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Influence of Twinline, an Elemental Diet, on the Generation of Nitric Oxide and Reactive-oxygen Intermediates from Mouse Peritoneal Macrophages and Polymorphonuclear Leukocytes

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Abstract

The influence of Twinline (SNN-6010), an elemental diet containing medium-chain triglycerides, on the generation of nitric oxide (NO) and superoxide ($O_2^{\cdot-}$) has been examined in mouse peritoneal macrophages and polymorphonuclear leukocytes (PMN).

When PMN and peritoneal macrophages obtained from untreated mice were cultured in medium containing 0·1% and 1% (v/v) Twinline for 48 h and stimulated with phorbol myristate acetate or *N*-formyl-methionyl-leucyl-phenylalanine, their chemiluminescence and $O_2^{\cdot-}$ generation were strongly suppressed, as was NO generation from peritoneal macrophages. PMN and peritoneal macrophages obtained from mice fed Twinline for 30 days generated much smaller amounts of $O_2^{\cdot-}$ and NO compared with PMN and peritoneal macrophages from control mice. In conjunction with this suppressed NO generation, inducible NO synthase and its mRNA expression in peritoneal macrophages were suppressed by Twinline both in-vivo and ex-vivo. Although phagocytosis of PMN and peritoneal macrophages was not suppressed by Twinline; their candida-killing activity was markedly suppressed.

These results indicate that Twinline suppresses the host-defence function of PMN and peritoneal macrophages by down-regulating their generation of reactive-oxygen intermediates and NO.

Elemental diets have been widely used for post-operative and post-traumatic nutritional management and for the nutritional treatment of patients with advanced cancer and immunosuppressive infection (Moore & Moore 1991; Moss & Naylor 1994; Gogos & Kalfarontzos 1995; Campos et al 1996). Because cellular and humoral immune function is usually suppressed in debilitated patients, and malnutrition induces further suppression of cellular immunity (Harbige 1996; Krenitsky 1996), immunosuppression is inadvertently induced if inappropriate nutritional management is maintained for a long period. Although elemental diets should be free from immunosuppressive activity, the influence of such diets on the immune system, especially on the function of phagocytes, has rarely been investigated. Apart from immunosuppression, elemental diets might create circumstances conducive to bacteria and it has been reported that elemental diet induced overgrowth of Gram-negative enterococci and reduced secretion of bile and pancreatic juice into the intestine, resulting in an increase of mucous membrane-penetrating enterococci (Shou et al 1991; Deitch et al 1993; Xu et al 1993). The results of an animal study indicate that alimentation with elemental diet carries a potential risk of bacterial dissemination.

Infections in immunosuppressed patients are life-threatening. In the management of patients undergoing nutritional treatment with elemental diet, fungal infections are one of the most serious problems (Alden et al 1989). Macrophages and polymorphonuclear leukocytes (PMN) play an important role in the host defence against microbial invasion by phagocytosis and the release of reactive-oxygen intermediates, nitric oxide (NO), and proteolytic enzymes (Babior 1978; Ferrante 1989). It has already been demonstrated that the bactericidal and fungicidal activity of phagocytes depends largely on their generation of reactive-oxygen intermediates (Sasada et al 1987; Ferrante 1989), activity which is regulated by a variety of cytokines and NO (Moncada et al 1991; Cenci et al

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1993). Acting together with cytokines, NO up-regulates the microbicidal activity of PMN and the activity of macrophages (Denis & Gregg 1990; Summersgill et al 1992; Simms & D'Amico 1997).

Reactive-oxygen intermediates are bactericidal molecules which react with NO. The reactant peroxinitrite ($ONOO^-$), which is formed from superoxide ($O_2^{\cdot-}$) and NO, has very strong bactericidal activity (Zhu et al 1992). However, reactive-oxygen intermediates such as the hydroxy radical ($^{\cdot}OH$), $O_2^{\cdot-}$ and peroxinitrite have potent cell- and tissue-damaging action. For example, damage of the lung, cerebrum and kidney by reactive-oxygen intermediates has frequently been reported (Halliwell 1992; Evans 1993; Laskin & Pendino 1995; Baliga et al 1997; Shiraishi et al 1997; Shohami et al 1997).

Although this background illustrates the importance of investigating the influence of elemental diets on the function of macrophages and PMN, few studies have been conducted (Scheurlen et al 1989; Shou et al 1991, 1994). We have, therefore, investigated the influence of an elemental diet containing medium-chain triglycerides on the generation of reactive-oxygen intermediates and NO from mouse peritoneal macrophages and PMN.

Materials and Methods

Mouse peritoneal macrophages and PMN

Male CBA/J mice, 6 weeks, were fed a conventional solid diet (CRF-1; Oriental Yeast, Tokyo, Japan) or Twinline (SNN-6010; Otuka, Tokyo, Japan) (Table 1) for 15 or 30 days. On the 15th or 30th day thioglycolate medium (2·5%, 2 mL; Difco, Detroit, MI) was injected intraperitoneally. Five days after injection, peritoneal macrophages were collected by washing out the peritoneal cavity with phosphate-buffered saline (PBS; 5 mL) containing heparin (5 units mL^{-1}). The collected cells were left to adhere to plastic Petri dishes for 2 h, and adherent cells were removed from the dish surfaces with ethylenediaminetetraacetic acid (0·02% w/v). Heparin (0·1 units mL^{-1}) was added to blood obtained from the jugular vein and PMN were separated by means of Ficoll-Hypaque gradients. Contaminated red blood cells were removed by hypotonic shock. After washing with PBS the cells were suspended in RPMI 1640 containing foetal bovine serum (2% v/v). Purity and viability > 95% were confirmed by Giemsa staining and trypan blue exclusion, respectively.

Assay of $O_2^{\cdot-}$ generation

$O_2^{\cdot-}$ was assayed spectrophotometrically by a cytochrome c reduction method by means of a Shimadzu (Shimadzu Seisakusho, Kyoto, Japan) UV-300 double-wavelength spectrophotometer, equipped with a thermostatted (37°C) cuvette holder. Peritoneal macrophages or PMN (1×10^7 cells mL^{-1}) suspended in Hank's balanced salt solution and 100 μM cytochrome c (type VI; Sigma, St Louis, MO) were poured into each cuvette, adjusting to a final concentration of 1×10^6 cells mL^{-1}. The reaction mixtures in the cuvettes were pre-incubated at 37°C for 1 min, and a stimulating agent (50 ng mL^{-1} phorbol 12-myristate 13-acetate (PMA, Sigma) or 10^{-7} M *N*-formyl-methionyl-leucyl-phenylalanine (FMLP; Sigma)) was added to the reaction mixtures. The

Table 1. Composition of Twinline.

Calories (kcal L^{-1})	1000
Protein (g L^{-1})	41·0
Fat (g L^{-1})	278·0
Vitamin A (Iunits L^{-1})	2070
Vitamin D (Iunits L^{-1})	135
Vitamin E (μmol L^{-1})	15·6
Vitamin B_1 (μmol L^{-1})	6·0
Vitamin B_2 (mmol L^{-1})	6·0
Vitamin B_6 (μmol L^{-1})	14·8
Vitamin B_{12} (nmol L^{-1})	2·4
Vitamin C (mmol L^{-1})	1·28
Pantothenic acid (μmol L^{-1})	42·9
Biotin (nmol L^{-1})	151·5
Folic acid (nmol L^{-1})	567
Ca (mmol L^{-1})	1·10
P (mmol L^{-1})	17·1
Mg (mmol L^{-1})	0·06
Na (mmol L^{-1})	28·8
K (mmol L^{-1})	30·2
Fe (mmol L^{-1})	0·11
Cu (μmol L^{-1})	3·6
Zn (μmol L^{-1})	145
Mn (μmol L^{-1})	29
Cl (mmol L^{-1})	30·2
Isoleucine (μmol L^{-1})	157·5
Leucine (μmol L^{-1})	274·8
Lysine (μmol L^{-1})	205·0
Methionine (μmol L^{-1})	119·4
Cystine (μmol L^{-1})	6·7
Phenylalanine(μmol L^{-1})	20·2
Tyrosine (μmol L^{-1})	73·8
Threonine (μmol L^{-1})	136·0
Tryptophan (μmol L^{-1})	27·8
Valine (μmol L^{-1})	214·3
Arginine (μmol L^{-1})	81·4
Histidine (μmol L^{-1})	75·7
Alanine (μmol L^{-1})	136·3
Asparagic acid (μmol L^{-1})	206·9
Glutamic acid (μmol L^{-1})	626·8
Glycine (μmol L^{-1})	97·1
Proline (μmol L^{-1})	270·8
Serine (μmol L^{-1})	208·1
Caprylic acid (mmol L^{-1})	131·4
Capric acid (mmol L^{-1})	1·8
Myristic acid (mmol L^{-1})	0
Palmitic acid (mmol L^{-1})	5·31
Stearic acid (mmol L^{-1})	3·0
Oleic acid (mmol L^{-1})	3·6
Linoleic acid (mmol L^{-1})	18·9
Linolenic acid (mmol L^{-1})	0
Other fatty acids (mg L^{-1})	0

kinetics of cytochrome c reduction were measured by measurement of absorbance change at 540–550 nm. The O_2^- concentration was calculated from the linear portion of the cytochrome c reduction curve using $\Delta\varepsilon_{550}$ cytochrome $c = 21{\cdot}1 \times 10^3$ mol L^{-1} cm^{-1}.

Chemiluminescence

Chemiluminescence was measured with a Jasco (Tokyo, Japan) CAF-100 calcium analyser. Cells suspended (5×10^5 cells mL^{-1}) in Hank's balanced salt solution containing 100 μM luminol were incubated for 1 min at 37°C, after which 50 ng mL^{-1} PMA or 10^{-7} M FMLP was added. Each activity was expressed as peak chemiluminescence intensity (mV).

NO (NO_2^-) determination

NO, quantified by accumulation of nitrite (NO_2^-) as a stable end-product, was determined by microplate assay (Cenci et al 1993). Briefly, pooled supernatant (100 μL) was incubated with an equal volume of Griess reagent (1% sulphanilamide, 0·1% *N*-1-naphthylethylenediamine dihydrochloride, 2·5% H_3PO_4) at room temperature for 10 min. The absorbance at 540 nm was determined with a Corona Electronic (Ibaraki, Japan) MTP-120 microplate reader. The nitrite concentration was calculated from a sodium nitrite standard curve.

Candida albicans

Sabouraud's broth medium (100 mL) was inoculated with *C. albicans* TIMM0134 (standard strain) and KSC1 (established from a patient with oral candidiasis) maintained on agar slants at 4°C, and then cultured for 24 h at 37°C. After three washes with pyrogen-free saline by centrifugation at 1500 *g* the cells in the yeast phase of growth were counted in a haemocytometer. They were resuspended in saline and the suspensions were then diluted to an appropriate concentration with saline. Viability was confirmed by plating serial dilutions on to Sabouraud's dextrose agar plates.

Phagocytosis

Peritoneal macrophages or PMN were co-incubated in Hanks' balanced salt solution with latex particles (diam. 1 μm) at a ratio of 1 : 100 for 1 h at 37°C. Phagocytized particles were studied by light microscopy. Peritoneal macrophages and PMN containing more than five particles were considered to be phagocytic cells. Phagocytic activity was expressed as the percentage of phagocytic cells in 400 cells observed.

Candida killing

Candida cells (TIMM0134 and KSC1) in the yeast phase of growth were labelled with ^{51}Cr for 1 h at 37°C at a concentration of 100 μCi/10^8 cells. After three washes candida cells and peritoneal macrophages or PMN in RPMI 1640 medium supplemented with 2% foetal bovine serum were mixed to give an effector/target ratio of 1 : 10 in a final volume of 0·2 mL/well in flat-bottomed 96-well plates. The plates were incubated for 4 h at 37°C, and the isotope activity of the supernatant (0·1 mL) from each well was counted by means of a gamma counter. The percentage cytotoxicity was calculated by use of the formula:

$$\%\ \text{cytotoxicity} = (\text{experimental release} - \text{spontaneous release})/(\text{maximum release} - \text{spontaneous release}) \times 100$$

where spontaneous release is the activity in target cells incubated without effectors, and maximum release is the activity in the supernatant after treatment of the candida cells with 0·1% (v/v) Triton X-100. All terms in the formula were expressed as counts min^{-1}.

Western blotting

Mouse peritoneal macrophages (2×10^6 cells mL^{-1}) were suspended in Hank's balanced salt solution and incubated at 37°C in the presence or absence of 0·1% Twinline. The reaction was terminated by adding ice-cold 15% trichloroacetic acid solution containing 2 mM phenylmethylsulphonyl fluoride and 1 mM sodium vanadate. The precipitate was washed with ice-cold ether–ethanol (1 : 1), dissolved in sodium dodecylsulphate (SDS) sample buffer, and subjected to SDS-polyacrylamide gel electrophoresis. After electrophoresis (30 mA, 3 h), the proteins were transferred to Immobilon-P filters (Millipore), by use of a Sartorius semi-dry blotting apparatus, and incubated with anti-inducible NO synthase (iNOS) monoclonal antibody (Transduction Laboratories, Lexington, KY) for 40 min after 60-min incubation in 5% powdered skimmed milk at room temperature. Antibody was detected with peroxidase-conjugated rabbit anti-mouse IgG, and peroxidase-positive bands were detected by means of an ECL Western blotting detection system (Amersham).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA from mouse peritoneal macrophages was isolated by standard procedures (Chomczynski & Sacchi 1987). The levels of iNOS-mRNA were

examined by the RT-PCR technique. Reverse transcription was performed at 42°C for 30 min and PCR was performed in a Perkin-Elmer (Vetterstetten, Germany) Cetus 480 thermal cycler. The cycle programme was: 30-s denaturation (94°C), 30-s annealing (60°C), and 90-s amplification (72°C) for 30 cycles with a final 2-min amplification. As a control, cDNA samples were amplified by use of β-actin primer. PCR samples electrophoresed on agarose gel were visualized by staining with ethidium bromide. Primers for iNOS and β-actin were purchased from Toyobo (Osaka, Japan). The primer sequences were: iNOS: sense 5′CCCTCCTCCCGAAGTTTCTGGCAGCAGC3′, antisense, 5′GGCTGTCAGAGCCTCGTGGCTTTGG3′; β-actin: sense, 5′ATCTGGCACCACACCTTCTACAAT GAGCTGCG3′, antisense, 5′CGTCATATCCTGCTTGCTGATCCACATCTGC3.

Statistical analysis

Results are expressed as means ± standard deviation (s.d.). Two mean values were compared with each other by use of Student's *t*-test; $P < 0.05$ was regarded as indicative of statistical significance.

Results

O_2^- generation and chemiluminescence

O_2^- generation from PMN and peritoneal macrophages obtained from Twinline-fed mice was slightly suppressed; significant suppression was observed when PMN and peritoneal macrophages obtained from mice fed Twinline for 30 days were stimulated with PMA (Figure 1A). In-vitro treatment of PMN and peritoneal macrophages with Twinline resulted in strong suppression of O_2^- generation (Figure 1B). Although 0·01% (v/v) Twinline led to slight suppression of O_2^- generation, 0·1% Twinline suppressed O_2^- generation to about two-thirds of the control level ($P < 0.05$ or 0·01) and 1% Twinline caused a suppression to about half the control level ($P < 0.01$ or 0·001).

Chemiluminescence was suppressed by in-vivo and ex-vivo treatment of PMN and peritoneal macrophages with Twinline (Table 2). The amount of the suppression was slight after 15-day Twinline feeding and with 0·01% Twinline in-vitro treatment, but strong suppression of chemiluminescence was observed after 30-day Twinline feeding and in-vitro treatment with 1% Twinline.

Phagocytosis and candida killing

Although a slightly lower amount of phagocytic cells was observed in peritoneal macrophages treated with 1% Twinline than in control peritoneal macrophages, phagocytosis of peritoneal macrophages was not suppressed by in-vivo treatment with Twinline (Table 3), neither was the phagocytosis of PMN suppressed by either in-vivo or in-vitro treatment.

The influence of Twinline on the candida-killing activity of PMN and peritoneal macrophages was similar to that on O_2^- generation (Figures 2A, B). Compared with the suppression of killing by in-vivo Twinline, the suppression of candida killing by 1% Twinline was greater. In both candida cell lines the fungicidal activity of 1% Twinline-treated PMN and peritoneal macrophages was reduced to approximately half of control activity.

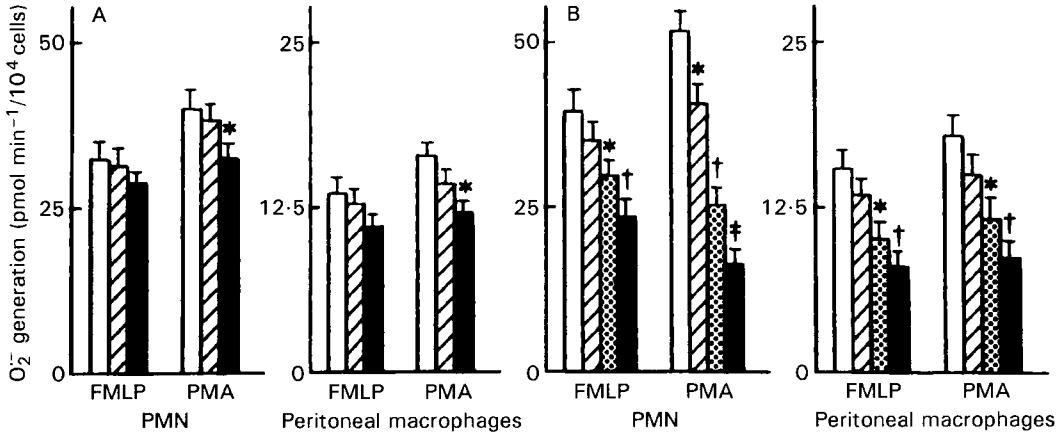

Figure 1. Influence of Twinline on in-vivo (A: □, before experiment; ▨, on 15th day; ■, on 30th day) and in-vitro (B: □, control (no Twinline); ▨, 0·01% Twinline; ▩, 0·1% Twinline; ■, 1% Twinline) O_2^- generation. Polymorphonuclear leukocytes (PMN) and peritoneal macrophages were obtained from mice fed a conventional diet (CRF-1) or Twinline for 15 or 30 days (n = 5 in each of four groups). PMN and peritoneal macrophages from Twinline-fed mice were cultured in medium without Twinline (A) whereas PMN and peritoneal macrophages from CRF-1-fed mice were cultured for 24 h in medium containing the indicated concentrations of Twinline (B). The cells were stimulated with *N*-formyl-methionyl-leucyl-phenylalanine (FMLP) or phorbol 12-myristate 13-acetate (PMA) and O_2^- generation was measured. *$P < 0.05$, †$P < 0.001$, ‡$P < 0.01$, significantly different from control result.

Table 2. Influence of Twinline on reactive-oxygen generation by mouse polymorphonuclear leukocytes and peritoneal macrophages in-vivo and in-vitro.

Treatment	Chemiluminescence (peak mV/5 × 10^5 cells)			
	Polymorphonuclear leukocytes		Peritoneal macrophages	
	FMLP (10^{-7}M)	PMA (50ngmL^{-1})	FMLP (10^{-7}M)	PMA (50ngmL^{-1})
In-vivo				
CRF-1 for 30 days	43·7±3·5	57·1±4·2	27·4±3·0	30·5±2·7
Twinline for 15 days	38·2±3·0*	44·7±3·8†	24·3±3·2	25·1±2·8*
Twinline for 30 days	33·7±2·9*	35·6±3·6†	20·5±2·1*	17·8±2·2†
In-vitro				
Twinline 0%	45·6±3·8	59·4±3·2	36·4±3·1	42·7±3·1
0·01%	41·8±3·2	57·1±3·0	33·5±2·7	39·1±2·9
0·1%	37·4±2·7*	53·1±2·6*	31·9±2·5	28·4±2·8*
1%	24·6±3·4†	39·5±2·8†	23·8±3·7*	22·1±2·5†

Polymorphonuclear leukocytes (PMN) and peritoneal macrophages were obtained from mice which had been fed a conventional diet (CRF-1) or Twinline for 15 or 30 days (n = 5 in each of four groups), and PMN and peritoneal macrophages from CRF-1-fed mice were cultured for 24h in medium containing the indicated concentrations of Twinline. The PMN and peritoneal macrophages were then stimulated with N-formyl-methionyl-leucyl-phenylalanine (FMLP) or phorbol 12-myristate 13-acetate (PMA) and chemiluminescence was measured for 10min. *$P < 0.05$, †$P < 0.01$, significantly different from control (CRF-1) result.

NO generation, iNOS and its mRNA

NO generation from peritoneal macrophages was suppressed by Twinline both in-vivo and ex-vivo (Figure 3A). When peritoneal macrophages were treated with 0·1% and 1% Twinline for 48h NO generation was reduced to 18·0±7·0 ($P < 0.05$) and 7·9±3·8μM ($P < 0.01$), respectively, compared with the control level (27·6±8·7μM). NO generation was markedly reduced in peritoneal macrophages obtained from mice fed Twinline for 30 days; peritoneal macrophages from these mice generated 13·8±3·9μM NO whereas peritoneal macrophages from control mice generated 23·4±4·2μM NO when 1000ngmL^{-1} lipopolysaccharide (LPS) was added ($P < 0.01$). In parallel with the suppression of NO generation, iNOS, a 130kDa protein, was not observed in peritoneal macrophages obtained from Twinline-treated mice but was observed in peritoneal macrophages obtained from control (untreated) mice. The protein was weakly expressed (20% of control) after the addition of 1μgmL^{-1} LPS even by peritoneal macrophages from mice fed Twinline (Figure 4A). The level of iNOS-mRNA was also reduced by in-vitro treatment of peritoneal macrophages with 0·1% Twinline; mRNA expression was not observed in 1% Twinline-treated peritoneal macrophages (Figure 4B).

Table 3. Influence of Twinline on phagocytosis by polymorphonuclear leukocytes and peritoneal macrophages.

Treatment	Phagocytosis (%)	
	Polymorphonuclear leukocytes	Peritoneal macrophages
In-vitro		
CRF-1 for 30 days	47·3±4·6	58·4±5·1
Twinline for 15 days	45·1±4·7	56·1±4·0
Twinline for 30 days	44·7±4·5	53·9±4·2*
In-vivo		
Twinline 0%	49·0±5·2	60·4±4·0
0·01%	48·2±4·8	57·3±3·8
0·1%	47·3±4·6	56·8±4·4
1%	44·5±4·7	54·7±3·7†

Polymorphonuclear leukocytes (PMN) and peritoneal macrophages were obtained from mice which had been fed a conventional diet (CRF-1) or Twinline for 15 or 30 days (n = 5 in each of four groups), and PMN and peritoneal macrophages from CRF-1-fed mice were cultured for 24h in medium containing the indicated concentrations of Twinline. Phagocytic cells were observed microscopically. Phagocytic activity is presented as the percent of phagocytic cells/400 cells. *$P < 0.05$, †$P < 0.01$, significantly different from control (CRF-1) result.

Discussion

Reactive-oxygen intermediates have a variety of biological activity associated with the microbicidal activity of phagocytes, including tyrosine phosphorylation of proteins, inactivation of proteolytic enzymes, impairment of DNA, and induction of apoptosis (Green et al 1971; Ferrante 1989; Bhatnagar 1994; DeFranco 1994; Shoji et al 1995). This microbicidal activity is most important in the nutritional management of post-operative, traumatized, organ-transplanted, virus-infected, and advanced cancer patients. It is well known that the bactericidal and fungicidal activity of phagocytes correlates closely with the generation of reactive-oxygen intermediates (Sasada et al 1987; Ferrante 1989) and so suppression of the generation of reactive-oxygen intermediates is potentially

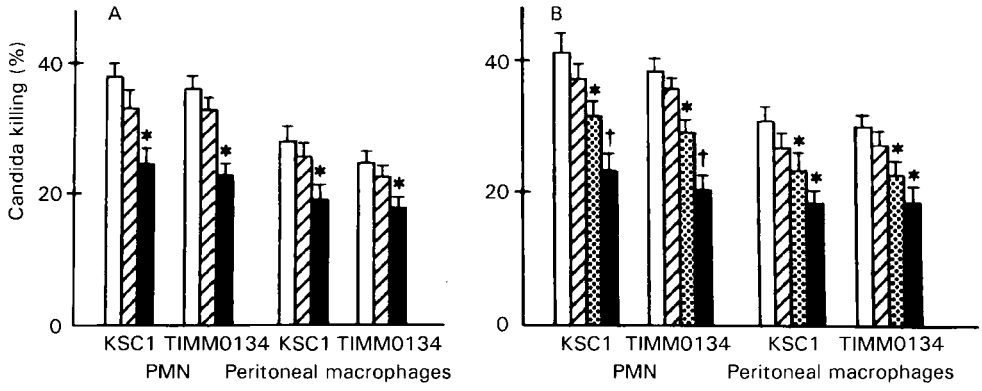

Figure 2. Influence of Twinline on the killing of candida cells (types TIMM0134 and KSC1) by polymorphonuclear leukocytes (PMN) and peritoneal macrophages obtained from mice fed CRF-1 or Twinline for 15 or 30 days (n = 5 in each of 4 groups). PMN and peritoneal macrophages from Twinline-fed mice were cultured in medium without Twinline (A: □, before experiment; ▨, on 15th day; ■, on 30th day), and PMN and peritoneal macrophages from CRF-1-fed mice were cultured for 24h in medium containing the indicated concentrations of Twinline (B: □, control (no Twinline); ▨, 0·01% Twinline; ▦, 0·1% Twinline; ■, 1% Twinline).

conducive to multiple infections. Because generation of extreme levels of reactive-oxygen intermediates and suppression of reactive-oxygen intermediate-scavenging enzymes might cause tissue damage (Halliwell 1992; Evans 1993; Laskin & Pendino 1995; Baliga et al 1997), intact regulation of the generation and scavenging of reactive-oxygen intermediates is very important for the maintenance of body integrity.

The influence of elemental diets on phagocyte function is poorly understood. Our study has shown that Twinline dose-dependently suppressed the generation of reactive-oxygen intermediates by mouse peritoneal macrophages although phagocytosis was not suppressed in-vitro. Corresponding with the suppression of $O_2^{\cdot-}$ generation and chemiluminescence, the candida-killing activity of peritoneal macrophages was reduced by Twinline. By use of other elemental diets (Nutrisourse Modular System) Shou et al (1991) also demonstrated suppression of the $O_2^{\cdot-}$ generation and the candida-killing activity of peritoneal macrophages. These results and ours suggest that elemental diets are likely to suppress the bactericidal activity of phagocytes by suppressing the generation of reactive-oxygen intermediates.

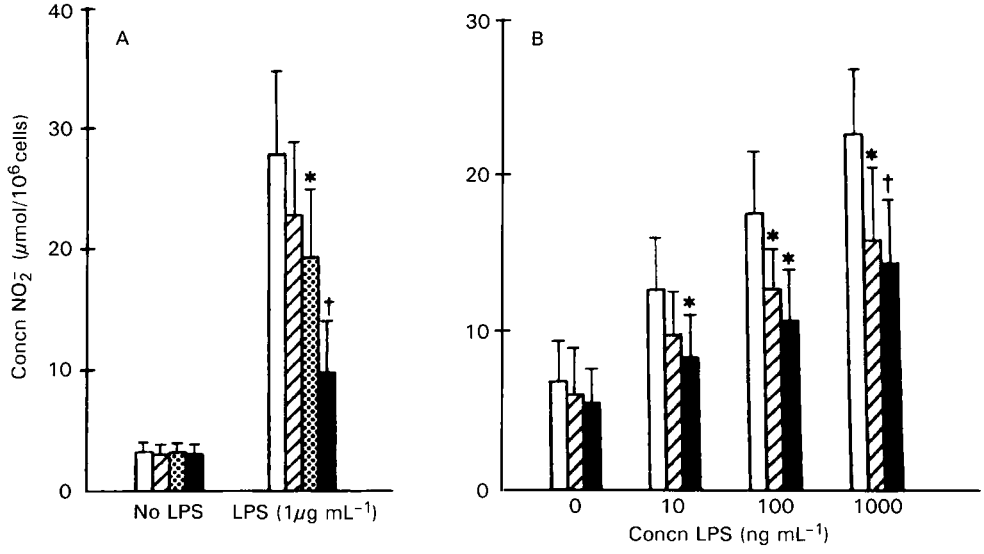

Figure 3. Ex-vivo (A: □, control (no Twinline); ▨, 0·01% Twinline; ▦, 0·1% Twinline; ■, 1% Twinline) and in-vivo (B: □, before experiment; ▨, on 15th day; ■, on 30th day) influence of Twinline on NO generation by mouse peritoneal macrophages. A: Peritoneal macrophages obtained from untreated mice (n = 4 in each group) were cultured for 48h in medium containing the indicated concentrations of Twinline and lipopolysaccharide (LPS). B: CBA/J mice (n = 4 in each group) were fed CRF-1 (control) or Twinline for 15 or 30 days, and peritoneal macrophages were collected and cultured for 48h in medium containing the indicated concentrations of lipopolysaccharide. Each bar indicates the mean ± s.d. of results from triplicate experiments. *$P < 0.05$, †$P < 0.01$, significantly different from control result.

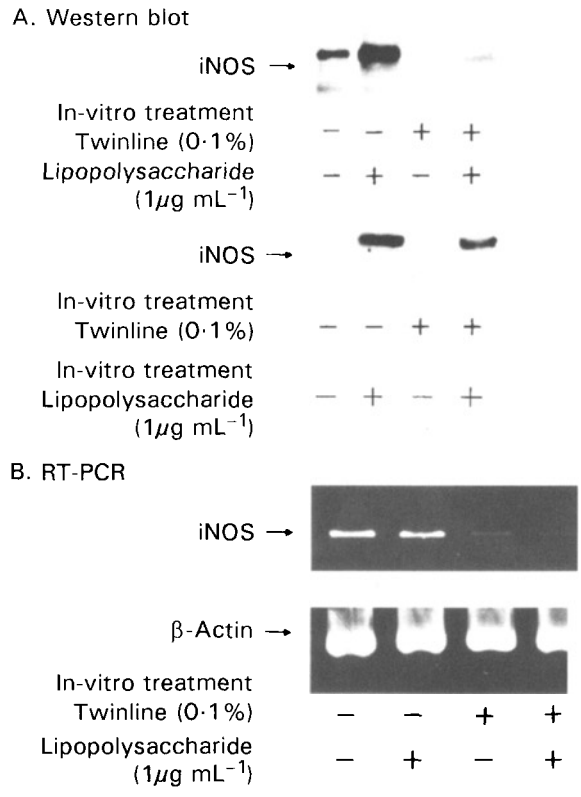

Figure 4. Influence of Twinline on inducible NO synthase (iNOS) and its mRNA expression in mouse peritoneal macrophages. Peritoneal macrophages obtained from Twinline-treated or -untreated CBA/J mice were cultured for 24h in medium containing the indicated reagents, and Western blotting (A) and 30 cycles of RT-PCR (B) were performed.

It has been reported that the serum level of peroxidized fatty acids in patients receiving Twinline was lower than that in patients receiving a conventional elemental diet containing long-chain triglycerides (Ueta et al 1997a). This difference seems to depend on the fatty-acid composition of Twinline and other elemental diets. In addition to active suppression of the generation of reactive-oxygen intermediates, the low level of peroxidized fatty acids seems to be advantageous for nutritional treatment of patients with ischaemic heart and brain disorders, because reactive-oxygen intermediates and peroxidized fatty acids are harmful in such disorders (Abadie et al 1993; Koudelova et al 1994).

Only slight suppression of $O_2^{\cdot-}$ generation was observed for peritoneal macrophages from mice fed Twinline for 15 days whereas $O_2^{\cdot-}$ generation and chemiluminescence were markedly suppressed for those from mice fed for 30 days. In addition to suppression of the generation of reactive-oxygen intermediates, human lymphocyte activity such as blastogenesis and generation of cytokines such as granulocyte macrophage-colony stimulating factor, tumour necrosis factor, and interleukin-2 were suppressed by in-vitro Twinline (data not shown). We have previously reported suppression of $O_2^{\cdot-}$ generation by PMN in Twinline-treated patients (Ueta et al 1997b). That finding and our current results suggest that Twinline has leukocyte-function-suppressing activity and so precautions against infection, especially fungal infection such as candidiasis, are essential during long-term administration of Twinline.

In conjunction with the suppression of the generation of reactive-oxygen intermediates, NO generation was also suppressed by Twinline. Suppressed NO generation was paralleled by reduced levels of iNOS protein and its mRNA. NO is synthesized from L-arginine with nicotinamide adenine dinucleotide phosphate and NOS as catalysts (Moncada et al 1991). Twinline contains less L-arginine than conventional elemental diets and it is, therefore, possible that the mechanism of the reduction of NO generation in peritoneal macrophages from Twinline-fed mice might depend on the small amount of L-arginine in the diet. It was recently reported that elemental diets containing large amounts of L-arginine, RNA and omega-3 fatty acid upregulate cellular proliferation, interleukin-2 production, and the cytotoxicity of T cells and macrophages (Daly et al 1992; VanMeter et al 1994; Kemen et al 1995; Senkal et al 1995). These reports suggest an advantageous elemental diet composition for the preservation of leukocyte function. Because Twinline downregulates leukocyte activity, including generation of cytokines, which upregulate NO generation (Liew 1995), there is a possibility that suppression of NO generation resulted, at least partially, from suppressed cytokine generation.

For nutritional management of patients, especially those in an immune-compromised condition, it is essential to recognize the physiological activity of their elemental diet. The results of this study reveal some of the characteristic effects of Twinline which might be important in its clinical use.

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