also were not due to a VT1-induced release of TNF-α from monocytes because addition of rboTNF-α (1–10,000 ng/ml) alone did not mimic the effect of VT1 and did not reduce the cellular metabolic activity (data not shown). Although rboTNF-α (10–1,000 ng/ml) led to a slight decrease ($P \leq 0.05$ for 100 and 1000 ng/ml rboTNF-α each versus VT1 + anti-VTB1) in the percentage of BoCD21$^+$ blast cells (Fig. 5), the percentages of BoCD8$^+$ and $Gb_3$/CD77$^+$ blast cells were not different ($P > 0.05$ for rboTNF-α versus VT1 + anti-VTB1) in rboTNF-α-treated and negative control cultures (Fig. 5).

Recombinant human interferon-α (rhuIFN-α; 1.5–1,500 units/ml) alone did not significantly alter the transformation and proliferation of PBMC subpopulations and the number of viable $Gb_3$/CD77$^+$ blast cells, but slightly depressed ($P \leq 0.05$) the metabolic activity of the cells at high concentrations (15,000 units/ml; data not shown). To test whether IFN-α was able to modulate the effect of VT1, the cells were incubated in the presence of 1.5–150 units/ml rhuIFN-α simultaneously to VT1. In this experiment, the metabolic activity of PBMC was reduced to a level similar to that when incubated with VT1 alone (Fig. 6). Moreover, rhuIFN-α augmented the VT1-induced reduction at higher concentrations. The addition of rhuIFN-α (15–1,500 units/ml) to VT1-treated cultures only marginally affected the transformation and proliferation of PBMC subpopulations (Fig. 7). In cultures with rhuIFN-α and VT1, the percentage of $Gb_3$/CD77$^+$ blast cells was reduced to a level similar to that in cultures treated with VT1 alone ($P > 0.05$ for VT1 + rhuIFN-α versus VT1).

**Effect of VT1 on Prestimulated Bovine PBMC.** The results described above suggest that induction of $Gb_3$/CD77 expression occurred before the inhibitory effect of VT1 when the toxin is present in the culture from the be-

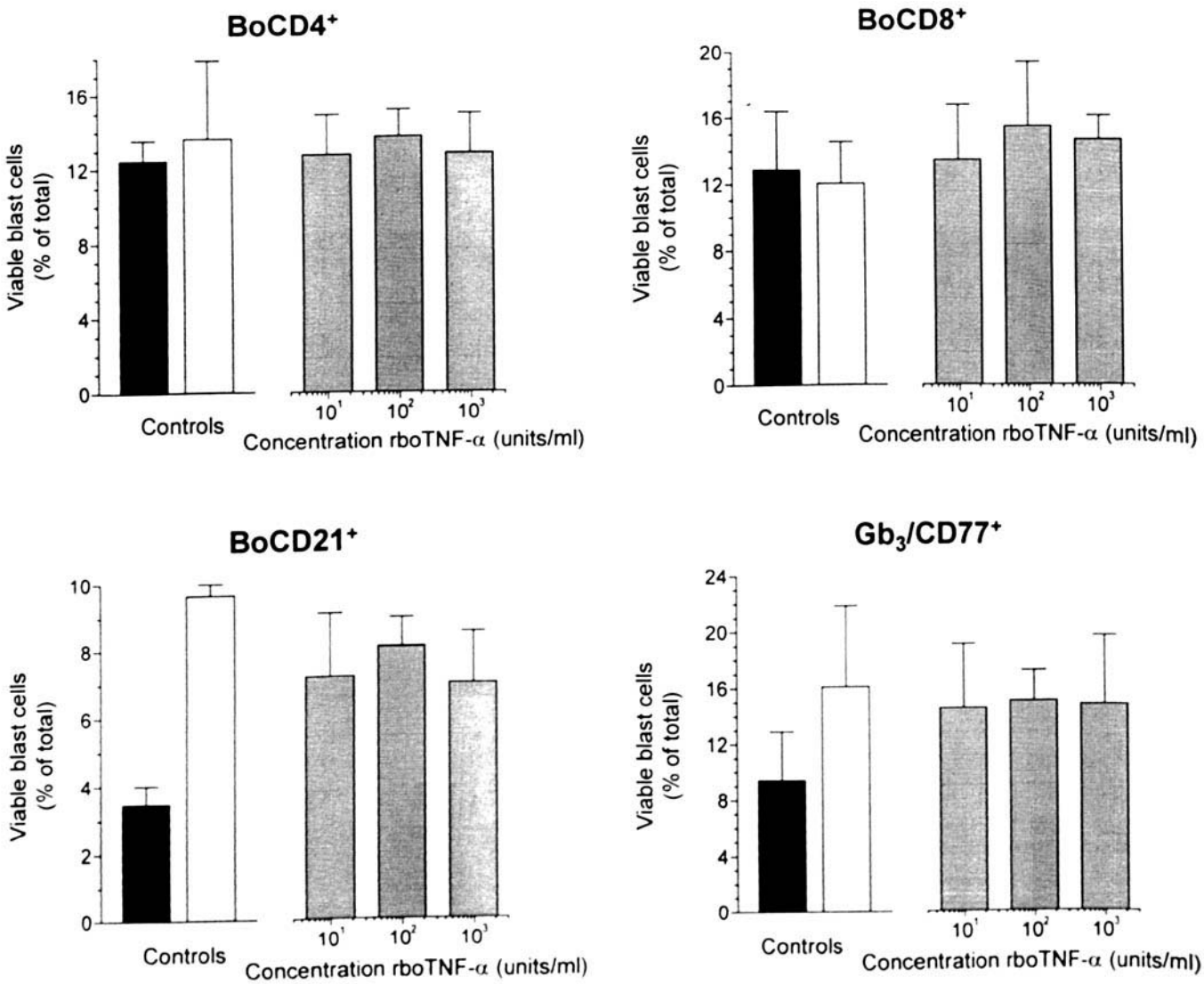


**Figure 5.** Effect of rboTNF-α on transformation and proliferation of PBMC subpopulations. Cells were either incubated with VT1 (200 $CD_{50}$/ml, quantified on Vero cells) with (open bars) or without (black bars) anti-VTB1 mAb 13C4 (1.5 μg/ml) or with rboTNF-α at the indicated concentrations (striped bars) at 37°C. Culture medium was supplemented with 5 μg/ml PHA-P. Lymphocyte subpopulations were identified by immunophenotyping after 96 hr of incubation, quantified by flow cytometry, and the percentage of viable blast cells belonging to the indicated subpopulation was calculated. Bars represent the mean ± SD of determinations from four cows.

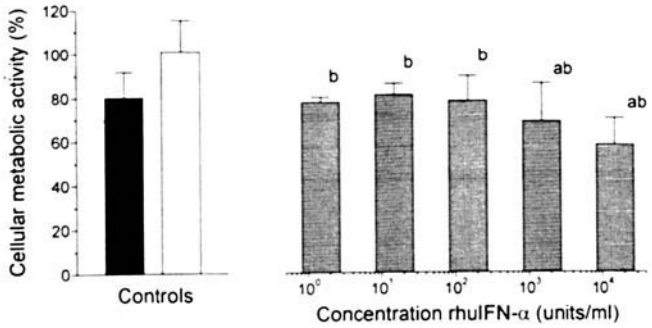


**Figure 6.** Effect of rhuIFN-α and VT1 on the cellular metabolic activity of bovine PBMC. Cells were either incubated with VT1 (200 $CD_{50}$/ml, quantified on Vero cells) with (open bar) or without (black bar) anti-VTB1 mAb 13C4 (1.5 µg/ml) or with VT1 together with indicated concentrations of rhuIFN-α (striped bars) at 37°C. Culture medium was supplemented with 5 µg/ml PHA-P. Cellular metabolic activity was determined by MTT reduction assay after 96 hr of incubation. Percentage activity was calculated and data represent mean ± SD of 12 determinations with cells from four cows. Differences ($P < 0.01$) to VT1 (a) and VT1 + anti-VTB1 (b) were detected.

ginning. Consequently, PBMC that were prestimulated to express $Gb_3$/CD77 at high levels before they experienced VT1 should have been much more sensitive to the toxin. We compared PHA-P-stimulated PBMC treated with VT1 from the beginning of the incubation (Day 0) until Day 4 with cells that received toxin after a 3-day prestimulation period and were characterized on Day 6 (Fig. 8). In fact, VT1 led to a more pronounced decrease in the number of viable $BoCD8^+$ cells and $BoCD21^+$ cells when added to prestimulated PBMC. However, reduction of cell numbers was not notably different from that achievable in cultures treated from Day 0 until Day 4 (Figs. 1–7). Even when VT1 was added to the cells on Day 3, a prominent number of $Gb_3$/$CD77^+$ cells were still detectable after 3 days of cultivation. Based on our data, susceptibility of bovine PBMC cultures for VT1 did not directly correlate with the percentage of $Gb_3$/$CD77^+$ cells.

## Discussion

Several studies using experimental infections and cultures of lymphoblastoid cell lines suggested that VT can cause immunomodulatory effects in animals infected with VTEC (16, 25–27). Based on our finding that VT1 inhibits bovine lymphocyte proliferation *in vitro* (15), we attempted to elucidate the mechanism underlying the immunomodulation by using primary cultures. Expression of $Gb_3$/CD77 (recognized as the VT1 receptor in other cell systems) in fact preceded the inhibitory effect of VT1 on bovine lymphocytes. After activation-induced $Gb_3$/CD77 surface expression, VT1 subsequently reduced the portion of $Gb_3$/$CD77^+$ cells. Inhibition by VT1 predominantly affects $BoCD8^+$ and $BoCD21^+$ cells (15), but $Gb_3$/$CD77^+$ cells were eliminated from all subpopulations identified. Therefore, the bovine immune system appears to be more sensitive to VT1 than the human immune system because in the latter, the activity of VT1 is restricted to the B cell compartment (25, 28). The significance of VT1 in bovine VTEC infections has been questionable since Pruimboom-Brees *et al.* (29) published information that cattle lack vascular receptors for VT. However, the present study indicates that in contrast to other species in which endothelial cells are the main targets for VTs, immune system cells are a predominant target for VT1 in cattle.

The impact of immunomodulatory effects caused by bacterial products *in vitro* on the course of bacterial infections can only be appropriately estimated if it is understood whether this effect truly results from a direct action on immune system cells or is rather due to a perturbation of cytokine profiles (30). Thus, we investigated the effects of selected cytokines in the presence and absence of VT1. First, TNF-α is secreted by human monocytes such as after stimulation with VT1 and sensitizes human endothelial cells by inducing $Gb_3$/CD77 (8). The PBMC preparations used in our studies consisted of up to 15% monocytes (15). Consequently, inhibition of lymphocytes could be secondary to a VT1-induced release of TNF-α from monocytes. However, the addition of recombinant bovine TNF-α (rboTNF-α) alone did neither augment the expression of $Gb_3$/CD77 nor did it mimic the effect of VT1 within a wide concentration range. Accordingly, we were not able to detect significant amounts of TNF-α in VT1-treated bovine monocyte cultures (C. Menge, unpublished data). Second, IFN-α only binds to its receptor (IFNAR) in its high-affinity conformation complexed to $Gb_3$/CD77 (10). Due to the partial homology between the bovine IFNAR and the receptor-binding B-subunit of VT1 (10), it appeared that IFN-α might have been able to block the activity of VT1 by stabilization of the $Gb_3$/CD77-IFNAR complex. However, the fact that rhuIFN-α augmented the inhibitory effect of VT1 in a concentration-dependent manner suggested that both substances use different signal transduction pathways. Third, because VT1 increased and prolonged expression of the bovine IL-2 receptor BoCD25 (15), we speculated that the effect of VT1 could have been due to a blockage of the paracrine boIL-2-release. This phenomenon is induced in human lymphocyte cultures (31) by a factor encoded for by the *lifA* gene of enteropathogenic *E. coli* (32). The fact that exogenous rboIL-2 was unable to overcome the effect of VT1 on bovine lymphocytes in a concentration range from 10 to 1000 units/ml suggests a direct effect of VT1.

An impact of cytokines cannot be ruled out completely because cytokines other than those used in our study may be involved, but VT1 drastically decreased the number of $Gb_3$/$CD77^+$ cells and predominantly acted as a leukotoxin rather than a modulin. Interestingly, low numbers of positive cells were still detectable during the entire cultivation period, even in the presence of VT1. Because VT1 also lowered the mean number of $Gb_3$/CD77 molecules on the surface of positive cells as concluded from a reduced mean fluorescence intensity (data not shown), VT1 seemed to induce a cytotoxic mode of action by eliminating $Gb_3$/$CD77^{high}$ cells.

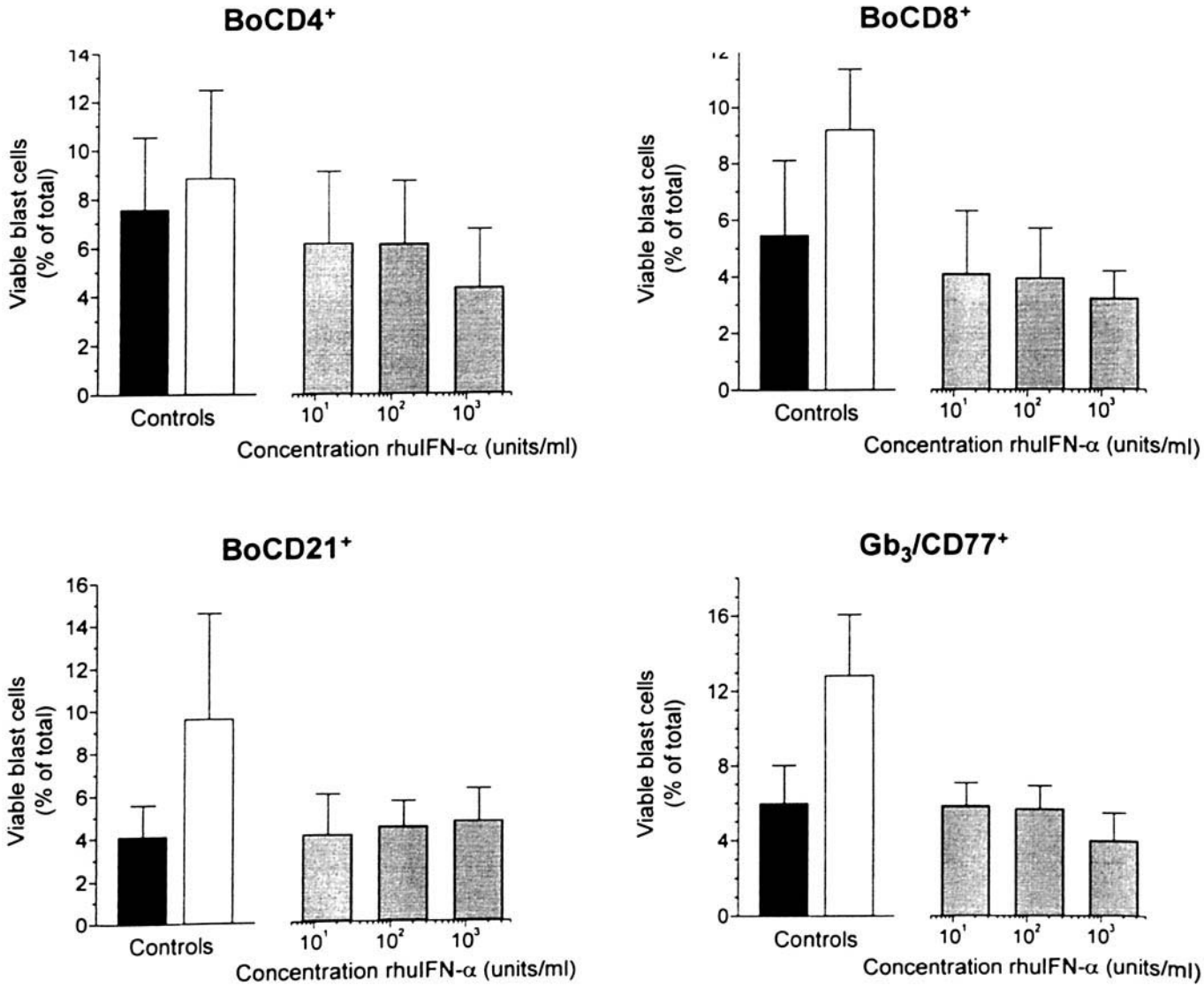


**Figure 7.** Effect of rhuIFN-α and VT1 on transformation and proliferation of PBMC subpopulations. Cells were either incubated with VT1 (200 $CD_{50}$/ml, quantified on Vero cells) with (open bars) or without (black bars) anti-VTB1 mAb 13C4 (1.5 µg/ml) or with VT1 together with various concentrations of rhuIFN-α (striped bars) at 37°C. Culture medium was supplemented with 5 µg/ml PHA-P. Lymphocyte subpopulations were identified by immunophenotyping after 96 hr of incubation, quantified by flow cytometry, and the percentage of viable blast cells belonging to the indicated subpopulation was calculated. Bars represent the mean ± SD of determination from four cows.

Such a correlation between the susceptibility of cells for VT1 and the number of $Gb_3$/CD77 molecules expressed on the cellular surface has already been described for other cell lines (33). However, there is evidence that the effect of VT1 on bovine cymphocytes is much more sophisticated. First, there is a marked contradiction between the prominent reduction in cellular metabolic activity caused by VT1 and the lack of a detectable increase in the number of apoptotic cells on Day 4 of culturing (15). Second, the percentage of $Gb_3$/CD77$^+$ cells was drastically decreased by VT1, even in those subpopulations of bovine lymphocytes proliferation of which was only marginally affected (Fig. 2). Third, the reduction of $Gb_3$/CD77$^+$ cells also was significant within the nonblast population of lymphocytes (Figs. 1 and 2) that expressed $Gb_3$/CD77 just at low to moderate levels (18). $Gb_3$/CD77 expression by bovine lymphocytes parallels the activation of the cells up to a certain stage characterized by moderate $Gb_3$/CD77 expression (18). At this point, the cells either survive and probably downregulate $Gb_3$/CD77 again or die from apoptosis expressing high levels of $Gb_3$/CD77. The effect of VT1 thus likely occurred early in cultivation on apparently small numbers of sensitive cells between the $Gb_3$/CD77$^{low}$ and the $Gb_3$/CD77$^{moderate}$ state of activation. These cells then end up in apoptosis after transient high-level $Gb_3$/CD77 expression. This might explain the significantly enhanced percentage of $Gb_3$/CD77$^+$ nonblast cells in VT1-treated cultures on Day 2 (Figs. 1 and 2). This notion is further supported by the fact that the addition of VT1 on Day 3 of the cultures still induced effects in a magnitude similar to that associated with addition of the toxin at the beginning. The cells must have become refractory again when they reached the $Gb_3$/CD77$^{high}$ state before experiencing VT1. Because bovine lymphocytes are VT1 sensitive only transiently during the activation process, VTEC

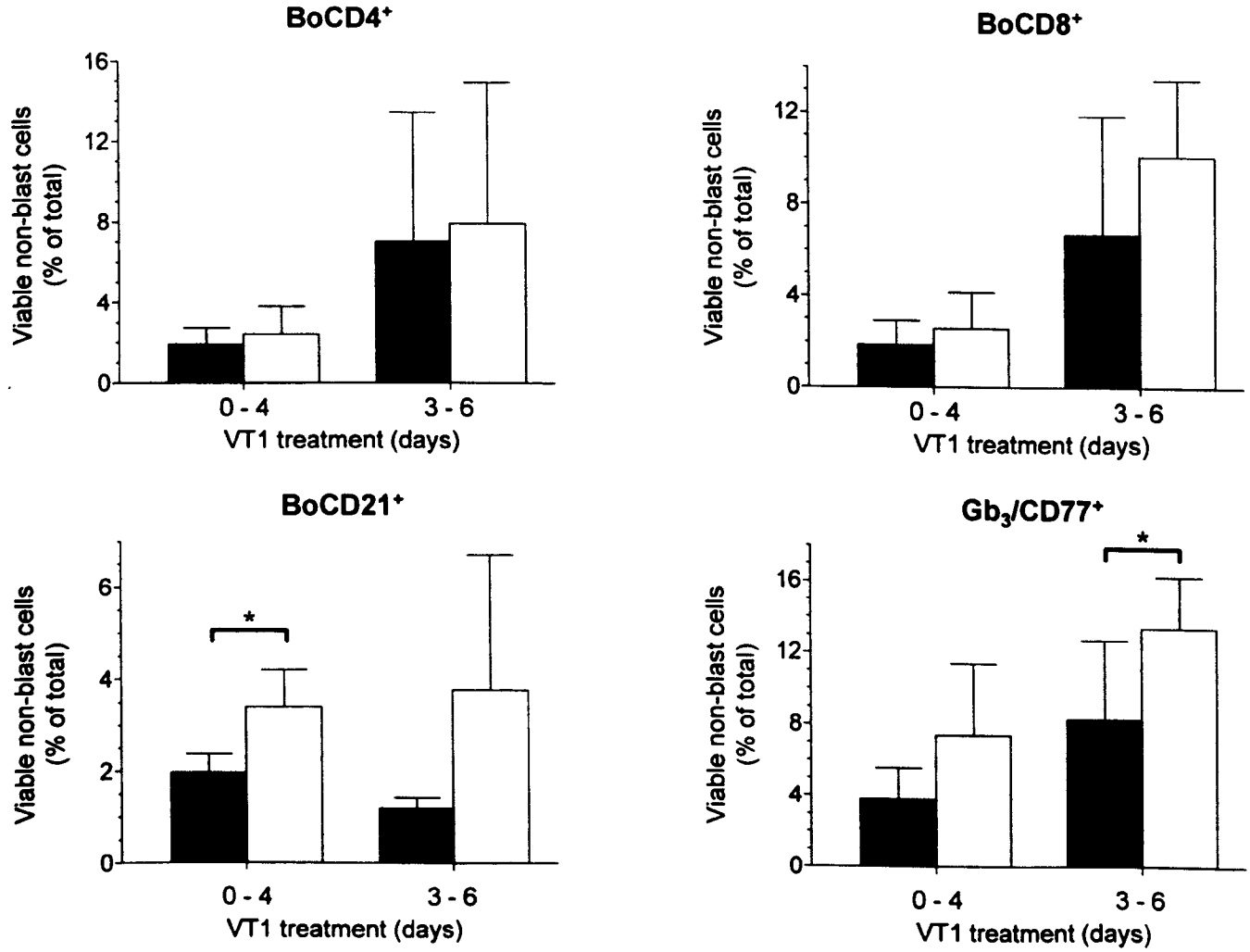


**Figure 8.** Effect of VT1 on prestimulated PBMC. Cells were incubated with VT1 (200 $CD_{50}$/ml, quantified on Vero cells) either with (open bars) or without (black bars) anti-VTB1 mAb 13C4 (1.5 μg/ml) at 37°C starting either on the day of PBMC preparation (Day 0) or on Day 3 of incubation. Culture medium was supplemented with 5 μg/ml PHA-P. Lymphocyte subpopulations were identified by immunophenotyping on Days 4 and 6 of incubation, respectively, quantified by flow cytometry, and the percentage of viable nonblast cells belonging to the indicated subpopulation was calculated. Bars represent the mean ± SD of determinations from four cows. Horizontal brackets enclose bars that are different ($P \leq 0.05$ [*]).

secreting VT1 predominantly block the onset of an immune response in their host rather than downregulating an established one. From the bacterial point of view, this strategy is highly efficient as less amounts of toxin are necessary to target smaller numbers of immune cells.

The fact that VT1 directly targets several subsets of lymphocytes at an early phase of activation underlines that the immunomodulation via secretion of VT1 is a very specific feature of VTEC. The impact of these findings on the course of VTEC infections can only be a matter of speculation. Currently, the impact of the interactions between VT and immunocytes on pathogenesis of HUS is under intense review (34–36). In cattle, Hoffman *et al.* (16) showed that experimental VTEC infection of calves led to a reduced mitogenic responsiveness of PBMC *in vivo*, confirming the relevance of our *in vitro* results. However, adult cattle are asymptomatic carriers of VTEC and frequently possess antibody titers against VT (37). Therefore, VT1 presumably modulates the host's immune response of adult cattle on a local level rather than causing a systemic immune deficiency. Because Paton *et al.* (38) showed that LPS-specific antibodies can prevent the adhesion of VTEC to epithelial cells, VT1 may facilitate the colonization of the bovine gut by acting on local B cells, hindering antibody production or secretion. This would explain why VTEC are shed for longer periods compared with the closely related but VT-negative enteropathogenic *E. coli* strains as it has been shown to occur in sheep (14). Accordingly, a prominent portion of bovine B cells separated from mesenteric lymph nodes are $Gb_3$/CD77$^+$ (18). Interestingly, the bovine intestinal intraepithelial lymphocytes also express $Gb_3$/CD77 *in vivo* and are sensitive to the proliferation inhibiting effect of VT1 *in vitro* (39). Further investigations on VT effects on mucosal immune cells will, therefore, be of considerable importance in increasing our understanding of VTEC's adaptation to their bovine reservoir.

We thank J. Naessens at ILRI (Nairobi, Kenya) for generously supplying hybridoma cell lines producing antibodies to bovine leukocyte antigens. R. Collins at IAH (Compton, UK) and R. Steiger at CIBA-GEIGY (Basel, Switzerland) are acknowledged for supplying recombinant bovine cytokines. We thank Heike Schoepe for performing the statistical analysis.

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