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SURFACE-ASSOCIATED PROTEIN FROM *STAPHYLOCOCCUS AUREUS* STIMULATES OSTEOCLASTOGENESIS: POSSIBLE ROLE IN *S. AUREUS*-INDUCED BONE PATHOLOGY

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SUMMARY

Objective. *Staphylococcus aureus* is the cause of bone destruction in osteomyelitis, bacterial arthritis and orthopaedic implant failure. We have previously shown that gentle saline extraction of *S. aureus* has revealed the presence of an extremely potent stimulator of osteoclast activation in both the murine calvarial bone resorption assay and the isolated chick osteoclast resorption assay. In order to investigate the mechanism of action of this surface-associated material (SAM), we have investigated its capacity to recruit osteoclasts.

Methods. The murine bone marrow osteoclast recruitment assay was used. The ability of the recruited cells to resorb dentine slices was also investigated.

Results. The SAM from *S. aureus* dose dependently stimulated tartrate-resistant acid phosphatase (TRAP)-positive osteoclast formation and pit formation on dentine slices. Neutralization of the cytokines tumour necrosis factor alpha and interleukin (IL)-6 totally inhibited, but antagonism of IL-1 only partially blocked, the stimulated maturation of osteoclast-like cells.

Conclusion. These findings suggest that bone destruction associated with local infection by *S. aureus* is due to the stimulation of osteoclast formation induced by the action of the easily solubilized SAM, and could explain the large numbers of osteoclasts found in infarcted bone in osteomyelitis.

KEY WORDS: *Staphylococcus aureus*, Osteomyelitis, Bacterial arthritis, Bone resorption, Osteoclastogenesis.

Staphylococcus aureus is a facultatively anaerobic Gram-positive bacterium found on the skin and in the anterior nares of 10–30% of healthy individuals. This organism is a principal cause of bone destruction in a number of lesions. Haematogenous infection with *S. aureus* is the major cause of pyogenic osteomyelitis [1] and it is the dominant organism associated with infected metal implants [2, 3]. *Staphylococcus aureus* is also the causative agent in $>60\%$ of cases of non-gonococcal arthritis [4]. In mice, injection of live *S. aureus* results in rapid destruction of the subchondral bone of diarthrodial joints [5].

In all these conditions, destruction of the calcified extracellular matrix of bone is rapid and severe. Whether the marked involvement of this particular bacterium in bone destruction is due to its greater propensity, relative to other bacteria, for colonizing bone, or to more active bone-modulating activity, is not clear. Indeed, the mechanism by which *S. aureus* stimulates bone destruction, and particularly that associated with osteomyelitis bone necrosis, is still far from clear.

We have been investigating the role of various Gram-negative bacteria in the bone destruction which accompanies chronic inflammatory periodontal disease (CIPD). We have shown that the surface-associated material (SAM) from these bacteria has the capacity to stimulate bone resorption *in vitro* [6, 7]. This very soluble, largely proteinaceous material, removed by a short period of gentle stirring in normal saline [6], is also a potent inhibitor of bone collagen synthesis [8], and has an effect on osteoblasts and other cell populations, including fibroblasts, epithelial cells, macrophages and neutrophils [9]. Indeed, crude extracts of these bacterial SAMs are some 2–3 log orders more active in stimulating bone resorption than the corresponding lipopolysaccharides (LPS) which are normally thought to be responsible for bone destruction in periodontal disease [6].

Staphylococcus aureus is a Gram-positive organism which, by definition, lacks LPS. We have extracted the SAM from this bacterium and have shown that the majority of the material solubilized was protein. The SAM has been shown to be a potent activator osteoclast in the murine calvarial bone resorption assay [10] and the isolated chick osteoclast assay [11]. The possibility that this material stimulates the maturation of osteoclast precursors has been investigated, and has revealed that this surface-associated fraction is a potent inducer of the maturation and activation of osteoclasts. The role of various mediators (cytokines and prostanoids) in the process of tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells (MNC) maturation has been investigated. The possibility that this material acts via synthesis of 1,25-$(OH)_2$ vitamin D_3 has also been investigated.

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MATERIALS AND METHODS

Growth and harvest of S. aureus

Staphylococcus aureus NCTC 6571 was cultured aerobically at 37°C on Wilkins–Chalgrens agar (Oxoid, Hampshire), containing 10% horse blood, for 24 h. The cultures were routinely Gram stained to detect contaminants and were then harvested by washing plates with sterile saline. The cells were then pelleted by centrifugation, washed once with saline and then freeze–dried.

Extraction of SAM

This was carried out as described by Wilson *et al.* [6]. Briefly, the freeze–dried bacteria were suspended (1 g/100 ml) in 0.85% (w/v) saline, and the SAM was removed by gentle stirring at 4°C for 1 h. The supernatant was collected after centrifugation at 3000 *g* for 1 h. The extraction process was repeated twice more, and the SAM was pooled, exhaustively dialysed against distilled water at 4°C, and lyophilized.

In experiments to determine the nature of the active constituents in the SAM, the extract was heat treated by boiling for 1 h or was exposed to trypsin (0.5 mg/ml trypsin incubated with 5 mg/ml SAM) in phosphate-buffered saline (PBS) for 4 h at room temperature. After incubation with trypsin, samples were diluted and soya bean trypsin inhibitor was added, and activity was compared to equivalent concentrations of SAM which had not been trypsin treated, but to which the soya bean inhibitor was added.

To exclude the effect of the medium the bacteria were grown on, a wash of the Wilkins–Chalgrens agar (Oxoid, Hampshire) containing 10% horse blood were extracted in a similar way and tested in the assay.

Electron microscopy

To check on the efficiency of extraction and the integrity of the resultant cells, bacteria were examined by transmission electron microscopy before and after saline extraction as described by Wilson *et al.* [6]. Briefly, a portion of the bacterial suspension was fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer for 1 h at room temperature (control cells). A second portion was fixed in the same manner, except that the buffer also contained 0.15% ruthenium red. Bacteria were then pelleted by centrifugation at low speed, the fixative decanted and the pellet washed in cacodylate buffer. Control cells were then fixed in 1% osmium tetroxide in cacodylate buffer for 2 h at room temperature in the dark. The test cells were treated identically, except that the buffer also contained 0.15% ruthenium red. The fixative was decanted after centrifugation and the pellets washed in cacodylate buffer. All specimens were then dehydrated in a graded series of concentrations of ethanol, and embedded in araldite. Sections were cut on an LKB ultramicrotome using a diamond knife and examined (unstained) in a JEOL 100CXII electron microscope.

Analytical procedures

The protein content of the SAM was determined by the Lowry method [12] and carbohydrate by the method of Dubois *et al.* [13] using glucose standards as a control. Lipids were extracted with methanol/chloroform (2:1 v/v), dried and weighed. The DNA content was estimated by UV absorption.

SAM was fractionated into >30 and <30 kDa fractions by passage through an Amicon PM30 membrane. The efficiency of fractionation was assessed by protein measurement and SDS–PAGE analysis; the bioactivity of each fraction was determined and compared with the starting material.

Bone marrow culture

The bone marrow cells were obtained using a modified method of Takahashi *et al.* [14]. Six-week-old MF-1 mice (Harlan Olac) were killed by cervical dislocation. Femora and tibiae were aseptically removed and dissected free of adherent tissue. The bone ends were removed, the bone was split lengthwise into two, and the bone marrow cells were scooped out of the cavity. The cells obtained were washed twice and suspended (4×10^6 cells/ml) in Dulbecco's Minimum Essential Medium (DMEM; ICN Flow) supplemented with 10% fetal calf serum (FCS; ICN-Flow), L-glutamine (2 mM; ICN-Flow) and penicillin/streptomycin (100 U/ml and 50 μg/ml; ICN-Flow). This suspension was placed (0.5 ml/well) in 24-well plates with or without dentine slices, and incubated at 37°C with various dilutions of SAM or SAM (at 1 or 10 μg/ml) plus added inhibitors. The cultures were incubated for a total of 10 days, the medium being replaced every 2 days.

Tartrate-resistant acid phosphatase staining

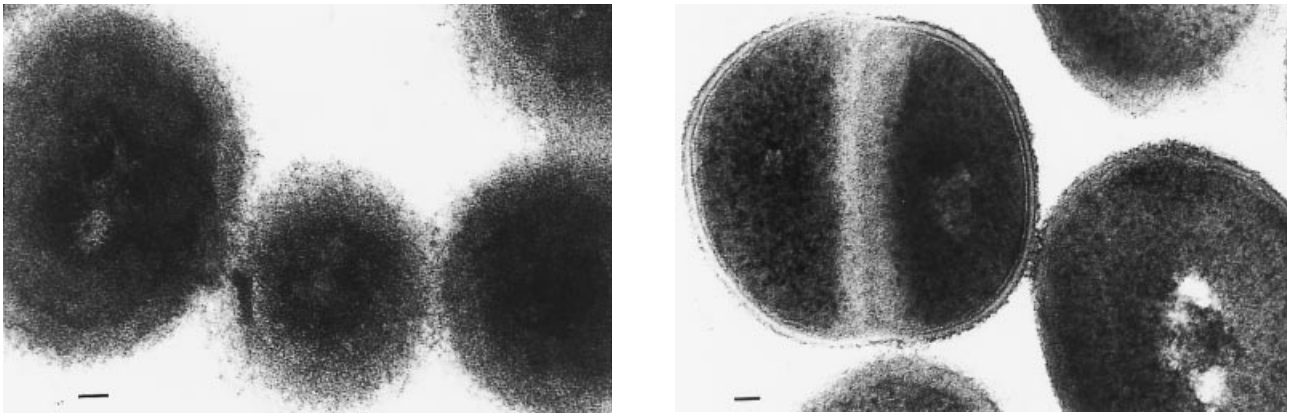
The cultures were fixed and stained for TRAP using a commercial histochemical staining kit (Sigma). All the TRAP-positive MNC with three or more nuclei were counted in four replicate wells for each treatment and results expressed as mean and S.D. Each experiment was repeated twice more to ensure that the results were reproducible.

Assessment of resorption activity in marrow cultures

The cells were removed from the dentine slices after 10 days, and the substrate was stained with toluidine blue and examined for the presence of resorption lacunae by light microscopy. The method used for the precise quantification of the resorptive capacity of the osteoclasts involved estimating their surface area by image analysis (Seescan, Cambridge).

Inhibitor studies

The role of prostaglandins in TRAP-positive MNC formation was tested by adding indomethacin (Sigma). The role of interleukin (IL)-1 was assessed by adding the IL-1 receptor antagonist (IL-1ra; Synergen, Boulder, CO, USA). The involvement of tumour necrosis factor alpha (TNF-α) was determined by use of the neutralizing monoclonal antibody TN3-19.12 (produced by Prof. Bob Schreiber, Washington University School of Medicine and manufactured by Celltech Ltd, Slough). The role of IL-6 was determined by adding



(a) (b)

FIG. 1.—Transmission electron micrographs showing the appearance of *S. aureus* prior to (a) and after (b) extraction of the surface-associated material. The ruthenium-stained surface-associated material that can be seen in (a) is virtually completely removed by the extraction with saline without any structural damage (b). Scale bar 0.1 μm.

a neutralizing rabbit antibody to murine IL-6 (Genzyme). These inhibitors were added to bone cultures at the same time as the SAM, which was used at a concentration of 10 μg/ml in all of these studies. Calcitonin (10^{-8} M) was also added to cultures to determine the role of this calciotropic hormone in the SAM-induced formation of osteoclast-like cells.

1,25 Dihydroxy vitamin D_3 assay

1,25-$(OH)_2$ Vitamin D_3 in the bone marrow cultures incubated with a range of concentrations of SAM over 4, 12 and 48 h time periods was assayed by radioimmunoassay [15].

Statistics

The statistical significance of the results was calculated by the use of Student's *t*-test.

RESULTS

Isolation of SAM

Staphylococcus aureus was seen to have a large amount of extracellular material when stained with ruthenium red and viewed by transmission electron microscopy (Fig. 1a). When the cells were extracted with saline, this material was virtually completely removed, as can be seen in Fig. 1b, and this extraction was not associated with the lysis of cells or any easily discernible damage to the cell structure.

Composition of SAM

The protein content of the saline-extractable SAM was 30% (w/w) and the carbohydrate content was 5%. Lipid and nucleic acids constituted only a few per cent of the SAM.

SAM-induced TRAP-positive MNC generation

The SAM dose dependently and reproducibly stimulated TRAP-positive MNC formation in murine bone marrow cultures. There was some variation between experiments in the maximal response seen, but the dose response was reproducible. In the data shown in Fig. 2, there was a 3-fold increase in TRAP-positive MNCs at 10 ng/ml and at 10 μg/ml the osteoclast-like cell numbers had increased 18-fold. Heat treatment or exposure to trypsin totally abolished the ability of the SAM to stimulate TRAP-positive MNC formation. Fractionation of the SAM into two molecular weight populations demonstrated that the TRAP-positive MNC-stimulating activity had a molecular weight of >30 kDa.

SAM also stimulated pit formation, i.e. resorption, on dentine slices in a dose-dependent manner, with a

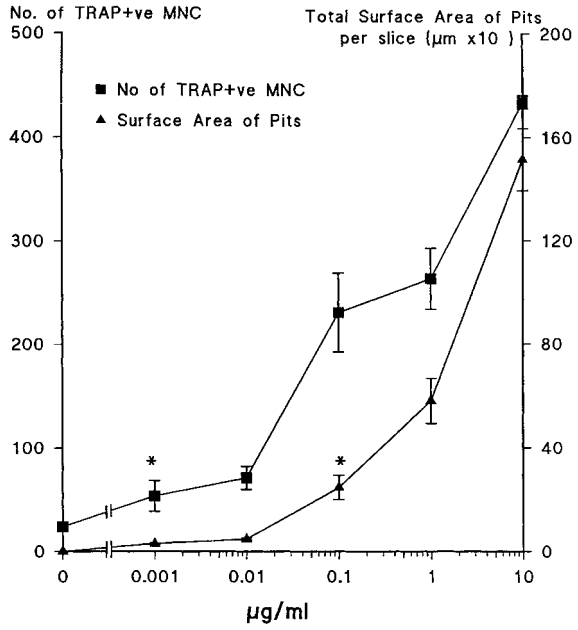


FIG. 2.—The effect of SAM from *S. aureus* showing the number of TRAP-positive multinucleated cells (MNC) in cultures of murine bone marrow following exposure to SAM (0.001–10 μg/ml) from *S. aureus* (■). The area of resorption of pits excavated by these cells on each dentine slice was quantified (▲). Results are expressed as the mean and S.D. of quadruplicate cultures ($^*P < 0.01$).

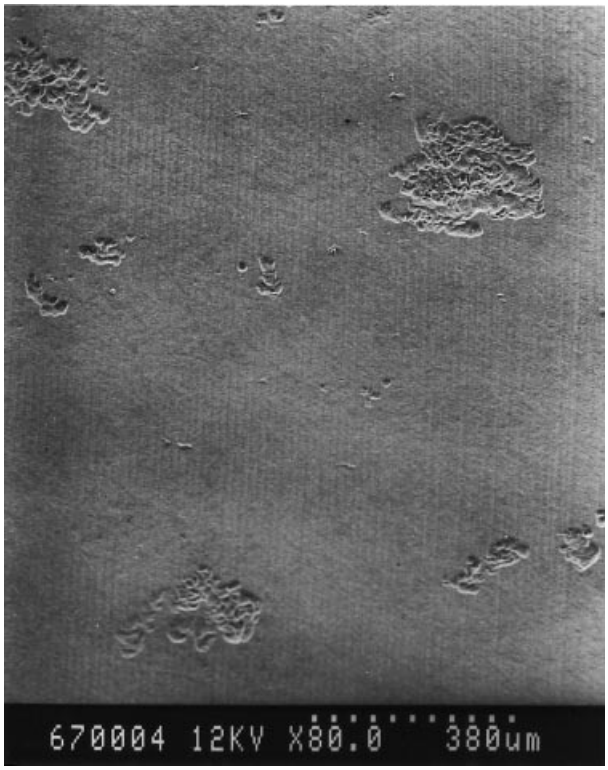


FIG. 3.—Scanning electron micrograph of a dentine slice on which mouse marrow cells were cultured. Mouse marrow cells were cultured with SAM (1 μg/ml) on the dentine slice for 10 days. A number of resorption pits are seen.

significant increase in pit area at a concentration of 100 ng/ml and above (Figs 2 and 3).

Effects of inhibitors

Calcitonin. This calciotropic hormone, when added at 10^{-9} M, inhibited TRAP-positive MNC formation by 50% ($P < 0.01$) (Fig. 4).

Neutralizing antibodies to cytokines. Inclusion of an anti-IL-6 antibody inhibited TRAP-positive MNC formation, with >50% inhibition being seen at 1/500 dilution of the antisera and complete inhibition at 1/300 dilution (Fig. 5). Similarly, the hamster anti-murine TNF-α antibody completely inhibited the formation of *S. aureus*-stimulated TRAP-positive MNC at 10 μg/ml with 50% inhibition being seen at 0.1 μg/ml (Fig. 6). Both antibodies also significantly inhibited the spontaneous generation of TRAP-positive MNCs in unstimulated cultures. However, neutralization of IL-1 by IL-1ra only inhibited TRAP-positive MNC formation by a maximum of 40% at the highest concentration used (100 μg/ml) (Fig. 7).

Inhibitors of prostanoid synthesis. In all experiments, indomethacin failed to inhibit the *S. aureus*-induced generation of TRAP-positive MNCs (Fig. 8).

1,25-(OH)$_2$ vitamin D$_3$

The media supporting bone marrow cultures, taken at 4, 12 and 48 h after initiation of culture, showed

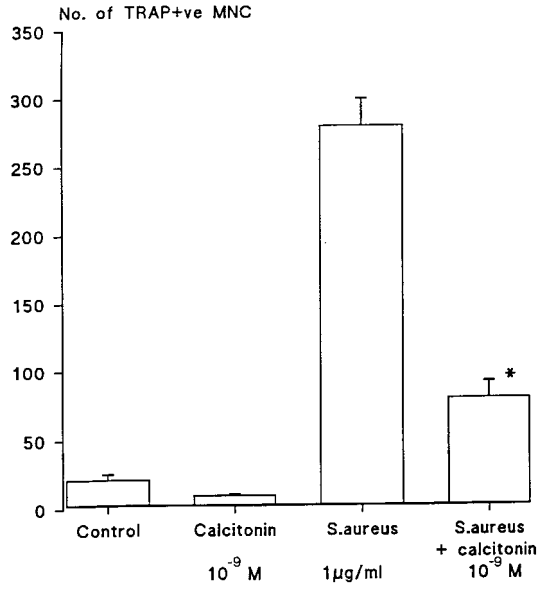


FIG. 4.—The effect of calcitonin (10^{-9} M) on SAM (1 μg/ml)-induced osteoclast formation. Data are expressed as the mean and S.D. of quadruplicate cultures (*$P < 0.01$).

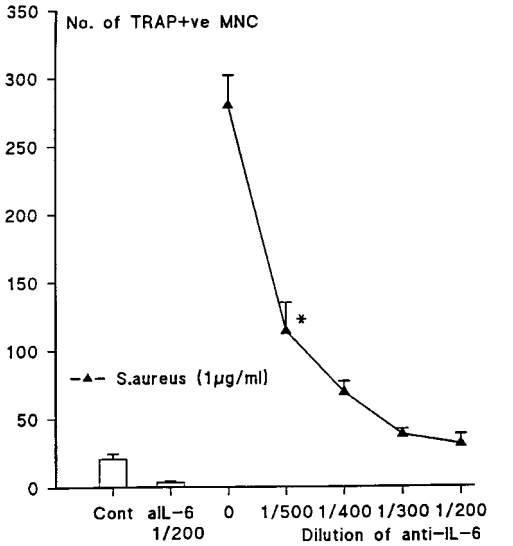


FIG. 5.—The dose-dependent inhibition of TRAP-positive MNC formation (induced by 1 μg/ml *S. aureus* SAM) by various dilutions of a neutralizing rabbit antibody to murine IL-6. Significant inhibition of TRAP-positive MNC formation was found at a dilution of 1/1000 (not shown) and on this graph >50% inhibition was seen with a 1/500 dilution of the antibody. Results are expressed as the mean and S.D. of quadruplicate cultures. The numbers of TRAP-positive MNCs in unstimulated cultures and in unstimulated cultures exposed to the highest concentration of antibody are also shown (*$P < 0.01$).

insignificant (5–7 pg/ml) levels of 1,25-(OH)$_2$ vitamin D$_3$.

DISCUSSION

Staphylococcus aureus is the major causative organism of acute and chronic osteomyelitis, and is also the causative agent in around 60% of cases of non-

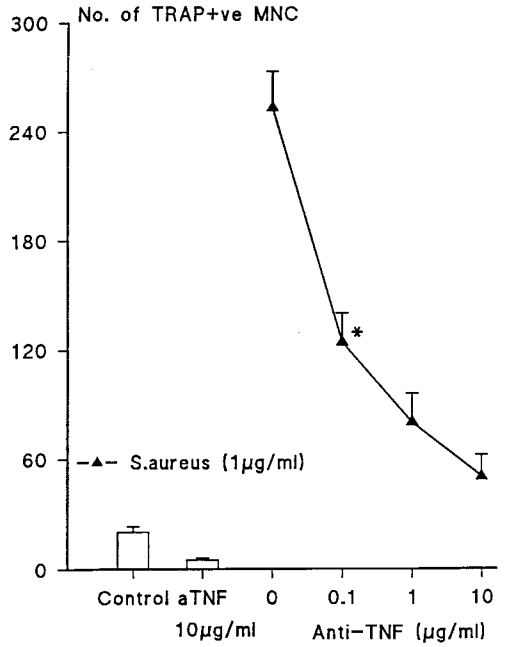


FIG. 6.—The dose-dependent inhibition of TRAP-positive MNC formation (induced by 1 μg/ml SAM) by a neutralizing anti-murine TNF antibody: TN3-19.12. Significant inhibition of TRAP-positive MNC formation was found at a concentration of 0.1 μg/ml of antibody. Results are expressed as the mean and S.D. of quadruplicate cultures. The numbers of TRAP-positive MNCs in unstimulated cultures and in unstimulated cultures exposed to the highest concentration of anti-TNF antibody are also shown ($*P < 0.01$).

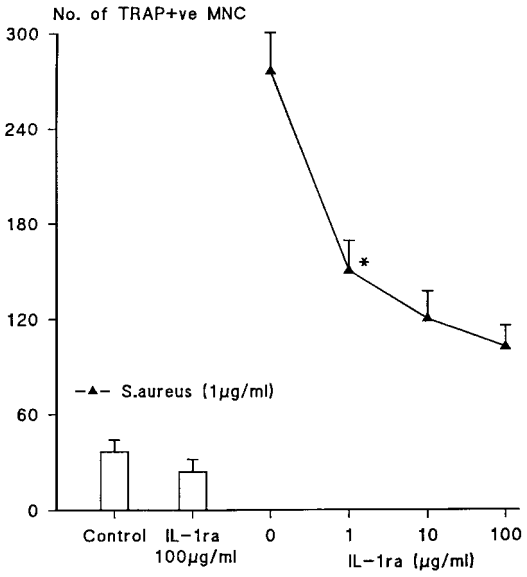


FIG. 7.—The dose-dependent inhibition of *S. aureus* SAM (1 μg/ml)-induced TRAP-positive MNC formation by interleukin-1 receptor antagonist (IL-1ra). Results are expressed as the mean and S.D. of quadruplicate cultures. The numbers of osteoclast-like cells in unstimulated cultures with no additives or those exposed to the highest concentration of IL-1ra are also shown ($*P < 0.01$).

gonococcal bacterial arthritis [4, 16] and of most cases of infected orthopaedic prostheses [3]. In all of these conditions, the major pathological change is the

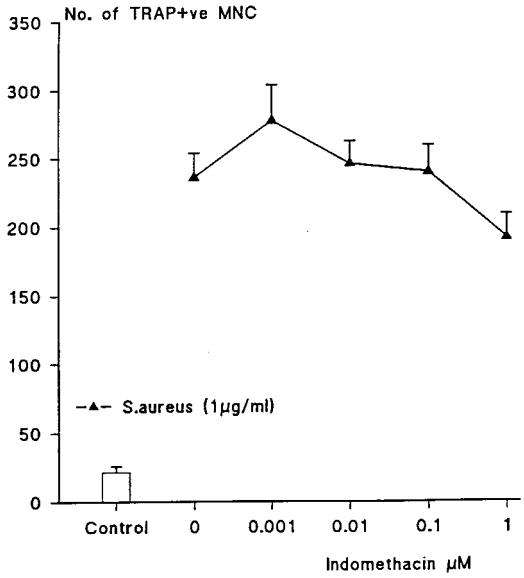


FIG. 8.—The effect of indomethacin on TRAP-positive MNC formation induced by 1 μg/ml of SAM from *S. aureus*. The concentration of indomethacin ranging from 10^{-9} to 10^{-6} M did not have a significant effect on the number of TRAP-positive MNCs. Results are expressed as the mean and S.D. of quadruplicate cultures.

destruction of bone, which can be a very rapid event. The mechanism of bone resorption induced by bacteria may either be due to the direct activity of bacterial constituents on bone cells or to an indirect effect resulting from the stimulation of the synthesis of osteolytic mediators by infiltrating leucocytes or mesenchymal cells. Very little is known about the mechanism of bone destruction induced by *S. aureus*. Previous studies from our group have concentrated on the osteolytic activity of SAM from Gram-negative anaerobic and capnophilic bacteria implicated in the pathogenesis of periodontal disease. In this condition, there is destruction of the alveolar bone supporting the teeth. The SAM from a number of, but not all, periodontopathic bacteria has been shown to be a potent stimulator of bone breakdown in the murine calvarial bone resorption assay [6, 7]. *Staphylococcus aureus* is a capsulated bacterium and we have now demonstrated that the SAM from this organism is an extremely potent bacterial osteolytic mediator capable of stimulating breakdown of neonatal murine calvaria at concentrations as low as 1–10 ng/ml [10, 11].

The classic finding of large numbers of osteoclasts at the periphery of the sequestrum in osteomyelitis led us to investigate the effect of SAM from *S. aureus* on osteoclast generation in bone marrow cultures. Addition of the SAM to bone marrow cultures produced significant and reproducible increases in TRAP-positive MNC at concentrations as low as 1 ng/ml and reproducible dose responses over the range 10 ng/ml–10 μg/ml. Activity was completely abolished by either heating the SAM or exposing it to trypsin, suggesting that the active component is proteinaneous. Fractionation of the crude mixture of surface components by Amicon filtration revealed that the active

constituent had a molecular weight >30 kDa and may be the 32–34 kDa protein described in our earlier study [10].

SAM from *S. aureus* stimulated TRAP-positive MNC formation in the absence of additional external factors, and appears to be a competence factor both for the proliferation and for the fusion of osteoclast precursors. This is in contrast to many agents, e.g. TGF-α, that increase MNC formation by stimulating proliferation of the osteoclast precursors [17], but depend on the addition of 1,25-$(OH)_2$ vitamin D_3 for the fusion of the precursors to form polykaryons [18]. We explored the hypothesis that components in the SAM may have been inducing 1,25-$(OH)_2$ vitamin D_3 synthesis, thus accounting for the effects seen. However, assay of the media from the bone marrow cultures revealed negligible quantities of 1,25-$(OH)_2$ vitamin D_3.

To determine the mechanism of osteoclast formation, bone marrow cultures were stimulated with a fixed concentration of the SAM and a range of concentrations of 'inhibitors' of major osteolytic mediators such as the prostanoids, IL-1, TNF-α and IL-6 [19]. The activity of the cytokines was inhibited by addition of neutralizing antibodies or IL-1ra. Cyclooxygenase activity was inhibited by addition of indomethacin. The osteoclastogenic activity of the SAM was significantly inhibited by the inclusion of the antibodies to the cytokines, TNF-α and IL-6. Neutralization of IL-6 and TNF generation in bone marrow cultures totally inhibited the generation of TRAP-positive MNCs. In contrast, IL-1ra was a fairly weak inhibitor of osteoclastogenesis. Despite the potent inhibitory activity of indomethacin in the murine calvarial assay [10], this compound had no effect on TRAP-positive MNC formation in bone marrow cultures. This may, in part, reflect intrinsic differences in both assay systems. However, it should be noted that TNF-α-induced MNC formation is not inhibited by indomethacin [20].

The nature of the active moiety in the SAM has not been defined and, given the complexity of the bone marrow assay, this may prove to be a difficult task. It is clear that the active agent is a protein of >30 kDa. Work is currently in progress to isolate this active constituent.

In conclusion, the SAM of the Gram-positive bacterium *S. aureus* is a potent inducer of osteoclast-like cell formation in the murine bone marrow cultures. Activity is seen at concentrations as low as 1 ng/ml (w/v), this has a molecular mass of >30 kDa; if one assumes that activity is due to one component, then the minimum effective concentration would be 25 pM. This activity is inhibited by antibodies to IL-6 and TNF, but is only partly inhibited by neutralizing IL-1 but not by the cyclooxygenase inhibitor indomethacin. The ability of the SAM from this bacterium to stimulate osteoclastogenesis, and the activity of these osteoclasts at extremely low concentrations, must contribute to the pathology of the various bone lesions associated with infection by *S. aureus*. The active constituent in this mixture of proteins and carbohydrate represents an important therapeutic target in view of the increasing numbers of isolates of *S. aureus* resistant to antibiotics [21].

Acknowledgements

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