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Investigation on the effect of *P. haemolytica* A1 leukotoxin on some cellular functions

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SUMMARY

The main virulence factor associated with P. haemolytica is believed to be a toxin (leukotoxin) which kills leukocytes of ruminants. Therefore, possible effect of the leukotoxin on the capacity of monocytic cell lines (MCL) to phagocytose latex beads, the susceptibility of mononuclear cells (MNC) and MCL to the leukotoxin of P. haemolytica A1, the suppressive affects of sublethal concentrations of the leukotoxin on MNC responses to phytohaemagglutinin (PHA) were aimed to investigate. As a result, leukotoxin derived from P.haemolytica A1 has no cytolytic effect on MCL derived from peripheral blood monocytes of lambs and calves while it had about 56% cytolytic activity (1/2 dilution) on fresh ovine MNC. Phagocytic activity of MCL were not depressed by the leukotoxin either. Sublethal concentrations of the leukotoxin depressed ovine MNC responses to mitogen, PHA and this depression was totally neutralised by the addition of RGF and partially neutralised by the addition of IL-1 and IL-2.

Key words: *P.haemolytica, leukotoxin, susceptibility, monocytic cell lines, LT responses, rat growth factor, interleukin 1,-2.*

P. haemolytica A1'den elde edilen lökotoksinin bazı hücresel fonksiyonlar üzerine etkilerinin araştırılması

ÖZET

Pasteurella haemolytica ile ilgili asıl virulens faktörün ruminantların lökositlerini tahrip eden bir toksin (leukotoxin) olduğuna inanılmaktadır. Bu nedenle, bu toksinin kuzu ve buzağıların monositlerinden elde edilen monositik hücre hatlarının (MCL) latex beads'lerini fagosite etmeleri üzerine olan etkilerini, toksine karşı bu hücre hatlarının ve mononükleer hücrelerin (MNC) duyarlılıklarını ve öldürücü dozun altındaki leukotoxin'in kuzuların mononükleer hücrelerinin bir mitojen olan phytohaemagglutinin (PHA)'e cevabı üzerindeki etkileri araştırıldı. Sonuç olarak, mononükleer hücreler P. haemolytica A1' den elde edilen leukotoxinin ½ sulandırmasına karşı bile yaklaşık % 56 oranında lize olurken, monositik hücre hatları üzerine herhangi bir sitolitik etkisi görülmedi. Aynı toksin bu hücre hatlarının fagositik aktivitelerini de deprese edemediği gözlendi. Öte yandan, leukotoxinin öldürücü dilüsyonlarının altındaki dilüsyonları, mononükleer hücrelerin bir mitojen olan PHA'ya cevabını deprese ettiği ve bu depresyonun rat growth faktörü (RGF) tarafından tamamen, inteleukin 1 ve 2 tarafından ise kısmen ortadan kaldırıldığı saptandı.

Anahtar kelimeler: *P.haemolytica, leukotoxin, duyarlılık, monositik hücre hattı, lenfosit transformasyonu, rat growth faktörü, interleukin 1, 2.*

INTRODUCTION

P. haemolytica type A1 is a major secondary respiratory pathogen in ruminants (14). It is one of the most common causes of bronchopneumonia in ruminants following viral infections (1,18,19). The main virulence factor associated with *P. haemolytica* is believed to be a toxin (leukotoxin) which kills leukocytes of ruminants (3,14). Leukotoxins are usually assayed using leukocytes freshly obtained from ruminants but it is not known whether cell lines derived from peripheral blood monocytes of ruminants are susceptible to the leukotoxin.

Mononuclear cell (MNC) responses to mitogens such as phytohaemagglutinin (PHA) and concanavalin A (ConA) have been extensively used as an index of T cell responses *in vitro* (7,21). Sublethal concentrations of *P. haemolytica* A1 leukotoxin have been shown to depress bovine lymphocyte responses to mitogens, ConA and PHA (4,10,11). This depression can be neutralized by the addition of interleukin 1 (IL-1) and interleukin 2 (IL-2) (11). However, the effect of rat growth factor (RGF) on depressed MNC responses has not been studied before

Monocytic cell lines (MCL) that remain most of their functional characteristics have been developed from the peripheral blood of several species (9,20). However, the behaviour of MCL derived from peripheral blood monocytes against leukotoxin of *P. haemolytica* has not been studied.

The purpose of the present study was to investigate the possible effect of leukotoxin on the capacity of MCL to phagocytose latex beads. The susceptibility of MNC and MCL to the leukotoxin of *P. haemolytica* A1 was also examined. The suppressive affects of sublethal concentrations of the leukotoxin on MNC responses to PHA were investigated whether it can be reversed or reduced by the direct addition of IL-1, IL-2 or RGF.

MATERIALS AND METHODS

This study was performed at the University of Liverrpool, Faculty of Veterinary Science, Department of Clinical Science, Leahurst, UK.

Cells

Heparinized blood samples, obtained from 21 cross-breed lambs between 4 and 8 weeks of age, were used to prepare MNC. The number of animals from which collected for each treatment (susceptibility, lymphocyte blastogenesis) are indicated in Tables 1,2,3 and 4. The MNC were separated from whole blood and used for lymphocyte blastogenesis as described earlier (17). Ovine and bovine MCL used for susceptibility test and phagocytosis were prepared as described earlier (9).

Leukotoxin

A reference strain of *P. haemolytica* biotype A, serotype 1 was donated by the Moredun Institute, Edinburgh. *P. haemolytica* leukotoxin was prepared by the method of O'Brien and Diffus (14). Briefly, *P. haemolytica* was subcultured onto blood agar plates overnight. Several colonies were then inoculated into 100 ml of tryptose phosphate broth containing 0.5% yeast extract, and incubated at 37°C on a horizontal platform at 20 revs./minute for 5 hours. Broth cultures were centrifuged at 800 g for 10 minutes at 4°C and the resulting pellet resuspended in 5 ml RPMI 1640 medium. After mixing with a vortex mixer, 5 ml of the suspension were added to 100 ml of RPMI 1640 medium containing 10 % foetal calf serum (FCS) and incubated for 1 hour at 37°C on the rotary platform. After centrifugation at 6000 g for 20 minutes, the supernatant was filtered through 0.45 µm and 0.22 µm membrane filters (Millipore, UK), and separated into one ml aliquots and lyophilized. The sterility of the leukotoxin produced was confirmed by overnight culture in tryptose phosphate broth and blood agar.

Cytokines and rat growth factor

Commercial preparations of recombinant human interleukin 1-α (10^8 units/mg specific activity; Genzyme, Cambridge, USA) and IL-2 (4×10^6 unit/ mg specific activity; Genzyme, Cambridge, USA) were used at various concentrations. RGF was generously donated by Dr A. K. Sharma. It was prepared from spleens of female Sprague-Dawley rats, as described earlier (8).

Susceptibility test

To investigate whether MCL retain their susceptibility to *P.haemolytica* leukotoxin, suspensions of MNC and MCL were assayed using the 3[4,5-dimethylethiazoyl-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma, England) dye reduction assay as described by Sharma and Woldehiwet (16). Briefly, one hundred microlitres of either MNC or MCL in RPMI medium were placed at concentrations 5×10^5 and 1×10^5 cells per well respectively. One hundred microlitres of serial two-fold dilutions of leukotoxin were added to either the MNC or MCL in triplicates and incubated at 37°C for 1 hour. Control wells for each plates included MCL and MNC without leukotoxin and wells without cells (RPMI 1640 medium only).

After incubation, 20 µl of the dye MTT were added to each well at a concentration of 5 mg/ml and the plates were incubated for 4 hours. One hundred microlitres of 0.04N HCl in isopropanol (acid isopropanol) containing 3% sodium dodecyl sulphate were added to each well to stop the reaction, and the absorbance of each well was then determined by a MR 700 Microplate Reader (Dynatech Lab. Inc., USA) at a test wavelenght of 570nm, a reference wavelenght of 630nm and a calibration setting of 1.00. The machine was blanked with controls for non-specific dye reduction. The Per cent killing was calculated as follows:

Killing(%)= {1-[(OD of leukotoxin treated cells)/(OD of untreated cells)]} × 100

OD= optical density

Phagocytosis

Pagocytic activity of ovine and bovine MCL was performed as described earlier (9). Briefly, latex beads (Difco Laboratories, USA) with a diameter of 0.81 µm were washed with phosphate buffer solution (PBS), resuspended in 500 µl of heat-inactivated FCS and incubated for 30 minutes at 37°C. After centrifugation at 2700 g for 10 minutes, the FCS was removed and the beads were transferred to universal bottles containing 10 ml RPMI 1640 medium with 10% FCS, to give a final concentration of 10^8 latex beads/ml. This suspension was stored at 4°C and used in all subsequent experiments. Before use, the latex bead suspension was warmed to 37°C for 30 minutes and thoroughly mixed; a 100-µl volume, containing 10^7 latex beads, was placed in each well, containing either ovine or bovine MCL to give a bead to cell ratio of 100:1. All assays were carried out in 24-well plates, each of which was provided with a cover glass. The plates were then incubated for 1 hour at 37°C and the wells were washed three times with warm PBS to remove free latex beads. Cells adherent to cover glass were stained with Diff-quick and 200 cells were examined microscopically. Those cells contained no latex beads were regarded as negative for phagocytosis. Phagocytosis was calculated as the percentage of cells containing one or more latex particles.

Mitogen

The mitogen phytohaemagglutinin (PHA, Sigma, UK) was diluted in RPMI 1640 medium to give a final concentration 40 µg/ml and used as described earlier (17).

MNC responses to mitogen PHA

The assays of MNC responses to PHA were performed as described by Sharma and Woldehiwet (17), with some modifications. Briefly, a suspension of ovine MNC (5×10^6 cells/ml) was dispensed in volumes of 50 µl into wells of flat-bottomed microtitre plates, followed by the addition of 50 µl volumes of various dilutions (sublethal concentrations) of leukotoxin; 50 µl volumes of different concentrations of IL-1, IL-2 and RGF suspension were then added, followed by 50 µl of PHA. Same amount of RPMI 1640 medium were added to the wells used for control PHA instead of leukotoxin and cytokines. The plates were incubated at 37°C in a humidified atmosphere containing CO_2 5% in air for 72 hours. Radio-labelling was carried out by adding 0.5 µCi of [^{3}H]-thymidine (Amersham, UK) per well in 50 µl of RPMI 1640 medium 24 hours before terminating incubation. The degree of blastogenesis was expressed as the ratio of [^{3}H]-thymidine incorporation in cultures stimulated by mitogen (PHA) to that of control cultures (Stimulation index, SI).

Analysis of Data

All values were expressed as the mean ± standard deviation. The significance of the differences between treatments was analysed by Student's *t*-test.

RESULTS

Susceptibility to leukotoxin

In this study, ovine MNC and ovine and bovine MCL were used to determine the effect of the leukotoxin on cell viability. There was about 56% reduction in ovine MNC viability at 1/2 dilution of the leukotoxin. The leukotoxic

effect disappeared when the leukotoxin diluted 1/32. However, MCL of both ovine and bovine origin were uneffected by the leukotoxin at any dilutions (Figure 1).

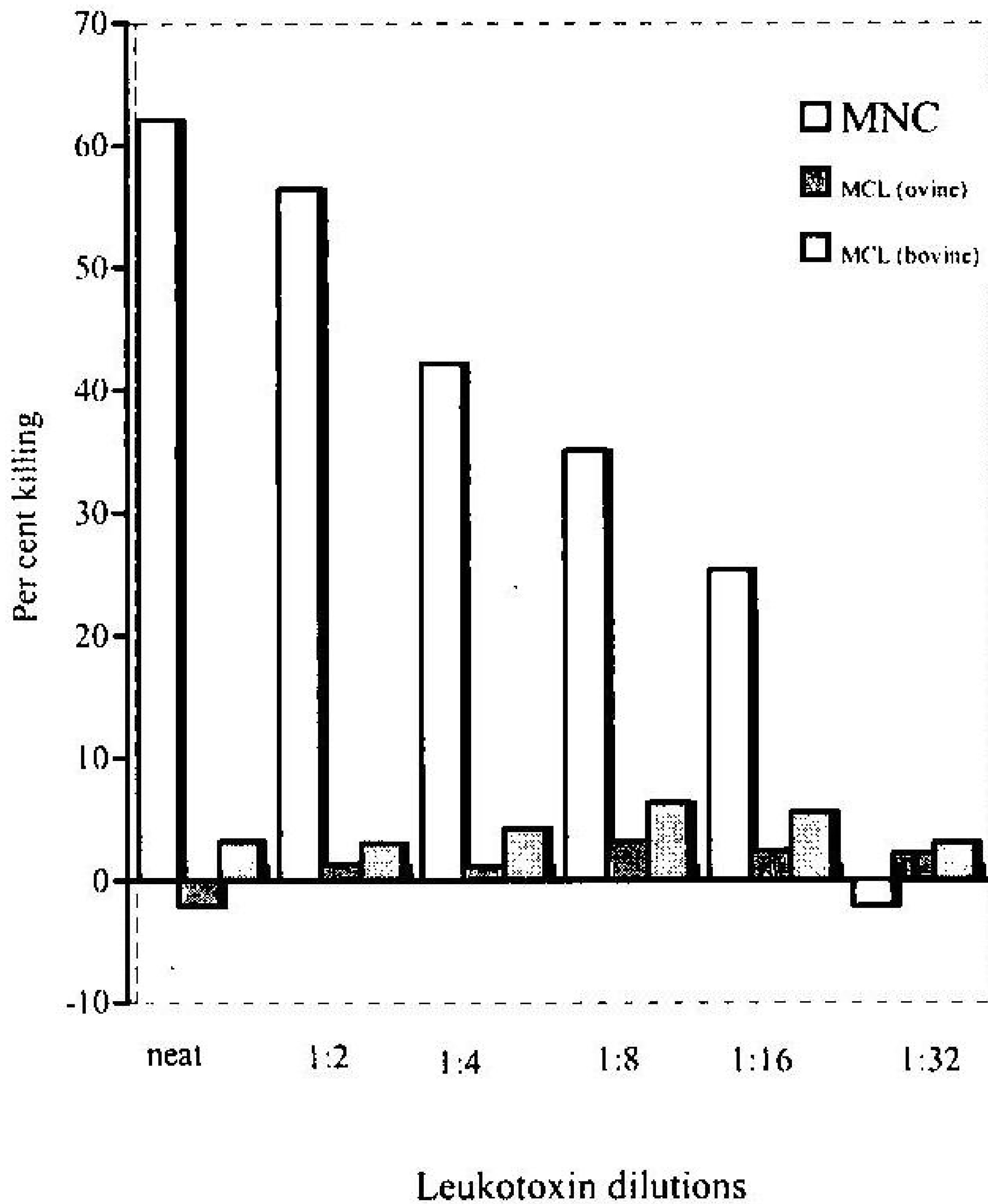

Figure 1: Susceptibility (% killing) of ovine MNC and ovine and bovine MCL to the leukotoxin of *P. haemolytica* A1 (n=6).

Effect of leukotoxin on phagocytic activity of MCL

Incubation of ovine and bovine MCL with *P. haemolytica* A1 leukotoxin significantly ($p<0.01$) increased the phagocytic activity of ovine MCL (Table 1).

Table 1: Effect of *P. haemolytica* A1 leukotoxin on the phagocytic activity (%) of ovine and bovine MCL.

	Control (% phagocytosis)	Leukotoxin added (% phagocytosis)
Bovine MCL (n=6)	89.75 ± 4.57	92.25 ± 3.76
Ovine MCL (n=6)	78.50 ± 2.89	90.00 ± 4.97*

*= p<0.01

Effect of sublethal concentration of the leukotoxin on ovine MNC responses to PHA

Sublethal concentrations (1/32, 1/64, 1/128) of *P. haemolytica* A1 leukotoxin were suppressive to ovine MNC responses to PHA (Table 2, Figure 2). These sublethal concentrations of the leukotoxin were not lytic to ovine MNC when tested by MTT and trypan blue method. Addition of RGF totally eliminated depression caused by sublethal concentrations of leukotoxin. Most significant increases in MNC responses to PHA were observed when RGF added together with depressive concentrations of *P. haemolytica* A1 leukotoxin (Figure 2, Table 2).

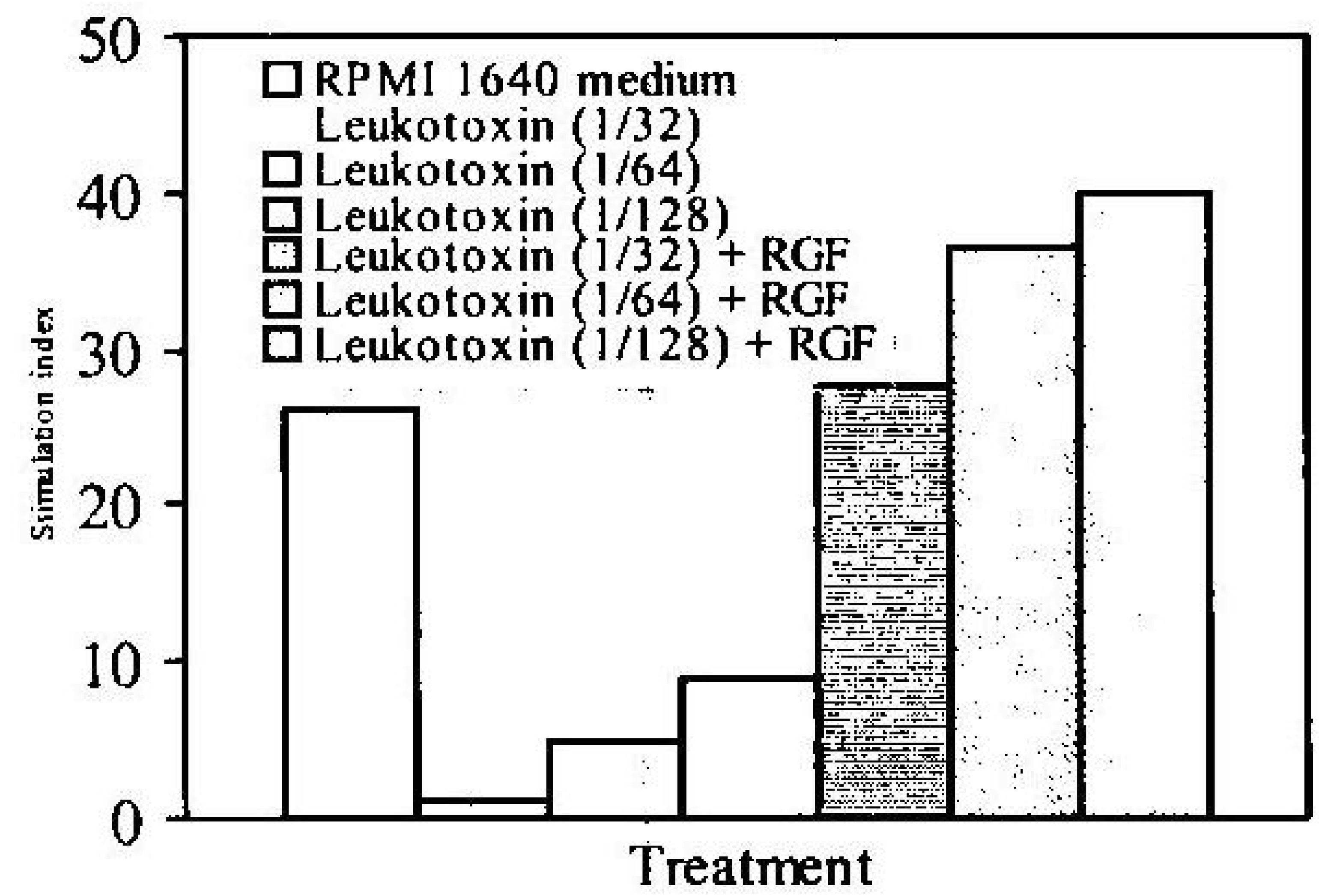

Figure 2: Effects of RGF and different dilutions of leukotoxin of *P. haemolytica* type A1 on ovine MNC responses to PHA.

Table 2: Effect of rat growth factor (RGF) on leukotoxin related depression of MNC responses to PHA.

n=12	SI (mean±sd)
PHA	26.13 ± 10.67
PHA+L (1/32)	1.18 ± 0.57
PHA+L (1/64)	4.77 ± 2.55
PHA+L (1/128)	9.04 ± 4.75
PHA+L (1/32) +RGF	27.67 ± 13.34
PHA+L (1/64) +RGF	36.56 ± 13.42
PHA+L (1/128) +RGF	40.04 ± 16.64

PHA= phytohaemaglutinin, L= leukotoxin (dilutions), RGF= rat growth factor, SI= stimulation index

Suppressive effects of the leukotoxin (1/32 dilution) were not eliminated totally by the addition of human recombinant IL-1, but the depressive effect was significantly ($p<0.05$) restored when IL-1 added together with sublethal concentrations of leukotoxin and PHA (Figure 3, Table 3).

Depression of ovine MNC responses to PHA caused by sublethal concentrations (1/32) of the leukotoxin were partially eliminated ($p<0.05$) when different concentrations of human recombinant IL-2 were added together with leukotoxin and PHA, but the restored MNC responses to PHA were not still as high as control MNC responses to PHA (Figure 4, Table 4).

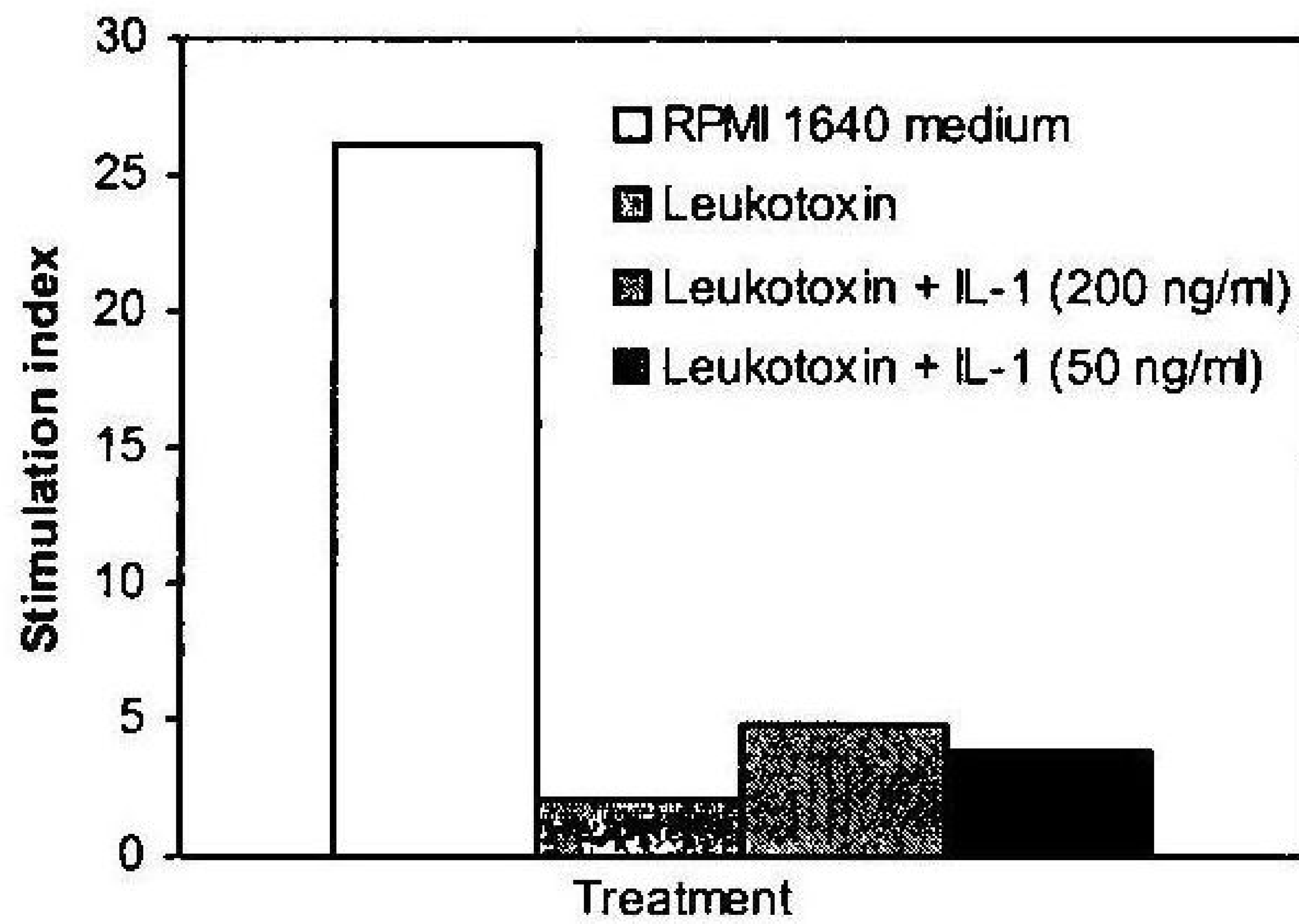

Figure 3: Effects of human recombinant interleukin 1 (IL-1) and leukotoxin of *P. haemolytica* A1 (1/32 dilution) on ovine MNC responses to PHA.

Table 3: Effect of addition of interleukin 1 (IL-1) on leukotoxin (L) related depression of ovine MNC responses to PHA.

n=7	SI (mean ± sd)
PHA	26.13 ± 10.67
PHA+L (1/32)	1.18 ± 0.57
PHA+L (1/32) + IL-1 (200 ng/ml)	4.90 ± 2.35
PHA+L (1/32) + IL-1 (50 ng/ml)	3.54 ± 1.45

PHA= phytohaemaglutinin, L= leukotoxin (dilutions), IL-1= interleukin 1, SI= stimulation index

Tabel 4: Effect of interleukin 2 (IL-2) on the leukotoxin related depression of ovine MNC responses to PHA

n=7	SI (mean±sd)
PHA	26.13 ± 10.67
PHA+L (1/32)	1.18 ± 0.57
PHA+L (1/32) + IL-2 (200 ng/ml)	9.23 ± 2.78
PHA+L (1/32) + IL-2 (50 ng/ml)	6.15 ± 1.18

PHA= phytohaemaglutinin, L= leukotoxin (dilutions), IL-2= interleukin 2, SI= stimulation index

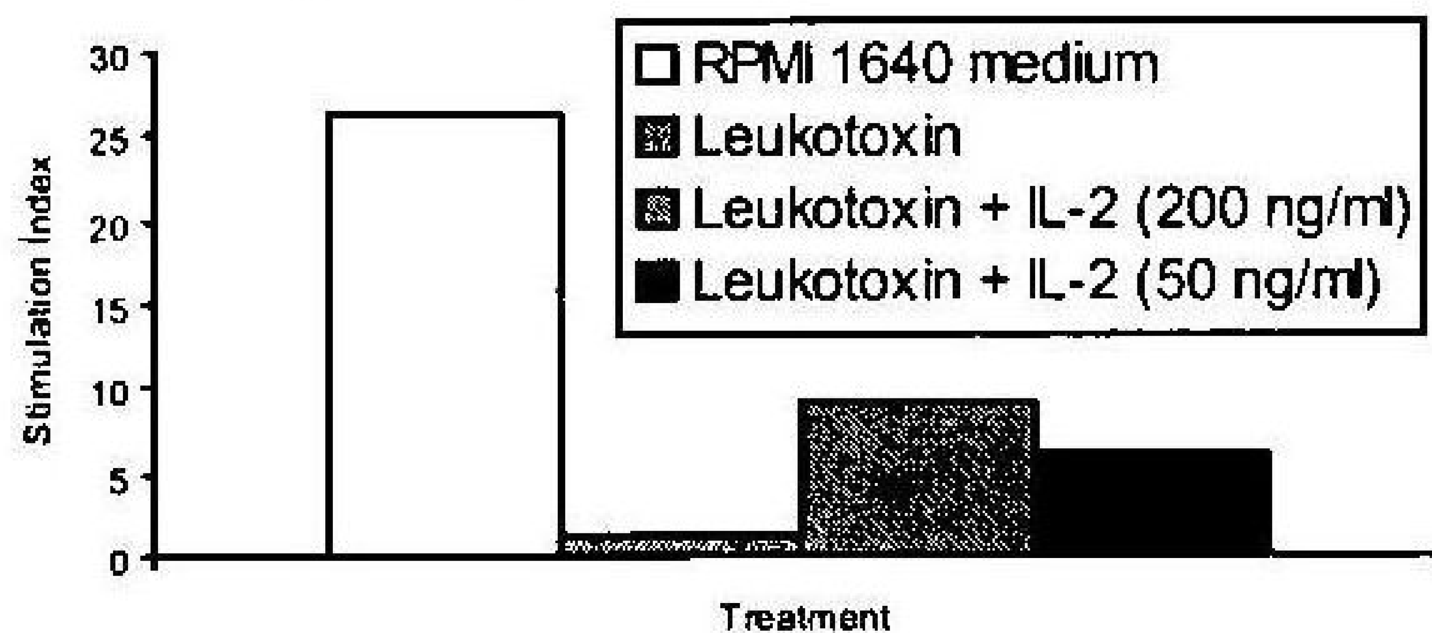

Figure 4: Effects of human recombinant IL-2 and leukotoxin of *P. haemolytica* A1 (1/32 dilution) on ovine MNC responses to PHA.

DISCUSSIONS

Monocytes/macrophages are important cells in defence system of an organism. Some of the important activities are phagocytosis, antigen presentation and releasing some madiators such as monokines. It has been reported that MCL derived from peripheral blood monocytes keep their phagocytic activity and antigen presenting capacity (9). Whether they release any mediators are not known. Their behaviour to the leukotoxin of *P. haemolytica* were not known either. *P. haemolytica* produces a subtance (leukotoxin) which is toxic to leukocytes of ruminant origin (3). This leukotoxin was reported to significantly accelerate the deterioration of alveolar macrophages without any apparent effect on the survival of parenchymal cells (22). It also severely damages bovine endothelial cell monolayers (2). In the present study, the leukotoxin had significant cytolytic effect on ovine MNC but had no effect on ovine and bovine MCL. It is difficult to explain the apparent stimulating effect of the leukotoxin on the phagocytic activity of ovine and bovine MCL but it could have arisen from the foetal calf serum used in the preparation of the leukotoxin. Alternatively, these results may suggest that although MCL retain most of the functional characteristics of macrophages (9), they may become less sensitive to certain toxins during cultivation.

MNC responses to mitogens have been used as a means to define cell-mediated immunity (15). Sublethal concentrations of *P. haemolytica* A1 leukotoxin have been shown to depress bovine MNC responses to mitogens, Con A and PHA (4,10,11). This depression can be neutralized by the addition of IL-1 and IL-2 (11). The results of the present study confirm previous observations that sublethal concentrations of leukotoxins of *P. haemolytica* have a depressive effect on ovine MNC responses (10,11). Furthermore, the leukotoxin-mediated depression seen in the present study was neutralised by the addition of optimum concentrations of IL-1, IL-2 and RGF. Blastogenic effect of IL-2 and RGF on ovine lymphocytes had also been demonstrated, earlier (8). The mechanisms of action of RGF are not clearly known but it is reported to contain several cytokines, including IL-1, IL-2, IL-3, IL-4, IL-5 and interferon-α (5,6,12,13).

In conclusion, leukotoxin derived from *P.haemolytica* A1 has no cytolytic effect on ovine and bovine MCL derived from peripheral blood monocytes of ovine and bovine respectively while it has about 56% cytolytic activity on fresh ovine MNC. Phagocytic activity of MCL were not depressed by the leukotoxin either. Sublethal concentrations of the leukotoxin depressed ovine MNC responses to mitogen, PHA and this depression was totally neutralised by the addition of RGF and partially neutralised by the addition of IL-1 and IL-2.

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