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Zinc regulates cytokine induction by superantigens and lipopolysaccharide

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SUMMARY

Zinc is known to be greatly involved in the regulation of immune functions. Pharmacological zinc supplementation, leading to serum zinc concentrations of more than 0·025 mM, has often been suggested to improve immune responses. However, the exact influence of elevated zinc level on immune functions has not yet been investigated. We found that zinc level selectively enhances cytokine induction by lipopolysaccharide (LPS) in a concentration-dependent fashion: as little as 0·0125 mM supplemental zinc led to nearly 50% elevated interleukin-1β (IL-1β) levels both in polymorphonuclear cells (PBMC) and whole-blood cultures. The secretion of interferon-γ (IFN-γ) could be increased more than 10-fold by 0·1 mM zinc. This could not be observed during stimulation with phytohaemagglutin (PHA). In contrast, zinc levels concentration-dependently down-regulated monocyte activation caused by the superantigens, staphylococcal enterotoxins A and E (SEA, SEE, more than 90% down-regulation by 0·1 mM zinc), the *Mycoplasma arthritidis*-derived superantigen (MAS), but not toxic shock syndrome toxin-1 (TSST-1), while T-cell response remained unaffected. This was not the result of chemical degradation of the superantigens. We assume that zinc concentration regulates interactions between SEA, SEE and MAS, but not TSST-1 and their major histocompatibility complex (MHC) class II-binding sites. Our data demonstrate that zinc levels control the secretion of IFN-γ and monokines after both LPS and superantigen challenge within a clinically relevant range of concentrations. This reveals new perspectives and indications for zinc supplementation and also indicates potential risks of therapeutic application of zinc.

INTRODUCTION

Physiological serum zinc levels (0·012–0·016 mM) are essential for the development and maintenance of immune functions.[1–5] We have recently shown that excess zinc (0·25 mM) induces the release of interleukin-1β (IL-1β), IL-6 and tumour necrosis factor-α (TNF-α) both in unstimulated peripheral blood mononuclear cells (PBMC) and separated monocytes,[5] indicating that the immunological role of zinc is most probably mediated by monocyte activation and monokine release. Zinc supplementation has often been suggested to improve the immune response of intensive care or multitransfused patients.[6–8] Pharmacological doses of zinc (1 mg/kg per day elemental zinc as $ZnSO_4$ orally) can lead to increased serum zinc concentrations (0·1 mM versus 0·015 mM before zinc treatment) without showing adverse side-effects.[9] However, not only supportive but suppressive effects of zinc supplementation on immune function *in vivo* have been described which have not yet been explained.[10]

The staphylococcal enterotoxins A and E (SEA, SEE) and the toxic shock syndrome toxin-1 (TSST-1) are exotoxins derived from *Staphylococcus aureus*, a common Gram-positive pathogen.[11–13] They belong to the family of superantigens, cause toxic shock syndrome and are often responsible for food poisoning or shock. MAS (*Mycoplasma arthritidis*-derived superantigen), the only established superantigen which is not produced by Gram-positive bacteria or retroviruses, induces synovitis in rats after intraarticular injection.[14–17] Superantigens do not require antigen processing, bind to major histocompatibility complex (MHC) class II molecules outside the conventional peptide-binding groove and interact with Vβ regions of the T-cell receptor in a manner different from conventional antigens or lectins.[18] The toxic effect of superantigens is mediated by excess release of TNF-α and IL-1β.[19,20] Binding studies suggest that the superantigens SEA and SEE, but not TSST-1, might be specifically regulated by zinc ions, while other bivalent cations show no effect.[21] It is not yet known, however, whether this observation has any relevance for a biological system. Furthermore, it has not been clarified whether zinc interferes only with the complex between the superantigen and MHC class II molecule or whether it is involved in the binding of the superantigen to the Vβ chains of the T-cell receptor as well. We therefore measured the secretion of IFN-γ as an indicator for T-cell activation and the release of the monokines TNF-α and IL-1β as a parameter for monocyte activation.

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Gram-negative infection or septicaemia are common problems in long-term intensive care and multitransfused patients. Lipopolysaccharide (LPS) is a bacterial endotoxin which plays a direct role in the pathogenesis of Gram-negative septicaemia by potent induction of monokines such as TNF-α and IL-1β.[22,23] LPS interacts with the LPS-binding protein (LBP), a serum acute phase protein, and leads to monocyte activation and polyclonal stimulation of the immune system.[23]

Here, we examined the influence of increased levels of zinc on relevant mechanisms of cytokine induction and the problems that may occur in further clinical application of zinc supplementation. We show that zinc levels within a clinically relevant range of concentrations control the secretion of IFN-γ and monokines both after LPS and superantigen challenge in an *in vitro* model, comparable to the situation *in vivo*.

MATERIALS AND METHODS

Isolation and culture of PBMC

PBMC were isolated from buffy coats of healthy blood donors by density centrifugation over Ficoll–Hypaque as previously described.[24] Cells were suspended in zinc-free RPMI-1640 medium (Biochrom, Berlin, Germany) supplemented with 10% heat-inactivated fetal calf serum (FCS, Gibco myoclone quality, Berlin, Germany; native FCS contains 0·01 mM zinc), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (all obtained from Biochrom). The cells were adjusted to a concentration of 2×10^6 cells/ml and seeded in samples of 0·8 ml into pyrogen-free 24-well culture plates (Falcon, Heidelberg, Germany). The cultures were incubated for 2 days at 37° in a 5% humidified CO_2 atmosphere, after the appropriate amount of zinc and mitogen had been added.

Whole-blood assay

Ten millilitres of whole blood was taken from healthy volunteers by venous puncture with a lithium–heparin monovette (Sarstedt, Nürtingen, Germany). The whole-blood samples were diluted with RPMI-1640 medium (containing penicillin and streptomycin in the same concentration as described above) in 1 : 8 ratio as previously described.[25] Four hundred microlitres of the diluted whole-blood samples was seeded into 5 ml test-tubes (Greiner, Nürtingen, Germany), and 50 μl zinc solution and 50 μl mitogen were added in the appropriate concentrations. Unsupplemented cultures received 50 μl of RPMI medium instead of zinc solution to obtain equal volumes of 500 μl. The incubation conditions were identical to PBMC cultures.

Mitogen and zinc preparations

Zinc sulphate ($ZnSO_4$) was dissolved under sterile conditions in non-supplemented RPMI-1640 medium. The solution was sterile filtered, autoclaved and then added to the cultures as indicated in volumes of 0·1 ml (PBMC) or 50 μl (whole-blood cells) leading to final concentrations of between 0·0125 mM and 0·1 mM. In non-supplemented controls, RPMI-1640 was applied instead.

Mitogens were dissolved in RPMI-1640 medium and added in volumes of 0·1 ml (50 μl, respectively) to the cultures leading to final concentrations as follows: 5 μg/ml phytohaemagglutinin (PHA; Wellcome, Dartford, UK), 1 μg/ml LPS (*Escherichia coli* isotype 0111 : B4; Sigma, Deissenhofen, Germany), SEA, SEE and TSST-1 0·5 μg/ml each (all obtained from Serva, Heidelberg, Germany).

MAS was obtained from cultures of *Mycoplasma arthritidis* Jasmin strain [American Type Culture Collection (ATCC), Rockville, MD, no. 14124] and one batch was used throughout. MAS preparation (free of LPS and mycoplasmas) was performed as previously described[17] and used in a concentration of 5% for stimulation.

Determination of cytokines

The culture supernatants were harvested after 24 hr (TNF-α), 48 hr (IL-1β) and 72 hr (IFN-γ, individual cultures for each cytokine) and stored at −80°. For quantification of the cytokine amount we used ELISA kits kindly provided by Dr H. Galatti (Hoffmann LaRoche, Basel, Switzerland); cytokine bioassays could not be performed because of the proliferative effect of zinc.[4] Results were measured in pg/ml at 450 nm using an ELISA plate reader (Anthos Labtec, Salzburg, Austria) and expresed in pg/ml or as a percentage of the amount detected in non-zinc-supplemented controls.

Statistical analysis

The results (except Tables 1 and 2) are expressed as mean values. The significance of differences in cytokine release between the non-supplemented cultures and the cultures supplemented with different concentrations of zinc was analysed by Friedman test (*$P < 0.05$; **$P < 0.01$).

RESULTS

Influence of zinc on the secretion of IL-1β and TNF-α

Our results demonstrate that secretion of both TNF-α (after 24 hr) and IL-1β (after 48 hr of culture) induced by LPS were significantly enhanced by the addition of zinc in a concentration-dependent manner (Table 1). In comparison to controls, supplementation of zinc at 0·0125 mM led to 39%, zinc at 0·025 mM to greater than 60% and 0·1 mM zinc to more than

Table 1. Influence of supplemented zinc (0·0125 mM, 0·025 mM, 0·05 mM, 0·1 mM) on the secretion of TNF-α in PBMC after stimulation with LPS (1 μg/ml). Supernatants were harvested after 24 hr and cytokine levels were measured by ELISA (experiments with PBMC of 10 individuals are presented)

	TNF-α (pg/ml)				
	No zinc supplement	Zinc 0·0125 mM	Zinc 0·025 mM	Zinc 0·05 mM	Zinc 0·1 mM
1	2062	2799	3141	4065	3696
2	923	1503	1502	1567	1839
3	2214	2691	2520	2757	2611
4	5520	5335	6954	9401	13359
5	1519	2151	2508	4259	5045
6	4430	5654	6754	9067	13737
7	1133	1869	2694	2301	2465
8	3717	5970	5510	6275	7160
9	3240	5815	7630	6328	10515
10	2189	2434	2757	3870	8615

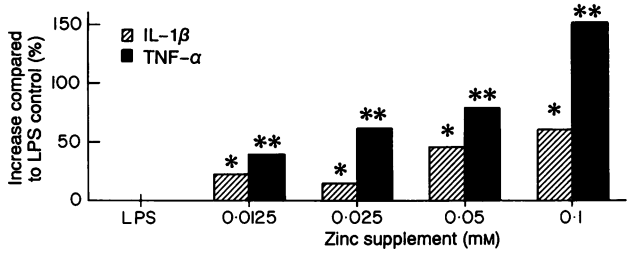

Figure 1. Zinc enhances monokine release after LPS challenge. PBMC supplemented with different concentrations of zinc were stimulated with LPS (1 μg/ml). TNF-α (after 24 hr) and IL-1β (after 48 hr) in supernatants were measured using ELISA. Mean values of 10 individual experiments are expressed as percentage increase compared to the LPS-stimulated controls without supplemental zinc. Significance of difference was analysed by the Friedman test (NS, not significant; $*P < 0.05$; $**P < 0.01$).

150% higher levels of TNF-α in the supernatants of PBMC (Fig. 1). Similar results were obtained for the secretion of IL-1β with augmentation of the cytokine amounts between 14% and 60% compared to the levels detected in non-zinc-supplemented PBMC. Release of IL-1β and TNF-α after PHA challenge, however, is not significantly influenced by low doses of supplemental zinc (less than 0·1 mM) and is only slightly enhanced by higher zinc levels (33% and 35%, with zinc 0·1 mM), compared to LPS treatment (Fig. 2). Investigating the influence of zinc on T-cell activation by LPS or PHA, we found that zinc ions potentiate T-cell activation induced by LPS in a dose-dependent manner (a greater than 10-fold increase in IFN-γ secretion with 0·1 mM zinc supplement; Table 2) whereas they have a weaker, but still distinctly supportive influence on the T-cell activation by PHA.

Zinc regulates monokine secretion in whole-blood cultures

To confirm the above findings under conditions closer to the *in vivo* situation, we performed a similar set of experiments in whole-blood assays. The influence of zinc on cytokine release in whole-blood assays was found to be comparable to the effect observed in PBMC: compared to non-supplemented cultures, zinc leads to an augmentation in the release of IL-1β between 58% (0·0125 mM zinc) and 100% (0·1 mM zinc) in LPS-stimulated cultures. After PHA challenge, we observed

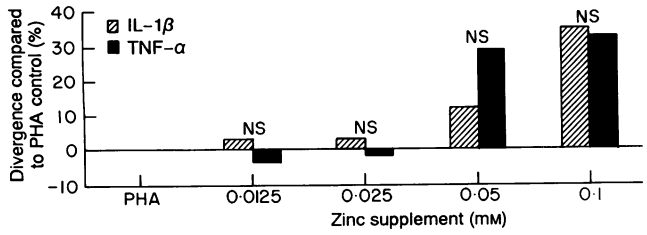

Figure 2. Influence of zinc on monokine induction by PHA. PBMC supplemented with different concentrations of zinc were stimulated with PHA (5 μg/ml). TNF-α (after 24 hr) and IL-1β (after 48 hr) in supernatants were measured using ELISA. Mean values of 10 individual experiments are expressed as percentage divergence from PHA-stimulated PBMC without supplemental zinc. Significance of difference was analysed by the Friedman test (NS, not significant).

Table 2. PBMC were stimulated with LPS (1 μg/ml), PHA (5 μg/ml), the superantigens SEA, SEE (0·5 μg/ml each) or MAS (5%), after addition of supplemental zinc (0·0125 mM, 0·025 mM, 0·05 mM, 0·1 mM). Control cultures received no zinc supplement. Supernatants were harvested after 72 hr of culture and IFN-γ was measured by ELISA (two individual experiments are presented)

	IFN-γ (pg/ml)				
	No zinc supplement	Zinc 0·0125 mM	Zinc 0·025 mM	Zinc 0·05 mM	Zinc 0·1 mM
Exp. 1					
LPS	392	753	778	1742	6122
PHA	27 850	28 550	35 050	36 100	43 100
SEA	38 525	37 100	46 725	45 975	45 201
SEE	29 100	33 425	32 405	33 842	29 975
MAS	8475	5650	4750	1204	550
Exp. 2					
LPS	424	577	857	973	6104
PHA	19 002	20 660	20 965	24 332	36 550
SEA	23 955	27 350	26 450	28 650	21 687
SEE	35 650	38 600	42 775	42 975	41 050
MAS	27 350	23 794	22 156	18 051	12 581

increases in monokine levels of 7% (0·0125 mM zinc) and 184% (0·1 mM zinc, not significant), respectively (Fig. 3).

Zinc specifically influences superantigen activity

To examine whether zinc up-regulates all mitogenic pathways, we studied the influence of zinc on cell activation by superantigens. In our experiments, zinc level specifically regulates the secretion of IL-1β induced by SEA, SEE and MAS in a dose-dependent manner, while the cytokine amount after stimulation with TSST-1 remains unaffected with even 0·1 mM zinc (Fig. 4, Table 3). We concentrated, therefore, on the superantigens SEA, SEE and MAS in further experiments. In contrast to the up-regulation of the monokine release after LPS challenge, zinc effectively down-regulated the monokine level induced by these superantigens: 0·0125 mM supplemental zinc reduced the production of IL-1β after stimulation with

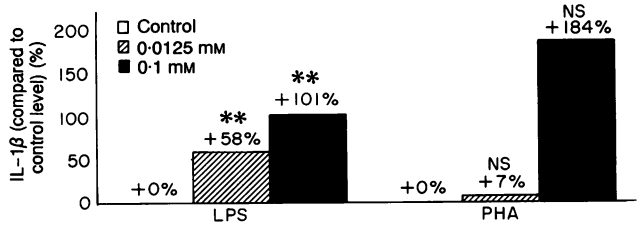

Figure 3. Zinc enhances monokine release in whole-blood cultures. Whole-blood cultures supplemented with different concentrations of zinc were stimulated with PHA (5 μg/ml) and LPS (1 μg/ml), respectively, for 48 hr. IL-1β from the supernatants was measured using ELISA. Mean values of 10 individual experiments are expressed in percentage divergence from PHA- or LPS-stimulated controls without supplemental zinc. Significance of difference was analysed by the Friedman test (NS, not significant; $**P < 0.01$).

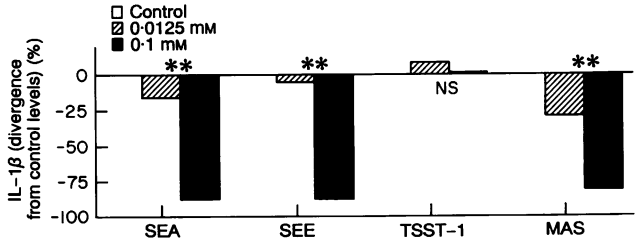

Figure 4. Influence of zinc on monokine induction by superantigens. PBMC were stimulated with the superantigens SEA, SEE, TSST-1 and MAS for 48 hr after varying concentrations of zinc (0·0125 mM and 0·1 mM) had been added, and IL-1β levels in the supernatants were measured. Controls received no supplemental zinc. Mean values of 10 experiments are expressed in percentage divergence compared to the controls without zinc. Significance of difference was analysed by the Friedman test (NS, not significant; $^{**}P < 0{\cdot}01$).

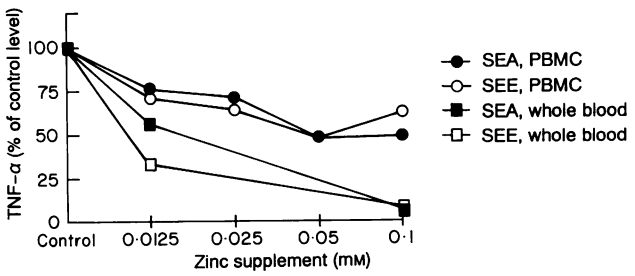

Figure 5. Monokine release induced by superantigens is reduced by zinc both in PBMC and whole-blood cultures. PBMC and whole-blood cultures were stimulated by the superantigens SEA or SEE for 24 hr after varying concentrations of zinc (0·0125–0·1 mM) had been added. Controls received no supplemental zinc. TNF-α levels in the supernatants were determined using ELISA. Mean values of 10 experiments are expressed as a percentage of the monokine levels detected in the control cultures without zinc (100%).

MAS, SEA or SEE in all samples tested. As much as 90% of the IL-1β level induced by SEA, SEE or MAS is inhibited by 0·1 mM zinc supplement.

However, neither 0·05 mM nor 0·1 mM zinc markedly changed the secretion of IFN-γ after stimulation with SEA or SEE (elevation of less than 20%, not dose dependent; Table 2), but inhibited nearly 90% of IL-1β release, whereas T-cell activation caused by MAS was dose dependently impaired by elevated zinc levels parallel to a reduction in IL-1β secretion.

Effect of zinc on SEA and SEE in whole-blood cultures

An effect similar to the antagonistic influence of zinc on IL-1β release in PBMC after stimulation with SEA or SEE was found when TNF-α levels in PBMC or whole-blood cultures were measured (Fig. 5; MAS was not studied as it was previously shown that MAS does not induce relevant amounts of TNF-α[17]). The influence of zinc was even more obvious in whole-blood assays than in PBMC. After supplementation with only 0·0125 mM zinc, less than 60% of the control levels in SEA-stimulated whole blood cultures were measured, whereas in samples stimulated with SEE as little as one-third the amount of TNF-α compared to the non-supplemented SEE controls was found after the addition of 0·0125 mM zinc. After 0·1 mM of zinc was added, less than 10% of the control level of TNF-α was detected after stimulation with SEA or SEE.

Table 3. PBMC were stimulated with LPS (1 μg/ml), PHA (5 μg/ml), the superantigens SEA, SEE, TSST-1 (0·5 μg/ml each) or MAS (5%) for 24 hr. The secretion of IL-1β in cultures without zinc supplement and in cultures with 0·1 mM supplemental zinc was compared. Mean values ± SD of 10 individual experiments are presented

	IL-1β (pg/ml)	
	No zinc supplement	0·1 mM zinc
No stimulus	0	0
PHA	17634 ± 6526	23225 ± 7474
LPS	10383 ± 5157	17076 ± 9177
TSST-1	6281 ± 5008	5069 ± 3556
SEA	4084 ± 2987	644 ± 799
SEE	2792 ± 1383	452 ± 531
MAS	2205 ± 1491	310 ± 176

DISCUSSION

Physiological serum zinc levels in humans range from 0·012 mM to 0·016 mM. In this study, zinc content of the cultures was adjusted to levels between 0·0125 mM and 0·1 mM, whereas controls remained unsupplemented. The zinc levels tested were, therefore, within a range which could be reached by the application of pharmacological doses of zinc *in vivo* and which are known to cause no adverse side-effects.[26] We measured secretion of the monokines IL-1β and TNF-α and the release of IFN-γ, and were able, therefore, to distinguish between the effect of zinc on either monocytes or T cells, respectively.

In our experiments, minimally elevated zinc levels ($< 0{\cdot}05$ mM) were found to enhance monocyte activation and monokine release triggered by LPS. This effect of zinc on monokine release was not observed after stimulation with PHA or superantigens and, therefore, seems to be specific to stimulation with LPS. PHA, superantigens and LPS induce monokine release by different mechanisms of polyclonal activation.[18] We therefore suggest that the serum zinc concentration enhances the signalling process for LPS activation by either stabilizing the LPS/LBP complex and its binding to the CD14 receptor or supporting second messenger processes, as previously suggested for other cell types.[27,28]

The up-regulation of monokine release after stimulation by LPS led to potentiation of T-cell activation with a more than 10-fold increase in IFN-γ amounts. Similar to the effect of zinc on monocytes, only T-cell stimulation by LPS, but not by PHA or superantigens, could be effectively enhanced by supplemental zinc ($< 0{\cdot}05$ mM). This, together with the parallel dose–response pattern for the induction of IL-1β and IFN-γ by LPS after addition of various amounts of zinc, indicates that the potentiation of the T-cell response after LPS stimulation is mediated by increased monokine secretion and subsequent T-cell activation as previously suggested[5] and not by the direct influence of zinc on T cells.

The supportive influence of elevated zinc levels on monokine release after LPS challenge was more obvious in

whole-blood assays cultured with native autologous serum than in PBMC cultures containing inactivated FCS, and was observed at zinc concentrations of 0·0125 mM. This underlines our hypothesis that zinc acts on the LPS/LBP complex because non-activated autologous serum contains more native LBP than heat-inactivated FCS.

Most symptoms of septicaemia are caused by excess cytokine release after LPS challenge.[22] Our data suggest that serum zinc levels may be an important regulatory element in immune response during Gram-negative infection. Furthermore, it appears that zinc levels represent relevant clinical parameters in the treatment of Gram-negative septicaemia. Based on our results, indications for zinc treatment in long-term intensive care or multitransfused patients, who are frequently exposed to Gram-negative bacteria or low doses of pyrogens,[8] should be carefully reassessed.

Zinc is involved in a completely different immunological pathway during stimulation with bacterial superantigens. Our experiments, both in PBMC and whole-blood cultures, show that monokine release triggered by the superantigens MAS, SEA or SEE, but not by TSST-1, can be effectively reduced, even at very low doses of supplemental zinc (0·0125 mM) in a concentration-dependent manner. This result is supported by the data of Fraser *et al.*,[21] who found that the binding of SEA and SEE, but not TSST-1, to isolated MHC class II molecules is regulated by zinc. This seems to be dependent on the different three-dimensional structure of TSST-1.[29] As our data show, the down-regulation of monocyte activation and monokine release caused by zinc did not influence the Vβ-specific T-cell stimulation by SEA and SEE and was not the result of chemical degradation of the superantigens themselves. The release of IFN-γ remained unchanged even at high zinc concentrations (0·1 mM). We, therefore, conclude that zinc ions exclusively regulate the binding of SEA and SEE to MHC class II and the subsequent immune response of monocytes, but have no regulatory influence on the binding of these superantigens to the Vβ chains of the T-cell receptor and T-cell activation.

The superantigens SEA and SEE seem to bind to an identical domain of class II MHC molecules whereas TSST-1 binds to a different domain.[30] The contrasting effect of zinc ions on cytokine release after stimulation with SEA and SEE or TSST-1 may be caused by a specific zinc-binding site in the SEA- and SEE-binding region of HLA-DR whereas the TSST-1 binding region of the MHC class II molecule may not interact with zinc ions. Excess zinc might saturate the zinc-binding regions of both the superantigen and class II MHC molecule and, therefore, inhibit binding and interaction between them. According to our results, the MHC-binding site of MAS seems to have a structure similar to the MHC-binding region of SEA and SEE, suggesting that the binding domain for SEE and SEA might not exclusively bind these superantigens. The mechanisms of T-cell stimulation by MAS differs from T-cell activation by SEE or SEA and may be regulated by zinc as well, because in our experiments zinc suppressed the release of IFN-γ induced by MAS depending on its concentration. The amount of monokines released after superantigen stimulation did not correlate with the induction of IFN-γ after superantigen challenge. In fact, an almost 90% reduction in IL-1β secretion after the addition of 0·1 mM zinc did not influence the amount of IFN-γ secreted by T cells under the same conditions.

The pathogenesis of SEA and SEE poisoning and shock *in vivo* appears to be mediated by the excessive cytokine release of TNF-α and IL-1β in particular. Our results show that supplemental zinc is able to reduce the secretion of TNF-α and IL-1β even at doses of the trace element which are close to its physiological serum level. Our data are supported by experiments performed in whole-blood cultures and, therefore, provide an interesting new clinical perspective for the treatment of food poisoning or staphylococcal infection.

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