SHORT ARTICLE

Haemolysin from *Mycobacterium avium* complex isolates from AIDS patients

R. G. DESHPANDE, M. B. KHAN, D. A. BHAT and R. G. NAVALKAR

Department of Microbiology and Immunology, Morehouse School of Medicine, 720 Westview Drive, Atlanta GA 30310, USA

Cell-bound haemolytic activity was observed in isolates of *Mycobacterium avium* complex (MAC) from AIDS patients. *M. avium* type strains showed negligible activity. None of the culture supernates exhibited any haemolytic activity. Zwitterionic detergent 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulphonate (CHAPS) was used to extract haemolysin from ethanol-treated *M. avium* complex strain 101 (MAC101) cells. Haemolysin was isolated from CHAPS extract (CE) by metal affinity chromatography and identified as a 32-kDa protein by polyclonal antibodies raised against *M. tuberculosis* haemolysin. Treatment of CE with trypsin resulted in reduction of haemolytic activity, whereas heating at 100°C for 10 min did not affect its activity. A similar 32-kDa haemolysin was extracted from cells of *M. avium* K128 which was isolated from a monkey infected with simian immunodeficiency virus (SIV). The haemolysin produced by *M. avium* strains isolated from AIDS patients may be associated with the pathogenesis of *M. avium* infection.

Introduction

Organisms belonging to the *Mycobacterium avium* complex (MAC) are the commonest cause of systemic bacterial infections in AIDS patients [1]. In the non-AIDS population, MAC usually causes pulmonary infection, which in some instances can be diagnosed with a combination of acid-fast staining and culture [2]. However, in immunocompromised patients, it causes a life-threatening tuberculosis-like pulmonary disease as well as local and disseminated infection [1, 3, 4]. The factors involved in causing disseminated infection in AIDS patients in contrast to the localised respiratory infection caused in non-AIDS patients, are not completely understood.

It is unclear what strategies *M. avium* uses to evade being killed by host cells. Cytolysins play an important role in virulence and intracellular survival of bacterial pathogens [5–7]. An earlier report described the isolation of a contact-dependent haemolysin/cytolysin from *M. tuberculosis* [8]. The presence of haemolytic activity in clinical strains of *M. chelonae* and *M. fortuitum* has been reported by Udou [9]. This study examined the haemolytic activity of *M.*

avium isolates from AIDS patients and from a monkey infected with simian immunodeficiency virus (SIV).

Materials and methods

Bacterial strains

Seven *M. avium* complex (MAC) isolates (MAC 101 – serotype 1; LR 535, LR 536, LR 541, LR 542, LR 559 and LR 562 – serotype 4) from AIDS patients were provided by Dr PRJ Gangadharam, University of Illinois, Chicago. Strains ATCC 25291 and 19075 were two *M. avium* type strains of serotype 2. *M. avium* strain K128 isolated from a monkey infected with SIV was kindly provided by Dr G. Newman, Morehouse School of Medicine, Atlanta.

Culture of strains and preparation of extracts

Each strain was inoculated into Middlebrook 7H9 Broth (Difco) supplemented with glycerol (without ADC enrichment) and incubated with shaking for 5–6 weeks at 37°C. The cells were harvested by centrifugation at 8000 *g* for 30 min, suspended in phosphate-buffered saline (PBS) and tested for haemolytic activity. At weekly intervals, culture samples of MAC101 and K128 were centrifuged at 8000 *g* for 30 min and the supernates (CS) were tested for haemolytic activity. The cell deposits were incubated in ethanol 50% v/v in 7H9 broth for 10 min at 37°C

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Corresponding author: Professor R. G. Navalkar.

E-mail: navlab@link.msm.edu

and centrifuged at 8000 *g* for 20 min. The supernate was discarded and the ethanol-treated cells were washed with PBS and extracted with CHAPS 1.5% in distilled water at 37°C for 24 h. The supernate (CE) obtained after centrifugation of the CHAPS-treated cells was stored at 10°C.

Assay for haemolytic activity

One ml of bacteria (10^{10} /ml) suspended in PBS was mixed with 1 ml of sheep red blood cells (RBCs) 0.7% in PBS containing bovine serum albumin 0.5%, co-sedimented by centrifugation at 8000 *g* for 10 min and incubated at 37°C for 12–16 h. The haemolytic activity of CSs and CEs was assayed by mixing 0.25 ml of CE or 1 ml of CS with RBCs and incubating the mixture at 37°C for 12–16 h. The samples were then centrifuged and the E_{540} of the supernates measured as an indicator of the release of haemoglobin from lysed erythrocytes with PBS as a blank. Appropriate controls (RBCs in PBS, RBCs mixed with 7H9 broth and RBCs mixed with CHAPS 1.5%) were treated similarly.

Immobilised metal affinity chromatography (IMAC)

Haemolytic activity was isolated from CE of MAC101 and K128 by IMAC with nickel nitrilotriacetic acid (Ni-NTA) resin under non-denaturing conditions [10]. One ml of unfiltered CE mixed with 1 ml of buffer A (0.5 M sodium phosphate buffer containing 0.5 M NaCl, pH 8.0) was loaded on to a Ni-NTA column. Bound protein was eluted with buffer B (10 mM potassium phosphate buffer containing 0.75 M NH_4Cl , pH 6.6) and collected in 1-ml fractions. Fractions (0.25-ml amounts) were tested for haemolytic activity with and without the addition of CHAPS 0.5%. Ten μl of fractions with haemolytic activity were analysed by SDS-PAGE (12%, Mini-Protean II ready gels, BioRad) followed by silver staining and immunoblotting.

Results

Cells of *M. avium* complex strain 101 (MAC101), *M. avium* K128 and six clinical isolates of MAC from AIDS patients showed strong haemolysis of sheep RBCs ($E_{540} = 0.3$ – 0.6) when compared to control RBCs incubated in PBS ($E_{540} = 0.06$). Both the *M. avium* serotype 2 strains showed negligible haemolytic activity ($E_{540} = 0.16$ and 0.18 , respectively). No haemolytic activity ($E_{540} = 0.12$ – 0.16) was demonstrated for CSs of any of the strains even after growth for 5 weeks as compared to the 7H9 broth control ($E_{540} = 0.13$). However, CSs from 6-week-old cultures lysed sheep RBCs ($E_{540} = 0.22$ – 0.29). At this time the cells had autolysed as determined by acid-fast staining. The haemolytic activity of MAC101 could not be solubilised by sonication or treatment with 8 M urea or ethanol or with detergents such as Tween 20 or Tween

80. The zwitterionic detergent CHAPS at a concentration of 1.5% extracted activity after incubation for 12 h at 37°C from MAC101 cells pre-treated with ethanol (E_{540} of CE = 0.6). CHAPS 1.5% alone did not lyse RBCs ($E_{540} = 0.08$). Cell debris obtained after treatment of ethanol-treated cells with CHAPS did not show any haemolytic activity ($E_{540} = 0.08$). Sterilising CE through 0.45- μm pore filters caused loss of haemolytic activity. However, addition of CHAPS 0.5% to the filtered CE restored activity. Therefore, all solubilised haemolysin preparations were used without filter-sterilisation. Heating CE at 100°C for 10 min did not affect activity ($E_{540} = 0.55$). Treatment of CE with trypsin (0.1 ml of 1 mg/ml in PBS, pH 7.6) at 37°C for 30 min caused a reduction in haemolytic activity ($E_{540} = 0.25$). CE of strain K128 prepared similarly also showed significant haemolytic activity ($E_{540} = 0.5$).

IMAC of MAC101 CE on Ni-NTA yielded a single peak eluting in the first two-to-three fractions. Each of these fractions showed strong haemolytic activity (E_{540} of 0.25 ml of each fraction = 0.3 – 0.4) only after addition of CHAPS 0.5%. Fractions tested for activity without the addition of CHAPS did not show any activity ($E_{540} = 0.09$). Elution of K128 CE from Ni-NTA column yielded two consecutive fractions, both of which were haemolytic (E_{540} of 0.25 ml of each fraction = 0.3 and 0.28). All the haemolytic fractions of MAC101 and K128 had identical SDS-PAGE profiles with protein bands in the M_r range *c.* 30 000 (data not shown). Therefore, the fractions of each strain were pooled. Silver staining of the pooled fractions after SDS-PAGE is shown in Fig. 1(A). Western blotting of the haemolytic fractions of strains MAC101 and K128 with polyclonal antiserum to *M. tuberculosis* haemolysin [8] showed a single band with M_r of *c.* 32 000 (Fig. 1B).

Discussion

The importance of MAC-associated disease has been magnified dramatically by the AIDS epidemic. During our investigation into the presence of haemolysin in mycobacteria, it was observed that MAC isolates from AIDS patients showed significantly more lysis of sheep erythrocytes than *M. avium* type strains. Furthermore, a strain of *M. avium* (K128) isolated from a monkey infected with SIV also showed strong haemolytic activity.

Both MAC101 and K128 demonstrated strong cell-associated haemolytic activity which could be extracted with CHAPS 1.5% only after pre-treating the cells with ethanol. Haemolytic activity was not secreted into the medium until after the onset of autolysis. MAC101 and K128 haemolysins share an epitope with *M. tuberculosis* haemolysin because they were recognised by polyclonal antiserum raised

Fig. 1(A). SDS-PAGE (12%) and silver staining of haemolytic fractions of (a) MAC101 and (b) K128 obtained from Ni-NTA column. The arrow indicates the 32-kDa band. **(B)** Immunoblot of haemolytic fractions obtained after IMAC of (a) MAC101 and (b) K128 with polyclonal antibody against *M. tuberculosis* haemolysin. The arrow indicates the 32-kDa band. Positions of the mol.wt markers (kDa) are indicated on the left in each panel (top to bottom: phosphorylase b, 97; BSA, 66.2; ovalbumin, 43; carbonic anhydrase, 31; soybean trypsin inhibitor, 21.5%; lysozyme, 14.4).

against *M. tuberculosis* haemolysin. However, the haemolysin of both the *M. avium* strains appear to be different from *M. tuberculosis* haemolysin, as they bound to Ni-NTA resin whereas the latter did not [8]. Because Ni-NTA binds proteins containing surface-exposed neighbouring histidine residues [11], it is probable that *M. avium* haemolysin possesses such residues. Ni-NTA resin can, therefore, be used as a quick step to isolate *M. avium* haemolysin and for differentiation between *M. avium* and *M. tuberculosis* haemolysins.

The exact location of haemolysin within the cell is not known. The difficulty in its solubilisation suggests that it is an integral membrane protein which can be extracted only after disruption of the lipid bilayer by ethanol. Most detergents even in combination with a denaturing agent such as urea were unable to solubilise it. Haemolysin of *M. avium* appears to be a very stable enzyme which retains its activity even after treatment with denaturing agents, detergents and organic solvents. Extended studies on MAC haemolysin, the gene(s) that encode it and its effect(s) on host cells may provide important insights into the pathogenesis and virulence of *M. avium* as haemolysins are important virulence factors among other intracellular pathogens [5, 7].

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