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Induction of tumor necrosis factor expression by a lectin from *Viscum album*

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Summary. A purified lectin (MLI) from *Viscum album* was used to test whether peripheral monocytes from human blood can be activated for the production of tumour necrosis factor (TNF). Cytotoxic activity was detected in the supernatant of MLI-stimulated monocyte cultures. This cytotoxic activity was completely inhibited by monoclonal antibodies to TNFα. Small amounts of soluble TNF protein were measured in a TNFα-specific enzyme-linked immunospecific assay system. Strong expression of TNFα mRNA was induced in human monocytes as well as in macrophage cultures from C3H/HeJ mice having a low response to endotoxin after 2 h of stimulation. Both chains of the MLI were found to induce TNF mRNA equally well in human monocytes. In macrophages of endotoxin-low-responder mice the toxic A chain was a better inducer of TNF mRNA than the galactose-specific lectin B chain. Thus, MLI has immunomodulating effects in activating monocytes/macrophages for inflammatory responses.

Key words: Tumour necrosis factor – *Viscum album* – Lectin

Introduction

Stimulation of the reticular endothelial system and especially of macrophages seems to be important in the defence of an organism against infections and tumours. Analysis of the mechanism and the structures involved in such activation of mononuclear phagocytes is required for the development of therapeutic applications. A great variety of biochemically different structures are already known for macrophage activation. Classical activating structures are bacterial lipopolysaccharide and interferon-γ (IFNγ) when the production of inflammatory monokines like tumour necrosis factor (TNF) or interleukin-1 (IL-1) is used as a parameter of macrophage activation [1, 3, 19]. Muramyldipeptide [23] and polyinosinic·polycytidylic acid [25] are also widely used for macrophage activation. Purified lipoarabinomannan from *Mycobacterium tuberculosis* has recently been reported to cause the release of TNF from human blood monocytes [21]. From plants, extracts from *Radix angelicae, Radix bupleuri, Rhizoma cnidii* and *Cortex cinnamomi* [14] and acidic arabinogalactan, a highly purified polysaccharide from cell cultures of *Echinacea purpurea* [17], were all able to activate macrophages effectively for TNF production.

For centuries the mistletoe *Viscum album* has been used therapeutically. In a series of papers it has been demonstrated that the main lectin from European mistletoe (mistletoe lectin I, MLI) belongs to the so-called toxic lectins besides ricin, abrin, modeccin and PCL, the lectin from *Phoradendron californicum* (for review see [6, 9]. These lectins all consist of an A and a B chain. The B chain of MLI is a galactose-specific lectin and the A chain acts enzymatically. Some years ago both chains were prepared without loss of biological activity [8, 22]. Together with Endo and his group we were able to show that the A chain of MLI is similar to the A chain of ricin: an *N*-glycosidase that releases adenine from position 4324 of 28S subunits of ribosomal RNA, thus serving as the toxophoric component [7]. Testing the biological activities of both A and B chains showed that the A chain is a mitogen. Moreover, it releases IL-1 and IL-2 from human lymphocytes [11]. The B chain activates macrophages both by direct interaction and by release of a macrophage-stimulating factor. Recently, Hajto et al. [13] published an account of a modulating potency of mistletoe extracts, which they explain by the activity of the B chain of MLI.

This investigation tested whether purified MLI or the isolated A or B chain was able to activate human blood monocytes or murine macrophages directly for TNF mRNA expression and TNF production.

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Materials and methods

Reagents. Lectin I (MLI) from *Viscum album* was prepared in the Staatliches Institut für Immunpräparate und Nährmedien, Berlin, FRG, according to the method described by Ziska et al. [26]. The MLI preparation (charge no. 000788) that was used for all experiments described in this manuscript contained 130 pg endotoxin/μg protein. A chain and B chain of MLI were prepared according to Franz et al. [8], using modifications by Pfüller et al. (manuscript in preparation). The A and B chain preparations contained 440 pg and 500 pg endotoxin/ μg protein, respectively, as determined by Dr. R. Urbaschek, Mannheim according to the method described in Ditter et al. [5]. Glutaraldehyde-fixed *Staphylococcus aureus* cells (Pansorbin, Calbiochem, Behring Diagnostics, La-Jolla, California) were used as a 0.1% (w/v) suspension. Recombinant human interferon γ (IFNγ) was provided by Dr. Adolf, Ernst Boehringer Institut für Arzneimittelforschung, with a specific activity of 2×10^7 U/mg. Lipopolysaccharide from *Salmonella minnesota* and *Polymyxin B* were purchased fom Sigma, Deisenhofen, FRG. Recombinant human TNFα (rhTNF) was supplied by Knoll/BASF AG, Ludwigshafen, FRG; the specific activity was 9×10^7 U/mg protein as measured in the bioassay. Monoclonal anti-rhTNF antibodies were obtained by fusing mouse spleen cells, from a mouse immunized with rhTNF, with AG8.653 myeloma cells by the polyethyleneglycol method and screening the hybridoma supernatants for neutralizing activity. The anti-TNF antibodies were used as a 1 : 1000 dilution of ascites fluid. The monoclonal anti-lymphotoxin antibody clone 9B9 and the monoclonal anti-rhTNF antibody clone 199 were a generous gift of Dr. A. Möller BASF, Ludwigshafen, FRG. Purified antibodies were used at a final concentration of 1 μg/ml.

Culture medium. The culture medium used was RPMI-1640 (Gibco) with 10% heat-inactivated fetal calf serum (Gibco), and gentamycin (50 μg/ml) (Sigma) unless stated otherwise.

Preparation of cells. Human peripheral blood mononuclear leucocytes were prepared from buffy coats of healthy blood donors by Ficoll-Hypaque density gradient centrifugation [2]. For the preparation of the adherent cell fraction the cells (3×10^6 cells/ml) were seeded in 24-well plastic tissue-culture plates (Falcon) and incubated for 2 h at 37° C in a humid atmosphere containing 5% CO_2. Nonadherent cells were removed by washing the cultures three times with culture medium. The remaining cell fraction consisted of over 90% monocytes as determined by morphology and phagocytosis. C3H/HeJ mice, having a low response to lipopolysaccharide, were purchased from the Hannoversche Versuchstieranstalt, Hannover, FRG. Peritoneal exudate cells were induced by injecting 1 ml phosphate-buffered saline i. p. 16 h before the peritoneal cells were washed out with cold medium. Samples of 5×10^6 peritoneal exudate cells were seeded in 24-well tissue-culture plates and the adherent fraction was obtained as described above.

TNF assay systems. The cytotoxicity assay for TNF was carried out on sensitive L929 mouse fibrosarcoma cells (2×10^4 cells/0.2 ml culture) in the presence of actinomycin D (2 μg/ml) (Sigma). The cultures contained serial dilutions of TNF samples. After 20 h of culture the surviving cells were fixed and stained with crystal violet (0.5% crystal violet, 3% v/v formaldehyde, 0.17% NaCl, 22% ethanol) for 15 min. Excess dye was washed off with water and the remaining dye solubilized in 33% acetic acid. Absorbance was measured at 540 nm. Concentrations of TNF are expressed as units indicating the reciprocal dilution of the samples in triplicate at which 50% survival was measured. Sensitivity of this assay varied between 1 ng and 0.5 ng rhTNF/ml.

TNF activity was also measured by an enzyme-linked immunospecific assay (ELISA) as described recently [18]. Plates (96-well flat-bottom, Titertek Immuno Assay Plate, Flow Laboratories, Meckenheim, FRG) were coated with affinity-purified (protein A-diasorb, Diagen, Düsseldorf, FRG), polyclonal rabbit anti-rhTNF antibodies 5 μg/ml in $NaHCO_3$ buffer (0.05 M, pH 9) for 16 h at 4° C. Serial dilutions of the test samples in phosphate buffer (0.1 M, pH 7.5, 2% EDTA, 1% bovine serum albumin) were applied to the plates for 2 h at room temperature after blocking with 1% bovine serum albumin in phosphate-buffered saline (PBS) for 2 h at room temperature. Plates were washed with PBS containing 0.05% Tween, and biotin (Sigma, Deisenhofen, FRG)-conjugated affinity-purified (protein-A – diasorb, Diagen, Düsseldorf, FRG) polyspecific rabbit anti-rhTNF antibodies (2.7 μg/ml) were added to the wells for 1.5 h at room temperature. After extensive washing with PBS containing 0.05% Tween, streptavidin-peroxidase complex (BRL, Karlsruhe, FRG) (1 : 2000 diluted) was applied for 30 min at room temperature. The plates were washed again with PBS containing Tween (0.05%) and the substrate solution [3,3′,-5,5′-tetramethylbenzidine, Miles Scientific, München, FRG, 10 mg in 100 ml sodium acetate/citric acid buffer (0.1 M, pH 4.9) and 14.7 μl 30% H_2O_2] was added to the complex. The reaction was stopped with 2 M H_2SO_4 and the absorption measured at 450 nm. The absorption curves obtained with the test samples were compared to a standard curve obtained with rhTNF and the TNF content was expressed as ng/ml. The sensitivity of this TNF assay was 0.5 ng rhTNF/ml.

Immunocytochemistry. The cells were fixed at room temperature with acetone for 20 min and treated with methanol containing 0.5% H_2O_2 for 3 min. Cell-associated TNF was determined with monoclonal mouse anti-rhTNF antibodies (clone 199) and peroxidase-coupled rabbit anti-(mouse Ig)antibodies (Sigma). The substrate 3-amino-9-ethylcarbazole (AEC, Sigma) was used. Controls were stained with an isotype-matched irrelevant first antibody.

RNA extraction and dot-blot analyses. The procedure has been described in detail recently [4]. Cells ($10^5 - 10^6$/culture) were solubilized with 1 ml 7.6 M guanidine/HCl in 0.1 M potassium acetate buffer pH 5, and DNA was sheared by aspirating five times through a 21-gauge needle. Ethanol (0.6 ml 95%) was added and RNA precipitated at –20° C for 12 h. RNA was pelleted by 20 min, centrifugation at 15000 *g*, the pellet dissolved in 150 μl 15% formaldehyde in water and 150 μl 20 × SSC [1 × SSC (standard saline citrate) is 0.15 M sodium chloride, 0.015 M sodium citrate] was added. The solution was heated 15 min at 50° C and chilled on ice. Aliquots or serial dilutions were applied to nylon filters (Compas, Genofit, Heidelberg, FRG) prewetted with water and then with 10 × SSC. The RNA was fixed on the nylon filters by exposure to ultraviolet light for 2 min and hybridization was performed according to the method described in detail by Khandjian [15] at 42° C in the presence of dextran sulphate. The filters were washed twice under high-stringency conditions (65° C, 30 min, 2 × SSC containing 1% sodium dodecyl sulphate). Probes were labelled with [^{32}P]dGTP and [^{32}P]dCTP (Amersham, Frankfurt, FRG, specific activity 3000 Ci/mmol) by the random-primer method using a hexamer (Pharmacia, Freiburg, FRG).

The TNF cDNA probe was a 700-base-pair(bp) *Eco* RI fragment of the coding region of human TNF. The human β-actin cDNA probe was described by Moos and Gallwitz [20] and consists of a 560 bp *Sal* I-*Eco* RI cDNA fragment. The murine β-actin cDNA probe was a 2000-bp *Pst* I fragment of the mouse β-actin gene. For quantitative analysis the developed X-ray films were densitometrically scanned and the absorption normalized to the absorption measured in the actin-hybridized filters.

Results

Human peripheral blood monocytes were stimulated with MLI in order to determine whether the purified protein preparation was able to activate the monocytes for TNF production. Concentrations of more than 1 μg/ml MLI induced release of cytotoxic activity (Table 1). In combination with human interferon γ (IFNγ) the MLI preparation was active at 10 ng/ml and more. When supernatants of identically stimulated cultures were tested in the TNF-specific ELISA system, TNF was detected at the highest concentrations of MLI used for stimulation. At the concentrations used in these experiments the lectin itself did not

Table 1. Induction of tumour necrosis factor (TNF) release in culture of human monocytes by mistletoe lectin I (MLI)

Stimulus	TNF[a]	
	Bioassay (unit)	ELISA (ng/ml)
MLI		
10 µg/ml	256	0.8
1 µg/ml	128	<0.5
100 ng/ml	4	<0.5
10 ng/ml	4	<0.5
1 ng/ml	2	<0.5
0.1 ng/ml	2	<0.5
MLI + IFNγ 100 U		
10 µg/ml	>256	0.8
1 µg/ml	256	0.5
100 ng/ml	64	<0.5
10 ng/ml	18	<0.5
1 ng/ml	8	<0.5
0.1 ng/ml	8	<0.5
IFNγ 100 U	8	<0.5
Staph. aureus 10 µg/ml	16	1.5
Unstimulated	2	<0.5

[a] Cytotoxic activity and TNF content were measured in the supernatant of monocyte cultures after 16 h of incubation. ELISA, enzyme-linked immunospecific assay; IFNγ, interferon γ

Table 2. Inhibition of cytotoxic activity with monoclonal antibodies to rhTNF

Stimulus	Antibody	TNF[a] (units)
TNF 10 ng/ml	–	64
	αTNF	<2
	αLT	64
MLI supernatants[b]	–	32
	αTNF	<2
	αLT	32
MLI + IFNγ supernatants[b]	–	32
	αTNF	<2
	αLT	32

[a] Cytotoxic activity was determined in the bioassay in the presence and absence of monoclonal antibodies to either rhTNF or recombinant human lymphotoxin (LT)

[b] Supernatants of monocyte cultures stimulated with MLI (10 µg/ml) or MLI (10 µg/ml) plus IFNγ (100 U/ml) were tested in the TNF bioassay

interfere with either the TNF bioassay or the ELISA system (data not shown). Activation of monocytes was reproducible but varied with monocytes from different donors.

Monoclonal antibodies to TNF and lymphotoxin were used to determine the contribution from TNF and from lymphotoxin to the cytotoxic activity measured in the bioassay. As can be seen in Table 2 antibodies to rhTNF completely neutralized the cytotoxicity of supernatants of monocytes stimulated with MLI or MLI plus IFNγ. In contrast, neutralizing antibodies to recombinant human lymphotoxin did not reduce the activity of the cytotoxic supernatants. This indicates that the cytotoxic activity can completely be attributed to TNF.

When RNA was extracted from human monocytes that had been stimulated for 2 h with MLI, TNF mRNA was

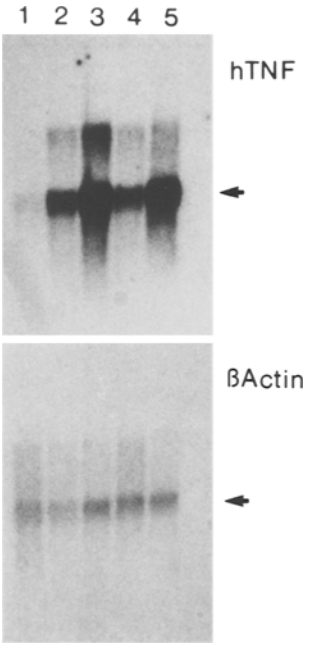

Fig. 1. Northern blot analysis of mRNA extracted from human monocytes cultured for 2 h without stimulus (*lane 1*), *Staph. aureus* 10 µg/ml (*lane 2*), mistletoe lectin I (MLI) 10 µg/ml (*lane 3*), interferon γ (IFNγ) 10 U (*lane 4*) or MLI 10 µg/ml plus IFNγ100 U (*lane 5*) probed with either a human tumour necrosis factor (*hTNF*) or a human β-actin cDNA probe

Table 3. Inhibition of TNF mRNA expression by the addition of polymyxin B for stimulation

Stimulus	Polymyxin B[a]	TNF signal[b] (absorbance)	Inhibition (%)
LPS	–	27.06	
	+	9.77	63.9
Staph. aureus	–	26.19	
	+	20.11	23.3
MLI	–	26.61	
	+	23.52	11.6

[a] mRNA extracted from human monocytes that had been cultured for 2 h either with lipopolysaccharide (LPS) of *S. minnesota* (50 µg/ml), *Staph. aureus* (10 µg/ml) or MLI (10 µg/ml) in the absence (–) or presence (+) of polymyxin B (20 µg/ml) was subjected to dot-blot analysis. Each stimulant together or without polymyxin B was kept at 37° C for 30 min before stimulation of the cells

[b] The absorbance of the total area of the signal obtained after hybridization with the TNF cDNA probe was normalized to the signal obtained with the actin cDNA probe on the same filter

clearly detectable by Northern blot analysis (Fig. 1). The signal induced by MLI stimulation of the monocytes was at least as strong as after stimulation with *Staph. aureus*.

Since the MLI preparation contained endotoxin, the monocyte activation could be due to this endotoxin even though the experimental system is not very sensitive to lipopolysaccharide [17]. To exclude lipopolysaccharide contamination of MLI as the activating signal, monocytes were cultured in the presence of polymyxin B. Dot-blot analysis demonstrated that *Staph. aureus*- and MLI-activated monocytes were rather resistant to the effect of poly-

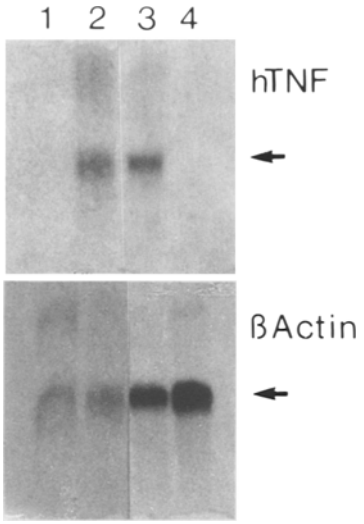

Fig. 2. Northern blot analysis of mRNA extracted from mouse (C3H/HeJ) peritoneal macrophages cultured for 2 h with either lipopolysaccharide of *S. minnesota* 10 µg/ml (*lane 1*), MLI 10 µg/ml (*lane 2*), *Staph. aureus* 10 µg/ml (*lane 3*) or without stimulus (*lane 4*) probed with either a hTNF or a murine β-acting cDNA probe

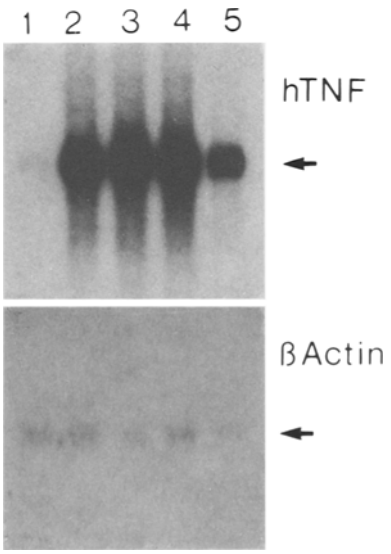

Fig. 3. Northern blot analysis of mRNA extracted from human monocytes cultured for 2 h without stimulus (*lane 1*), MLI 10 µg/ml (*lane 2*), A chain 10 µg/ml (*lane 3*), B chain 10 µg/ml (*lane 4*) or *Staph. aureus* 10 µg/ml (*lane 5*) probed with either a hTNF or a human β-actin cDNA probe

myxin B whereas lipopolysaccharide activation of the monocytes was clearly reduced in the presence of polymyxin B (Table 3).

To exclude further a stimulating effect of lipopolysaccharide macrophages of mice having a low response to lipopolysaccharide (C3H/HeJ) were stimulated with MLI. As can be seen in Fig. 2 only *Staph. aureus* (lane 3) and MLI (lane 2) but not lipopolysaccharide (lane 1) were able to induce the expression of TNF mRNA in these cells, demonstrating the direct activating effect of MLI on macrophages.

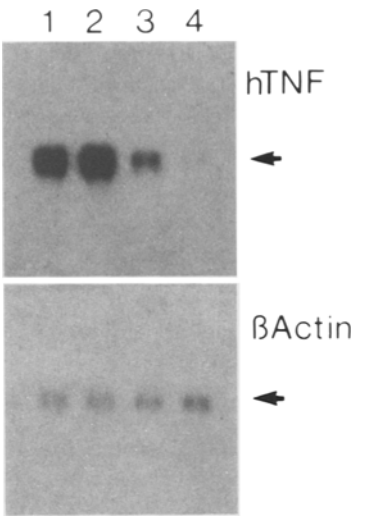

Fig. 4. Northern blot analysis of mRNA extracted from mouse (C3H/HeJ) peritoneal macrophages cultured for 2 h with either MLI 10 µg/ml (*lane 1*), A chain 10 µg/ml (*lane 2*), B chain 10 µg/ml (*lane 3*) or lipopolysaccharide of *S. minnesota* 10 µg/ml (*lane 4*) probed with either a hTNF or a murine β-actin cDNA probe

Monocyte cultures were stimulated with MLI and in parallel with the isolated A chain and B chain. As can be seen in Fig. 3 all three agents were equally able to induce TNF mRNA. Both chains were also active on peritoneal exudate cells of C_3H/HeJ mice (Fig. 4). The A chain, however, induced a much stronger TNF mRNA expression in murine macrophages than did the B chain.

Culture of peripheral blood lymphocytes with either the A or B chain of MLI for 16 h clearly led to production of cell-associated TNF (Fig. 5). No positive staining was obtained in unstimulated cells or cells stained with an irrelevant first antibody for control.

Discussion

The biochemical characterization of a biologically active component of *Viscum album* made it possible to analyse the immunomodulary mechanisms of this substance on the molecular level. Purified MLI, one of three known mistletoe lectins consisting of two disulphide-linked subunits, was tested for its monocyte activating capacity in vitro. This investigation was prompted by the observation that MLI given intraperitoneally produced C-reactive protein in mice [10].

Generation of cytotoxic activity was found in supernatants of human monocyte cultures stimulated with MLI. The levels of cytotoxic activities induced by MLI varied from blood donor to blood donor as already noted with other stimuli [18]. The stimulant itself did not account for the observed cytotoxic activity in the TNF bioassay, since the amount of MLI used for monocyte stimulation did not itself affect the viability of the TNF indicator cells. In addition, soluble TNF protein was detectable in the TNFα-specific ELISA system after MLI stimulation of the monocytes. The addition of IFNγ only slightly increased the amount of TNF released from monocytes indicating that

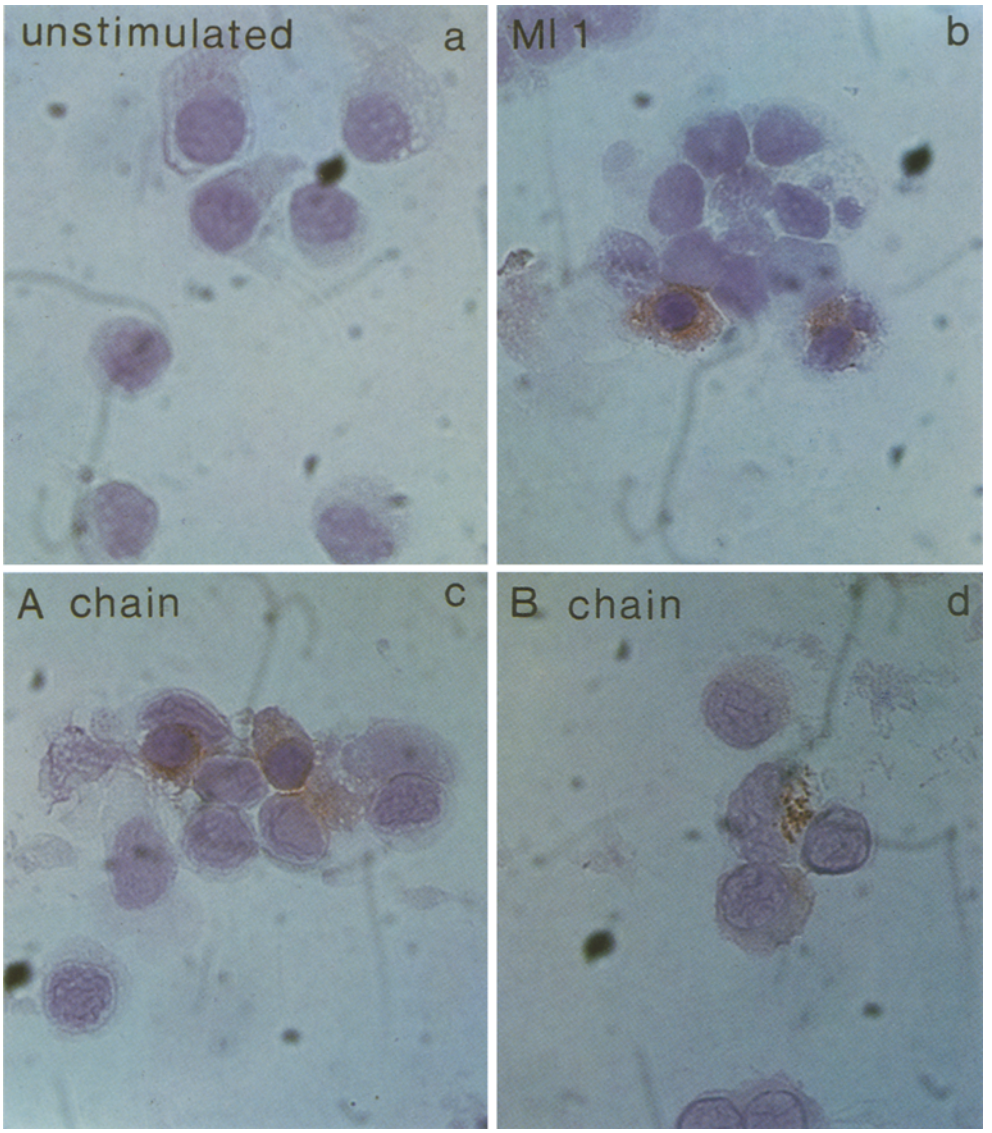

Fig. 5. Peripheral blood monocytes were cultured for 12 h without stimulant (**a**), or with either MLI 10 μg/ml (**b**), A chain 10 μg/ml (**c**), or B chain 10 μg/ml (**d**) and stained for cell-associated TNF. The photomicrographs show the stained cells at a 1000× magnification

MLI like IFNγ provides a stimulating signal for TNF production rather than a trigger for TNF release. The induction of TNF mRNA by 10 μg/ml MLI was comparable to the signal induced by 100 U IFNγ in the monocyte activating system. The relative insensitivity of the system to stimulation with lipopolysaccharide [18] already indicated that endotoxin contamination was not responsible for the observed monocyte activation. Pretreatment of MLI with polymyxin B and the presence of polymyxin B during stimulation of the monocyte cultures did not abolish the activating capacity of MLI. Furthermore, peritoneal exudate macrophages from C3H/HeJ, mice having a low response to lipopolysaccharide, were activated by MLI for TNF mRNA expression. Thus, the contaminating endotoxin did not account for the stimulatory capacity of MLI, which must then be an intrinsic activity of the MLI molecule. It is possible, therefore, that the release of mediators like TNF is involved in the mechanism of the impressive toxicity of MLI after intraperitoneal application. This is in agreement with recent histochemical results [12]. After application of lethal doses of MLI into mice the glycogen disappeared completely from hepatocytes, and the activities of both thiamine phosphatase and non-specific alkaline phosphatase increased. However, when we injected a lethal dose of MLI into C_3H/HeJ mice having a low response to endotoxin and injected at the same time a large dose of murine TNF-neutralizing monoclonal antibody, no protective effect was achieved by these antibodies. No difference in the behaviour of the animals nor in the histological evaluation of sections from spleen, liver, kidney or lung was seen. Neither TNF nor interleukin-6 was detectable in serum or in supernatants of cultured peritoneal exudate cells from these mice (unpublished results).

Experiments to determine whether the enzymatic activity of the MLI A chain and/or the carbohydrate-binding activity of the MLI B chain was responsible for the monocyte activation demonstrated that both chains idependently were able induce TNF mRNA. The expression of IL-1β mRNA was also strongly enhanced (data not shown). Experiments with peritoneal exudate cells from mice having a low response to endotoxin showed that TNF mRNA expression by the toxic A chain of MLI was stronger than by

the galactose-specific B chain. This indicated that either the endotoxin contamination, although by itself inactive, contributed and synergized with the MLI B chain or that stimulation requirements for murine macrophages are quantitatively different from human monocytes. The possibility of the MLI B chain binding to a D-galactose-containing receptor structure of the monocyte/macrophage might give further insight in the activation process and the involved membrane structures. Considering a recent report on the activation of human blood monocytes by an oligosaccharide from *Viscum album* extracts [16], we can not exclude the possibility that the carbohydrate moiety of MLI also has a biological activity. In view of the therapeutic application of MLI or other lectins from *Viscum album* (MLII, MLIII) in cancer treatment, there are at least three possible opportunities: (a) direct killing of the malignant cells by the cytotoxic lectin, (b) induction of endogenous mediators of the immune system, and (c) use of the A chain of MLI as an alternative component for the preparation of immunotoxins [24].

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