A competitive immunosorbent assay for detection of *Pseudomonas pseudomallei* exotoxin

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Summary. The development of monoclonal antibody and enzyme-linked immunosorbent assay (ELISA) techniques has made possible the detection of specific antigens at extremely low concentrations. Diagnosis of recalcitrant diseases such as melioidosis depends upon either early isolation and identification of the causative organism or the identification of a specific marker antigen, *Pseudomonas pseudomallei* exotoxin, in serum; the latter is better because it allows more rapid and simple diagnosis. A method of detecting exotoxin concentrations of > 16 ng/ml by an ELISA based on a monoclonal antitoxin is here described; it is significantly more sensitive than the mouse lethality test (lower threshold $30 \mu\text{g/ml}$) currently in use and an in-vitro cytotoxicity test (lower threshold $10 \mu\text{g/ml}$) that we have developed and describe here.

Introduction

Pseudomonas pseudomallei causes melioidosis, a fulminating and usually fatal disease of man and animals in South East Asia and tropical Australia. The ability of *P. pseudomallei* to produce lethal toxic filtrates has been demonstrated by several investigators (Liu, 1957; Colling *et al.*, 1958; Heckly and Nigg, 1958; Heckly, 1964). The extent to which the toxic product of *P. pseudomallei* is involved in human and animal infections remains unclear. The uncertainty concerning the role of exotoxin in the pathogenesis of melioidosis stems in part from the lack of a simple, precise, highly sensitive test for *P. pseudomallei* exotoxin.

At present, the only assay systems for detecting *P. pseudomallei* exotoxin are bioassays, the most common of which is the mouse lethality test (Liu, 1957). This method is cumbersome, requires large numbers of mice, is at best only semi-quantitative, and so is impracticable for many routine laboratories. Recently, we have partially purified the *P. pseudomallei* exotoxin and used this relatively pure material to develop a serological test for the exotoxin by competitive inhibition of an enzyme-linked immunosorbent assay (ELISA). We have also used this sensitive and specific assay to study the effect of culture conditions and medium on the production of exotoxin by the organism.

Materials and methods

Bacteria

P. pseudomallei was obtained from the Microbiology Department, Faculty of Medicine, National University of Malaysia, Kuala Lumpur. The strain was isolated from a male patient who died of melioidosis. The organisms were propagated on Brain Heart Infusion Agar (BHIA) slants and stored at 4°C .

Determination of bacterial growth

The bacterial cell mass was pelleted by centrifugation of the culture and was resuspended in an equal volume of phosphate-buffered saline, pH 7.4 (PBS). A sample of the resulting suspension was diluted further in PBS to OD_{575} values in the range 0.1–0.8. An OD_{575} of 0.3 represented

Table. Exotoxin production by *P. pseudomallei* in BHIB in various conditions

Conditions of culture and medium	Incubation temperature	Growth—mean dry weight of cells, mg (SEM)	Exotoxin production—ng/ml (SEM)
BHIB	RT	0.76 (0.05)	86 (9)
BHIB + glycerol 2%	RT	0.81 (0.07)	157 (13)
BHIB	37°C	2.83 (0.08)	112 (8)
BHIB + glycerol 2%	37°C	3.07 (0.12)	244 (11)

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BHIB = Brain Heart Infusion Broth; RT = Room Temperature.

a concentration of organisms equal to 0.35 mg (dry weight)/ml.

Isolation of P. pseudomallei exotoxin

P. pseudomallei was grown in Brain Heart Infusion Broth (BHIB) containing mucin 1% and glycerol 2% at 37°C in static conditions for 7 days. The culture was harvested by centrifugation at 32 000 *g* for 40 min (Sorvall RL-53 Superspeed; 14 000 rpm, Rotor GSA). The resultant supernate was centrifuged at 55 000 *g* for 40 min (Beckman L 3-50 Ultracentrifuge; 30 000 rpm, Rotor 50.2 Ti). The supernate was then membrane-filtered (0.45 µm; Millipore) to remove any remaining cells. The cell-free filtrate was dialysed twice against PBS for 24 h. The resulting crude toxin extract was concentrated to a volume of 20–30 ml through an ultra-filtration unit (Cole Palmer), at a cut-off point of 10 000 mol. wt, with a nitrogen pressure of not more than 20 p.s.i. At the end of the concentration step, the sample was lyophilised and the powder stored at –20°C. In the final step of exotoxin isolation, the lyophilised sample was resuspended in PBS and applied to a G100 Sephadex column. The single peak that showed toxic activity in the mouse lethality test was collected and stored at –20°C. SDS-polyacrylamide gel electrophoretic (SDS-PAGE) analysis showed only one major band at mol. wt *c.* 31 000 (Ismail *et al.*, in press).

Test of toxicity

Toxicity of the exotoxin preparation was determined at each stage of the purification by intraperitoneal injection of 0.5 ml of serially diluted test samples into groups of mice. Mortality was recorded for 7 days and the LD₅₀ was calculated by the method of Reed and Muench (1938).

Antiserum production

Mice were immunised with a solution of exotoxin preparation 1.0 mg/ml mixed with an equal volume of Freund's complete adjuvant. The immunisation schedule used was that described by Galfré and Milstein (1981). Mice were test-bled routinely and those producing high titres were selected for hybridoma production. Sera from exsanguinated mice were collected and used as positive controls in subsequent assays.

Hybridoma production was by a modification of the polyethylene glycol technique of Kohler and Milstein (1975). The modifications were based on those outlined by Fazekas de St Groth and Scheidegger (1980). Antibody-producing hybrids were selected by cloning in soft agar and the clones expanded *in vitro*. Spent culture medium containing antibody activity was used in all assays.

ELISA method

The ELISA was performed by a modification of the method of Voller *et al.* (1976). Polystyrene flat-bottomed 96-well microtitration plates were used for the assay (NUNC Immunoplate II, Gibco Laboratories, Grand Island, NY, USA). Wells were coated with 100 µl of purified exotoxin (0.5 µg/ml) in carbonate-bicarbonate binding buffer, 0.1 M, pH 9.6. After overnight incubation at 4°C, the plates were washed five times with PBS containing Tween 80 0.05% (PBST) and stored at 4°C. A 100-µl sample of antiserum diluted in PBS was added to each appropriate well and the plates were incubated, with shaking, at room temperature for 30 min. After incubation, the plates were washed five times with PBST and 100 µl of peroxidase-labelled rabbit anti-mouse immunoglobulin G (Sigma Chemical Co., St Louis, MO, USA) diluted 1 in 200 in PBST was added. The plates were incubated for 20 min with shaking at room temperature, washed five times with PBST after which 100 µl of substrate solution was added. The substrate solution was H₂O₂ 0.012% in citrate buffer (pH 5.0) containing 2,2'-azino-bis (3-ethylbenz-thiazoline sulphonic acid (ABTS) 6 mg/ml. Reaction mixtures were incubated with shaking at room temperature for 10 min. Control wells were treated identically, except that PBS replaced the serum. The OD was read at 405 nm in a Microelisa Reader (Dynatech).

Competitive ELISA Test

A competitive ELISA test was performed by the method of Yolken *et al.* (1977). A dilution of the test serum in PBS was selected that gave an absorbance value of 1.00 to 1.200 in wells coated with exotoxin. The serum was preincubated with increasing concentrations of exotoxin or lipopolysaccharide for 30 min at 37°C, and then tested in the ELISA system. The reduction in absorbance by the addition of exotoxin was expressed as a percentage of the OD reading for a negative control. To determine exotoxin production in a culture of *P. pseudomallei*, 100 µl of cell-free supernate of a 48-h broth culture was preincubated with the test serum and then used in the ELISA. Control wells contained test serum preincubated with broth culture that had been inoculated with non-viable formalin-treated *P. pseudomallei*.

Cytotoxicity Assay

Cell-culture techniques were similar to those previously described (Allen *et al.*, 1979). EB2 cells were seeded in 96-well flat-bottomed tissue culture plates and allowed to grow for 24 h at 37°C in an atmosphere of air + CO₂ 5%. All tissue-culture media contained fetal calf serum, L-glutamine and antibiotics (Gibco Lab., Grand Island, NY, USA). After 24 h, increasing concentrations of exotoxin were added and the plates were incubated overnight. Radionucleotides and labelled amino acid (Radiochemical Centre, Amersham) were added to

appropriate wells. After incubation for 4 h, the cultures were harvested by a Titertek cell harvester and radioactivity determined by liquid scintillation counting. All cultures were tested in quadruplicate. To determine the effect of exotoxin on thymidine uptake by cells during the early period of incubation, cultures were harvested at hourly intervals after the addition of exotoxin.

Results

Lethal toxicity in mice

Preliminary studies were performed to determine the LD₅₀ for mice of the purified exotoxin preparations. The LD₅₀ of different batches was fairly uniform; the mean value was 29.5 ± 8.0 μ g of protein.

Titration of hybridoma supernate (CB4)

Titration studies with hybridoma supernate showed a linear relationship between supernate dilution and net OD (fig. 1). The supernate from the hybridoma culture (CB4) gave a high titre with *P. pseudomallei* exotoxin in a direct ELISA for antibody to toxin. Indeed, a significant positive ELISA reaction was observed even at dilutions of 1.6×10^4 , when the minimal usable absorbance value was fixed at a reading equivalent to twice that

Fig. 1. ELISA reactivity (OD₄₀₅) at serial twofold dilutions of clone CB4 anti-exotoxin hybridoma supernate (—●—) and adsorbed supernate (—○—).

obtained from negative control assays. The reactivity of antibody-containing supernates was unaffected by pre-absorption with bacterial growth medium (BHIB, mucin 1% and glycerol 2%).

The titration results showed that the optimum antibody dilution for use in an inhibition assay for exotoxin would be 1 in 2000. This dilution was then used in all our studies and a positive control at this dilution was included in every test plate, giving a standard reference value for all the results.

Competitive ELISA for *P. pseudomallei* toxin

Fig. 2 shows the results of a typical competitive ELISA in which *P. pseudomallei* exotoxin was used as the competitor antigen. The binding of the positive control supernate was inhibited 94% by purified exotoxin at a concentration of 50 μ g/ml. Similar concentrations of lipopolysaccharides of *Salmonella* sp. and *E. coli* produced inhibitions of 5–17%. Pre-incubation of the positive control supernate with bacterial growth medium also failed to cause a decrease in ELISA reactivity in this system.

Exotoxin levels in culture supernates

The table shows exotoxin production by *P. pseudomallei* grown under different conditions. The competitive ELISA technique was used to determine the concentration of exotoxin in culture supernates. The amount of exotoxin was calculated from a standard curve generated for each microtitration plate by plotting the absorbance against

Fig. 2. Quantitative inhibition of ELISA reactivity by purified exotoxin (—●—), *Salmonella* sp. lipopolysaccharide (—△—), *E. coli* lipopolysaccharide (—▲—) and BHIB + glycerol + mucin (—○—). The reference hybridoma supernate was used at a dilution of 1 in 2000.

concentrations of purified exotoxin added to wells (50 ng–1.0 µg/well). Exotoxin production by *P. pseudomallei* was enhanced in the presence of glycerol 2%. The addition of glycerol significantly increased the production of exotoxin in static cultures at room temperature and at 37°C. This enhancement of exotoxin production was not due to a difference between growth rates because the addition of glycerol to the growth medium did not significantly increase the dry-weight yield of the cultures. However, significantly different growth rates and exotoxin production were observed in cultures grown at room temperatures and at 37°C. Growth and exotoxin production were increased in cultures at 37°C.

Correlation between competitive ELISA and tissue-culture cytotoxicity assay

The titration curves for the exotoxin obtained by ELISA and by cytotoxicity tests are shown in fig. 3. Different concentrations of purified exotoxin were tested and there was close correlation of ELISA reactivity with cytotoxicity as determined by the effects on EB2 cells in culture. In the competitive ELISA test, inhibition of reactivity was observed at exotoxin concentrations as low as 16 ng/ml. However, cytotoxicity, as measured by radio-labelled thymidine incorporation was not observed until the concentration of exotoxin was *c.* 2.0 µg/ml. Above this threshold concentration, increases in exotoxin concentration to 10 µg/ml and 50 µg/ml

resulted in 50% and 90% inhibition of ³H-thymidine uptake respectively.

Discussion

Our results show that *P. pseudomallei* exotoxin can be measured by a competitive ELISA technique. The specificity of the assay for anti-exotoxin antibodies is confirmed here and elsewhere (Ismail *et al.*, in press) and is supported by the fact that the exotoxin preparation used for coating the micro-titration plates showed a single major protein band on SDS-PAGE. The competitive ELISA reported here showed that anti-exotoxin activity could not be inhibited by purified lipopolysaccharides of *E. coli* or *Salmonella* sp., confirming that the exotoxin preparation was not significantly contaminated with bacterial endotoxins. Growth medium initially seeded with non-viable *P. pseudomallei* also failed to show any effect on the ELISA reactivity, further confirming that our antibody preparations were specific for the active exotoxin and that the antibody did not cross-react with other antigens present in cell pellets or culture broth.

Our studies also show that the assay could be used to detect exotoxin in culture supernates. Exotoxin production in cultures was enhanced in the presence of glycerol. The increase in exotoxin concentration was clearly not due to an increase in bacterial growth. This lack of correlation between bacterial growth and toxin production implies that the lethal activity observed in all toxin-positive culture supernates was not due to endotoxin because the yield of lipopolysaccharide should parallel bacterial growth. Whether the increased exotoxin concentration observed in growth medium containing glycerol was due to the specific induction of exotoxin synthesis by the cells or to increased secretion of intracellular exotoxin into the surrounding medium is not clear at present. Enhancement of *P. aeruginosa* exotoxin A production in the presence of exogenously added glycerol has been reported previously (Liu, 1973). The availability of the competitive ELISA technique described here for quantification of *P. pseudomallei* exotoxin should provide a sensitive and specific tool for future studies of the factors influencing, and mechanisms of, exotoxin production by this organism.

We have also shown that the competitive ELISA procedure for exotoxin measurement compares favourably in sensitivity and specificity with that of an in-vitro cytotoxicity assay. Indeed the ELISA technique described was considerably more sensitive than the toxicity assay in detecting toxin levels

Fig. 3. Relationship between ELISA reactivity (—●—) and cytotoxic activity (—○—) for exotoxin.

of >16 ng/ml, compared with the lower detection limit of the cytotoxicity assay of >10 µg/ml.

The role of exotoxin in the pathogenesis of *P. pseudomallei* infection is not yet clear. If the toxin is responsible for the lethal effects of melioidosis, a survey of the distribution of toxigenic strains of *P. pseudomallei* in its natural environment could provide important information about the epidemiology, dissemination and mode of infection of melioidosis in endemic regions. Moreover, the ELISA technique described here could be routinely used to determine the occurrence of toxin-producing strains in clinical isolates and further clarify the role of exotoxin in the pathogenesis of melioidosis.

Most cases of melioidosis have been reported in developing countries such as Burma, Malaysia and Vietnam where facilities for a comprehensive

survey on the distribution of *P. pseudomallei* are limited. The ELISA method offers several advantages to such countries. The reduced cost and convenience of performing this assay in microtitration plates make it potentially useful as a research tool in screening for non-toxigenic strains or mutants of *P. pseudomallei* for future vaccine development. ELISA is sensitive and specific and uses stable and inexpensive reagents and simple equipment. Once appropriate conditions are set, the assay may be performed simply and reproducibly.

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REFERENCES

- Allen J C, Smith C J, Hussain J I, Thomas J M, Gaugas J M 1979 Inhibition of lymphocyte proliferation by polyamines requires ruminant-plasma polyamine oxidase. *European Journal of Biochemistry* **102**: 153–158.
- Colling M, Nigg C, Heckly R J 1958 Toxins of *Pseudomonas pseudomallei*. I. Production *in vitro*. *Journal of Bacteriology* **76**: 422–426.
- Fazekas de St Groth S, Scheidegger D 1980 Production of monoclonal antibodies: strategy and tactics. *Journal of Immunological Methods* **35**: 1–21.
- Galfrè G, Milstein C 1981 Preparation of monoclonal antibodies: strategies and procedures. In: Langone L J, Van Vunakis H (eds) *Methods in enzymology* 73. Academic Press, New York, pp. 3–46.
- Heckley R J 1964 Differentiation of exotoxin and other biologically active substances in *Pseudomonas pseudomallei* filtrates. *Journal of Bacteriology* **88**: 1730–1736.
- Heckley R J, Nigg C 1958 Toxins of *Pseudomonas pseudomallei*. II. Characterization. *Journal of Bacteriology* **76**: 427–436.
- Ismail G, Allen J C, Smith C J Enzyme-linked immunosorbent assay for antibody to *Pseudomonas pseudomallei* exotoxin (in press).
- Kohler G, Milstein C 1975 Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* **256**: 495–497.
- Liu P V 1957 Survey of hemolysin production among species of pseudomonads. *Journal of Bacteriology* **74**: 718–727.
- Liu P V 1973 Exotoxins of *Pseudomonas aeruginosa*. I. Factors that influence the production of exotoxin A. *Journal of Infectious Diseases* **128**: 506–513.
- Reed L J, Muench H 1938 A simple method of estimating fifty percent end points. *American Journal of Hygiene* **27**: 493–497.
- Voller A, Bidwell D, Bartlett A 1976 Microplate enzyme immunoassays for the immunodiagnosis of virus infections. In: Rose N, Friedman H (eds) *Manual of clinical immunology*. American Society for Microbiology, Washington, pp. 506–512.
- Yolken R H, Greenberg H B, Merson M H, Sack R B, Kapikian A Z 1977 Enzyme-linked immunosorbent assays for detection of *Escherichia coli* heat-labile enterotoxin. *Journal of Clinical Microbiology* **6**: 439–444.