



AG 85, a major secretion protein of *Mycobacterium tuberculosis*, can be identified in ancient bone



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S U M M A R Y

Keywords:

Extracellular bone matrix proteins
Ag 85
Western blot

For the confirmation of Ag 85 in ancient and recent ECM of native macerated human bone, five cases were investigated. In three individuals, highly positive results for Ag 85 were identified in Western blot: 1) a male from Arzhan, South Siberia, dating from the 7th century BC, 2) a male from Kirchberg in Hesse, Germany, dating from the 10th – 12th century AD and 3) a recent female with a proven diagnosis of TB. As a negative control, a recent male is presented who did not suffer from TB. In another recent male, Ag 85 could be identified only very weakly. From cases in the literature it is well-known that highly positive results for Ag 85 indicate active TB, however, weakly positive results indicate a silent initial infection with Mtb. Thus, apparently, also in ancient individuals, it might well be possible to differentiate between diseased persons and disease carriers using paleoproteomic techniques.

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1. Introduction

Tuberculosis (TB) has been known to civilization since prehistoric times. An indication of the antiquity of this disease was the finding of specific morphological changes consistent with tuberculosis in a fossil of *Homo erectus* from Turkey dating from the middle Pleistocene (490–510,000 years BP) [1]. Although TB can affect many parts of the human body, this commonly found disease most frequently affects the lungs. Nowadays, TB is one of the most common infectious diseases known to man. Nearly one third of the human population is infected with the TB pathogen [2]. Approximately nine million of these infected people develop active disease and more than two million of them die each year [2].

Of the many mycobacterial strains which mainly exist as soil bacteria, only some strains are infectious agents for human tuberculosis. These four strains, termed *Mycobacterium tuberculosis*, *M. bovis*, *M. africanum*, and *M. cannetti*, lead to an identical clinical pattern [3]. The pathogenic species are able to survive and grow within macrophages which enable them to evade the host immune

system. An active cell-mediated immune system is required to contain and kill the tubercle bacilli. The extremely high level of latent infection is an indication of long-term co-existence of human host and bacterial pathogen [4].

A characteristic feature of bone which differentiates it from other connective tissues is the mineralized extracellular matrix (ECM). The living ECM consists to about 70% of inorganic molecules (calcium phosphates similar to hydroxyapatite), 5–10% of water and to about 20% of organic molecules. Most of the organic molecules are proteins; 90% of the ECM-proteins are collagen type I and 10% are non-collagenous proteins (NCPs). The NCPs bind very intensively to hydroxyapatite and/or to collagen, and if the bone is well preserved, the NCPs are also present after death in the same place as in life time.

A new discipline has started to develop in the field of paleopathology, called paleoproteomics. The paleoproteomic investigation of intact ECM-proteins of bone from individuals who lived hundreds or thousands of years ago belongs to the field of biochemistry [5,6]. In ECM-proteins, however, there is almost no contamination risk as in aDNA because, as a rule, these proteins are bound very tightly to the hydroxyapatite scaffold as in the living organism.

A great challenge in paleoproteomics is the identification of ECM-proteins of the normal bone metabolism and also of proteins which are typical for disease. Principally, there are two different approaches: investigation of disease-specific proteins which are

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built by the bone cells of the infected individual or proteins which are built by cells of other organs of the same infected individual. These proteins are transported by the bloodstream to the bone ECM, such as PSA, a marker for prostate cancer [7]. If pathogens have infected an individual, the proteins secreted by the pathogens can also bind to the ECM of bone.

The proteins of the antigen 85 complex (Ag 85) are major secretion products of *M. tuberculosis* (Mtb) and have been studied by independent investigators for at least two decades [8,9]. The key antigenic targets recognized by the T-lymphocytes were identified as Ag 85 and ESAT-6, belonging to the group of secreted proteins [10]. These secreted extracellular antigens are important in the induction of protective immunity [11,12], especially during the early phase of infection. Up to 30% of Mtb culture filtrate proteins are composed of Ag 85 [9], a family of three highly homologous 30–31 kDa proteins, Ag 85A, Ag 85B and Ag 85C. Each of these Ag 85 is associated with mycolyl transferase activity *in vitro* [13] suggesting their essential involvement in the characteristic cell wall of mycobacteria.

2. Materials and methods

2.1. Bone samples

2.1.1. For this study, five specimens were available

Case 1: Male, 22–24-year old, Burial 20, SK-1, excavated from kurgan 2 (= burial tumulus) at the archaeological site of Arzhan (South Siberia, Russia), 7th century BC. The sample was taken from the right femur as dry bone, stored at room-temperature.

Case 2: Male (40) 45–59-year old, Burial G 19, excavated from the archaeological site of the churchyard of Kirchberg (Germany), 10th–12th century AD. The sample was taken from the right parietal as dry bone, custody of sample: room-temperature.

Case 3: Male, 38-year old, body donator from the Department of Anatomy, University Medical School Göttingen (Germany), suffered from cancer, no clinical diagnosis of TB known. The sample was taken from the right femur, custody of sample: directly frozen.

Case 4: Female, adult, Pathology Collection of the University of Göttingen (Germany), late 19th or early 20th century, known clinical diagnosis of TB, sample taken from the left radius as dry bone, custody of bone: room-temperature. This sample was used as a positive control.

Case 5: Male, 76-year old, body donator from the Department of Anatomy, University Medical School Göttingen (Germany), no clinical diagnosis of TB known. The sample was taken from the right femur, custody of sample: directly frozen. This sample was used as a negative control.

For the microscopic analyses, thin-ground sections were prepared by suitable techniques [14,15].

2.2. Preparation of bone samples

Cleaning, degreasing and powdering of bone samples were carried out as previously described [5,6].

2.3. Extractions of bone matrix proteins

About 1 g of mineralized bone powder was extracted with 5 ml buffer A (4 M guanidine-HCl, 20 mM NaH₂PO₄, 30 mM Na₂HPO₄, pH 7.4) under permanent stirring for 24 h (4 °C). After centrifugation (10,000 g, 30 min, 4 °C), the supernatant was removed. The pellet was extracted with 5 ml buffer B (buffer A and 300 mM EDTA) under constant stirring for 24 h (4 °C). From the resulting extract, chelated calcium ions and salts were removed by rinsing (35,000 g, 30 min) three times with a 30x excess of autoclaved

distilled water. The pellet, containing the bone matrix extract (BME), was lyophilized and then again powdered under permanent cooling in liquid nitrogen in the Mikro-Dismembrator U (B. Braun Melsungen AG) at a shaking frequency of 1600 rpm for 2 min [5,6].

The BME can be stored for a long time at –20 °C. Each extraction step was carried out with a combination of protease inhibitors, 5 mM benzamidine, 1 mM PMSF, 2 mM aprotinin, 50 mM leupeptin, and 10 mM EDTA. All procedures were performed with gloves, autoclaved instruments, and autoclaved or sterile filtered solutions [5,6].

2.4. Western blot analysis

Solubilized denatured protein samples were separated by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli 1970 [16] (10% T and 2.5% C) and electro-transferred to polyvinylidene fluoride (PVDF) membranes. The PVDF-membrane was blocked using starting block-buffer (Firm Perbio Science, Germany). The first antibody used was against Ag 85 from Mtb, dilution 1:200 (mouse monoclonal antibody subclass: IgG/k, HYT 27, Firm DIANOVA, Hamburg, Germany). The immunogen was a bacterial press extract from *M. tuberculosis* H37Rv absorbed onto aluminum hydroxide gel.

As secondary antibody, horseradish peroxidase linked anti-mouse was used at a dilution of 1:15,000; bands were visualized by the enhanced chemiluminescence-Plus detection system (Firm Millipore, Temecula CA, USA). Between the different steps, a rinsing procedure with phosphate-buffered saline was carried out.

3. Results

3.1. Macroscopy

The first archaeological case is the young adult Scythian warrior from Arzhan. In his vertebral column, the 6th–9th and, particularly, the 7th and the 8th vertebral body exhibit vestiges of serious osteolytic destruction (Figure 1A). The 2nd to the 12th rib show pronounced vestiges of osteoblastic activity in the form of periosteal layers due to chronic pleurisy (Figure 1B). Similar, however, slighter changes are seen in some right ribs. Additionally, there are resorption cavities in the vertebral end of the left 7th rib and in the rim of the right acetabulum. The external surfaces of both tibiae show a coarse striation. Accordingly, slighter changes are seen on the surfaces of both femora (Figure 1C). These vestiges, particularly the changes in the vertebrae and the ribs, are pathognomonic for TB. The changes in the long bones and the pelvis suggest the existence of milary tuberculosis [17].

The second archaeological case is the old mature male from Kirchberg. Here, vestiges of chronic pleurisy are observable; however, there is also evidence of chronic inflammatory processes in the nasal cavity, the paranasal sinuses and the middle ear region (otitis media, mastoiditis) [18]. Additionally, at the skull base, vestiges of meningeal reactions in the form of branched or net-shaped blood vessel impressions which are characteristic of a meningeal process and round to oval impressions (“gruebchen”) (Figure 2A) apparently caused by pressure atrophy probably due to tubercles suggest the existence of *Leptomenigitis tuberculosa* [18,17].

In the third case of an old adult body donator from the anatomy department of the University of Göttingen, no macro-pathological changes were found which might be associated with TB. However, there was evidence of metastasizing cancer.

The fourth case is an exhibit from a pathology collection with known clinical diagnosis of tuberculous infection of the left elbow joint of an adult female. The character of the predominantly

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