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BRIEF COMMUNICATION

Characterization of the major neutrophil-stimulating activity present in culture medium conditioned by *Staphylococcus aureus*-stimulated mononuclear leucocytes

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SUMMARY

Culture medium conditioned by stimulating human mononuclear leucocytes (MNL) with killed *Staphylococcus aureus* (Scm) was found to contain a substantial amount of tumour necrosis factor-alpha (TNF-α) but no detectable tumour necrosis factor-beta (TNF-β). Culture medium conditioned by MNL in the absence of bacteria contained no TNF-α activity. When Scm was fractionated by high-performance liquid chromatography (HPLC) on Bio-Sil TSK 250, TNF-α co-eluted with neutrophil-stimulating activity measured by chemiluminescence. Similarly, the ability of neutrophils to kill opsonized *S. aureus* was enhanced in fractions that contained this neutrophil-stimulating activity. The stimulating activity could be almost completely removed by pretreatment of the Scm with a TNF-α-specific monoclonal antibody (mAb). The ability of neutrophils to kill *S. aureus* in response to Scm was also substantially reduced by mAb to TNF-α. These results demonstrate that bacterial interaction with MNL leads to the release of neutrophil-stimulating activity that consists predominantly of TNF-α.

Previous studies from our laboratory have demonstrated that Scm augments the ability of neutrophils to release both oxygen-derived reactive species and enzymes from azurophilic and specific granules in response to N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP), phorbol myristate acetate (PMA)[1,2] and opsonized *S. aureus*.[3] These studies also showed that this Scm was able to potentiate the ability of neutrophils to kill *S. aureus*.[3] Stimulation of mononuclear leucocytes (MNL) can lead to the release of a number of cytokines capable of modulating neutrophil function. The potential importance of T cell and macrophage–neutrophil interactions in anti-microbial immunity has recently been addressed.[4] In the present investigation we have attempted to determine whether tumour necrosis factor-alpha (TNF-α) is the main neutrophil stimulating factor present in the Scm.

Leucocytes were prepared from heparinized blood of healthy volunteers as described previously[5] and washed three times in Medium 199 (Flow Laboratories, Sydney, Australia). The MNL were resuspended finally in RPMI-1640 (Flow Laboratories). Neutrophils were resuspended in Medium 199 or Hanks' balanced salt solution (HBSS) for bactericidal or chemiluminescence assays, respectively. The MNL-conditioned media were prepared as described previously.[1] Medium conditioned by bacteria-stimulated MNL was termed 'stimulated conditioned medium' (Scm), medium conditioned by MNL alone was termed 'non-stimulated conditioned medium' (NScm) and medium cultured without MNL or bacteria was termed 'non-conditioned medium' (Ncm). HPLC gel filtration was performed as described previously[2] using a 300 × 7·5 mm I.D. Bio-Sil TSK 250 column with guard column (Bio-rad Labs, Sydney, Australia). Elution buffer consisted of 0·1 M Na_2HPO_4—NaH_2PO_4, 0·3 M NaCl, pH 7·0. Fractions (0·4 ml) were collected into siliconized tubes at a flow rate of 1 ml/min. The column was calibrated with the following molecular weight (MW) markers: γ-globulin (158,000), ovalbumen (43,000), myoglobin (17,000) and Vitamin B_{12} (1350), from Bio-rad Labs, and albumin (67,000), from Pharmacia (Sydney, Australia). Aliquots of fractions from HPLC runs were analysed on the same day for neutrophil-stimulating activity by chemiluminescence. The remainder of the fractions were stored at −70° after addition of 0·1% (v/v) human heat-inactivated AB serum. After thawing, fractions were concentrated using Centricon 10 micro-concentrators (Amicon, Danvers, MA.) and the retentate solvents exchanged for HBSS by repeated concentration and reconstitution with HBSS. These were used to determine bactericidal activity and TNF-α levels. TNF-α and TNF-β were measured by ELISA, as described recently.[6]

Neutrophil-stimulating activity was measured by chemiluminescence. For HPLC analyses, neutrophils (100 μl; 5×10^5) were preincubated for 30 min at 37° with 100 μl column fractions, 100 μl HBSS and 100 μl doubly distilled water before addition of 500 μl luminol (0·25 mg/ml HBSS; Sigma, St Louis,

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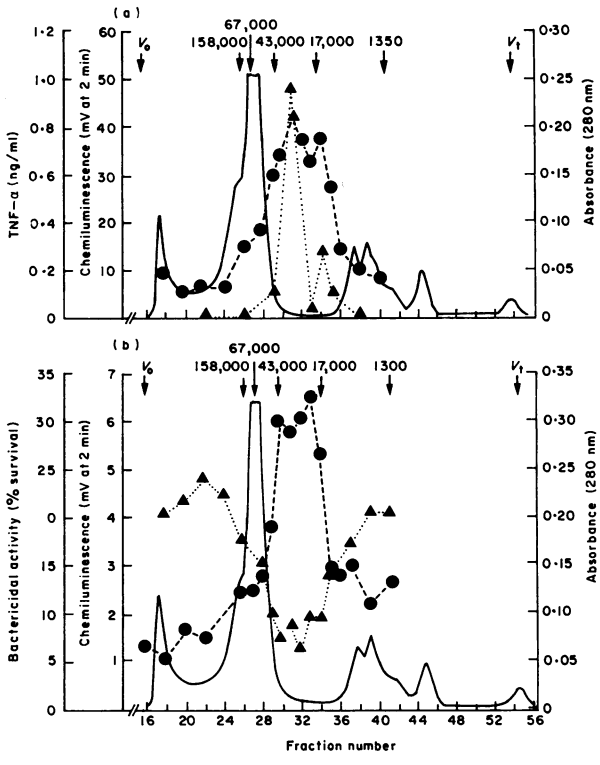

Figure 1. HPLC gel filtration of Scm. Scm was fractionated by HPLC and fractions analysed for neutrophil stimulating activity (●) by chemiluminescence. (a and b) represent separate experiments. In (a) fractions were also analysed for TNF-α (▲) and in (b) for bactericidal activity (▲). The absorbance at 280 nM was monitored continuously (solid line).

MO) and 100 μl FMLP (final concentration 1×10^{-6} M; Sigma). The resultant light output was recorded in mV in a luminometer (Model 1250; LKB, Wallac, Finland). Values represent 2-min responses.[2] In all other experiments neutrophils (100 μl; 1×10^6) were preincubated for 15 min at 37° with 200 μl-pretreated Scm or Ncm before addition of 50 μl HBSS, 500 μl lucigenin (0·25 mg/ml HBSS; Sigma) and 150 μl of heat-killed opsonized *S. aureus* (5×10^7). The resultant light output was recorded as above. Values represent peak responses. Bactericidal activity against *S. aureus* was measured as described previously;[3] neutrophils being preincubated for 30 min with HPLC fractions or mAb-pretreated Scm or Ncm. The pretreatment of Scm or Ncm (diluted 10-fold) was for 15 min at 37° with 10 μl of mAb (400 ng) to TNF-α or to IFN-γ in a final volume of 200 μl. The mAb to TNF-α was produced by Genentech Inc. (San Francisco, CA) and kindly provided by Dr G. R. Adolf from Ernst-Boehringer Institute, Vienna, Austria. Murine mAb (IgG1) to recombinant human TNF-α was purified from ascites fluid, reconstituted at 1·7 mg/ml in PBS and had a neutralizing capacity of 6000 units TNF/μg. GZ-4 murine mAb (IgG1) to human IFN-γ was produced by Boehringer Ingelheim (Vienna, Austria) and purified from ascites fluid, reconstituted at 2 mg/ml in water and had a neutralizing capacity of 1000 units IFN-γ/mg. Statistical significance was evaluated by a two-tailed *t*-test for paired data.

When MNL were stimulated with *S. aureus*, TNF-α production was evident within 2 hr of culture and reached maximum

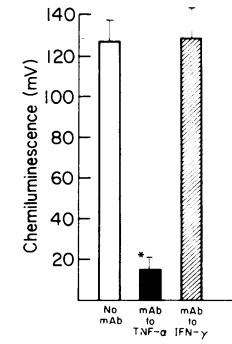

Figure 2. Effect of mAb to TNF-α on Scm-enhanced chemiluminescence in response to *S. aureus*. Scm was treated with mAb to TNF-α or IFN-γ before use in chemiluminescence assays. Results are calculated after correction for effects of mAbs without Scm but in the presence of *S. aureus*. Results are expressed as mean ± SEM for three separate experiments. *Indicates $P < 0.005$ compared to incubations in the absence of mAb.

levels (10–20 ng/ml) by 1 or 2 days of culture and remained at high levels over a 7-day culture period. There was no detectable TNF-β over this period. TNF-α was not detectable in the NScm even after prolonged culture. When Scm was subjected to HPLC, the main peak of neutrophil-stimulating activity eluted with an apparent molecular weight of 15,000–40,000 (Fig. 1). TNF-α co-eluted with this activity (Fig. 1a). The ability of neutrophils to kill *S. aureus* was enhanced in fractions containing neutrophil-stimulating activity (Fig. 1b). When NScm was fractionated by HPLC, bactericidal activity (percentage survival) was unchanged over the column, being 22·3 ± 1·1% (mean ± SEM) for the 14 fractions assayed. There was no evidence of neutrophil-stimulating activity in the same fractions (1·65 ± 0·12 mV, mean ± SEM, $n = 14$). Pretreatment of the Scm with mAb to TNF-α resulted in almost complete removal (89%; Fig. 2) of neutrophil-stimulating activity. A mAb to IFN-γ of the same isotype and concentration was without any effect (Fig. 2). Pretreatment of Scm with mAb to TNF-α also substantially reduced (by 64%; $P < 0.05$) the enhanced bactericidal activity normally seen with Scm (percentage enhancement of killing by Scm in the absence and presence of mAb to TNF-α was 28·7 ± 14·3 and 78·9 ± 4·3, respectively; mean ± SEM, $n = 3$). The Scm was not itself bactericidal.

Our data demonstrate that TNF-α is a major contributor to the neutrophil-stimulating activity present in Scm. TNF-α has stimulatory effects on many neutrophil functions.[7–11] Our data here demonstrate that it can also enhance the ability of neutrophils to kill *S. aureus*.

The macrophage mediator IL-1 is also present in Scm.[12] The inability of the mAb to TNF-α to abolish neutrophil-stimulating and bactericidal activities completely may indicate that other cytokines, such as IL-1 also exert some effect under these conditions, since both IL-1α and IL-1β augment neutrophil functions.[9,13] Since the Scm has been shown to augment the killing of *S. aureus* by neutrophils,[3] cytokine interactions between cells may be of fundamental importance *in vivo* in staphylococcal infections.

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