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CORRESPONDENCE

To the Editor: In 1973 a new group of DNA binding proteins were copurified with histones from cell nuclei and termed high mobility group (HMG) proteins because of their rapid mobility on electrophoresis gels (1). HMG box protein 1 (HMGB1) has a variety of functional activities in addition to its DNA binding properties including the enhancement of neurite outgrowth, a potential promoter of tumor invasion, a role in murine erythroleukemia, activation of osteoblasts, and a function as a late mediator of systemic inflammation and endotoxin lethality associated with sepsis (2–4).

In a recent article in this journal Zetterström *et al*. (5) attributed HMGB1 with an additional antibacterial activity against the airway pathogen Moraxella catarrhalis and also Escherichia coli and Bacillus megaterium. These authors used HMGB1 purified from human adenoid glands and recombinant HMGB1 and described them as having an antibacterial activity, which eradicated more than 95% of bacteria in cultures within 5 min.

In contrast to these findings in our hands HMGB1 proteins from different sources had no bactericidal activity against *Escherichia coli*. We cloned the human and murine HMGB1 gene into the pCAL-n vector containing a N-terminal CBP tag (Stratagene, La Jolla, CA, U.S.A.) as described (4) and in addition the human HMGB1 gene into the pETBlue vector (Novagen, Madison, WI, U.S.A.) with a C-terminal linkage to a His tag. The recombinant proteins were expressed fused to the respective tags and purified to high homogenicity (>95%) using the respective affinity columns followed by gel filtration in a HiLoad 26/60 Superdex 200 prep grade column (Amersham Biosciences, Uppsala, Sweden). HMGB1 is known to undergo extensive post-translational modification (2). Therefore native HMGB1s could have different functional activities. Taking this into consideration we extracted HMGB1 two times from calf thymus tissue independently according to methods originally described by Goodwin *et al*. (1) and Adachi *et al*. (6). Essential purification steps were a gel filtration in a HiLoad 26/60 column prepacked with Superdex 200 prep grade and ion-exchange chromatography (HR 5/5 column prepacked with Mono Q, Amersham Biosciences). The identities of all HMGB1 proteins were verified by N-terminal amino acid sequencing and the purities were checked in a SDS-PAGE gel. The endotoxin contents of the HMGB1 preparations as determined with a standard endotoxin-specific LAL reagent (Coachrom, Charleston, SC, U.S.A.) were below 50 ng/mL. The different HMGB1s were functionally active as shown in a modified assay for induction of tumor necrosis factor-α (TNF-α) (7). We added the HMGB1 proteins in decreasing concentrations to 1 mL heparinized whole human blood and used a sandwich ELISA kit (Biosource International, Camarillo, CA, U.S.A.) to determine the amount of TNF-α released after 3 hours. All preparations of native as well as recombinant prokaryotic HMGB1 induced TNF-α release in a dose-dependent manner. The lipopolysaccharide (LPS) content of diluted HMGB1 samples inducing TNF-α release was calculated to be far below the amount of LPS required for TNF-α release. In addition, Polymyxin B, which inhibits LPS did not reduce the amount of released TNF-α.

The antibacterial activity of the HMGB1 proteins was first examined in an inhibition zone assay for *Escherichia coli*. Sterilized filter papers with a diameter of 5 mm were added to Lauria-Bertani (LB) broth agar plates containing log phase *Escherichia coli* DH-α. Samples of 5 mL (5–10 mg) HMGB1 (recombinant human HMGB1 with Cal tag or His tag, two different preparations of HMGB1 from calf thymus, and recombinant murine HMGB1 with CPB tag) and 2 mL (2 mg) ampicillin (control) were loaded onto the filter paper and the plates were incubated for 2 days at 37°C. We observed a large inhibition zone of approximately 1 cm in diameter on the plates with ampicillin but no inhibition of bacterial growth was detectable on the plates with HMGB1 samples. We then added 10 mL of native HMGB1 in a concentration of approximately 50 mM to 90 mL LB medium containing about 10,000 *Escherichia coli* DH-α cells and incubated this culture at 37°C. After 0, 20, and 60 min aliquots of 10 mL were removed, mixed with 90 mL LB and spread on LB plates. After an overnight incubation at 37°C the bacterial colonies were counted. At every time point the number of colonies was approximately 1,200 with no differences observed.

Our data indicate that HMGB1 does not exert antibacterial activity, at least for *Escherichia coli* DH-α. We checked the purity of our HMGB1 proteins and proved their functional activity. The Cal-tagged HMGB1 we used has the same amino acid sequence as that used by Zetterström *et al*. The discrepancies between the results of the two studies are therefore enigmatic. Conceivably differences in the expression and purification procedures of the HMGB1 proteins are responsible or possibly antibacterial activity of HMGB1 is restricted to specific bacterial strains. Nevertheless an expression yield of pure HMGB1 of up to 200 mg/L bacterial culture obtained under optimized conditions (8) argues against an overall antibacterial activity of HMGB1. Clearly more detailed studies are necessary to elucidate a potential role of HMGB1 in antibacterial defense.

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