Possible antimicrobial compounds from the pouch of the koala, \textit{Phascolarctos cinereus}

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Summary

The antimicrobial activity of secretions of the pouch of the koala, \textit{Phascolarctos cinereus} has been documented in the presence and absence of young. These secretions have been analysed by polyacrylamide gel electrophoresis, high performance liquid chromatography and mass spectrometry (MS) including time of flight (TOF), MS-MS and Edman sequencing. Gel electrophoresis of reduced samples revealed the presence of a four polypeptides in active and inactive secretions. The sequence data obtained from all four do not appear to be either defensin or cathelicidin-like peptides and show little homology with known antimicrobial protein/peptide sequence available on public access databases. It is proposed that marsupials regulate microbial populations in the pouch via cleavage of large, inactive molecules to produce small active peptides.

Introduction

Marsupials offer a unique biological opportunity to study the early, innate immunological defence strategies of mammals. They are born after a short period of gestation ranging from 15 days in the bandicoot (\textit{Isoodon macrourus}) to 35 days in the koala (\textit{Phascolarctos cinereus}) [1]. At the time of birth they have no functional lymphoid tissue, and subsequent development occurs in the environment of an external pouch laden with micro-organisms [2]. The immunological protection of the young animal during this period relies on a combination of maternal strategies and the development of its own immunocompetence, which occurs during the first three to four months after birth [3].

These documented strategies include pre and post natal transfer of maternal immunoglobulins, high levels of neutrophils in neonatal blood and lymphoid cells in early mammary gland secretions [4, 5, 6, 7]. The possible secretion of antimicrobial substances by the marsupial pouch was first suggested nearly 30 yr ago [8]. Yet, to date, despite tantalising data on changes in bacterial numbers and identity over oes-
In the koala the presence of micro-organisms in the maternal pouch has been linked to the subsequent death of pouch young although this has not been demonstrated in other marsupials [14,2]. To investigate the possible role of antimicrobial secretions in the koala we have undertaken a longitudinal study, covering several breeding cycles, to document and isolate potential antimicrobial compounds. This paper reports on our progress to date.

Methods

Animals

Samples were collected over a three year period from six mature females maintained in a captive breeding population at Taronga Zoo, Sydney. During the period of study all females gave birth to young, one of the females died and one was moved to another zoo.

Sample collection and preparation

Samples of pouch secretions were collected as pouch washes from animals every two to four weeks during the breeding season, from December to May and at eight weekly intervals at other times over the three year study period. The process involved a lavage with 1 ml sterile dH2O, which was stored at -20°C until analysis. Prior to analysis the wash was lyophilized and resuspended in 25-50 μl sterile 0.01% acetic acid. In some animals, it was possible to directly collect secretions from the surface of the pouch using a microcapillary tube.

Antimicrobial assays

Two test organisms were used to assess antimicrobial activity – gram negative Escherichia coli and gram positive Staphylococcus aureus. To prepare the test culture tryptic soy broth (1.5% TSB) was freshly inoculated (1/50) with an overnight culture of the respective test organism and allowed to grow to mid log phase for 2.5 hrs. The culture was centrifuged at 900g at 4°C and washed with cold 10 mM Na PO4 buffer pH 7.4 (NAPB). The cells were then resuspended in NAPB containing 0.03% TSB, 0.02% Tween 20 (NAPBTT) to give an absorbancy at 620 nm of 0.05 (E. coli) and 0.1 (S.aureus). This was determined to give approximately 1.2 x 10^6 CFU ml⁻¹. The assays were undertaken in 96 well plates using 50 μl of bacterial culture. Five to twenty five microlitres of pouch wash concentrate in a total volume of 50 μl NAPBTT was added and the plates incubated, with shaking, for 1 hr at 37°C. After this time 100 μl TSB was added to all wells and the plates were further incubated for 3 hr. Similarly, controls consisting of sterile 0.01% acetic acid and the commercially available antimicrobial, cecropin (Sigma) (0.3 to 12.5 μg ml⁻¹) were prepared. Absorbance was measured at 595 nm and percentage inhibition of growth compared to control wells was recorded.

Sample analysis

Wash concentrates, which displayed antimicrobial activity, were analysed as follows:

**High performance liquid chromatography (HPLC)**
Reverse phase HPLC separation of samples was performed on a Hewlett Packard Series 1100 HPLC with a diode array detector with routine monitoring at 215 and 280 nm. Separation was performed on a Merck Lichrocart 250–4 HPLC cartridge packed with Lichrosphere 100 RP-18 (5 μm) at a flow rate of 1 ml min⁻¹ and a gradient of 1–100% Buffer B over 30 min. Buffer A was 0.1% Trifluoroacetic acid (TFA) in dH2O and Buffer B was 0.1% TFA in acetonitrile.

HPLC was also conducted on samples using the Pharmacia SMART system with a C2/C18 PC3.2/3 column at a flow rate of 250 μl min⁻¹ over 30 min and a gradient of 5–55% Buffer B over 30 mins.

One minute fractions were collected, lyophilised and subjected to gel electrophoresis.

**Gel electrophoresis**
Wash concentrates and HPLC fractions were analysed on 17.5% tris-tricine polyacrylamide gels under both reducing and non-reducing conditions. Protein bands were detected by staining with Sypro Ruby (Biorad) for up to 24 hr and selected components were excised for sequencing.

**Mass spectrometry**
Mass spectrometric (MS) analysis was conducted on samples which underwent a 16 hr in gel tryptic digest at 37°C. The resulting peptides were concentrated and desalted using a C18ZipTip. The samples were analysed by ESI-TOF MS/MS using a Micromass Q-TOF MS equipped with a nanospray source using borosilicate capillaries at a source temperature of 70°C and a drying gas flow rate of 40L/hr. A potential of 850V was applied to the capillary tip combined with a back