CHARACTERIZATION OF *PSEUDOMONAS PSEUDOMALLEI* ANTIGENS BY SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS AND WESTERN BLOT

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Abstract. Immunological characterization of various *Pseudomonas pseudomallei* preparations was carried out by SDS-PAGE and Western blot using sera from infected humans and from patients with other bacterial infections. Somatic (SOM) and partially purified cell extracts (PCE) gave more complex SDS-PAGE patterns: Mr ranged from 86 to 12.7 and 48 to 10 kDa, respectively. The culture-filtrated antigens (CF) from 3 different kinds of synthetic media consisted of fairly simple profiles with common bands Mr of 40, 26 and 16 kDa. PCE and CF reacted specifically with infected human sera; SOM did not. The components with Mr of 40 kDa in CF reacted consistently with all infected sera but failed to react with sera infected with *Escherichia coli, Enterobacter* spp., *Klebsiella pneumoniae, Proteus mirabilis, Salmonella* spp., *Staphylococcus aureus, Streptococcus* spp., *Pseudomonas aeruginosa* and *P. stutzeri.* This peptide was demonstrated to be a major component in CF thus suggesting its potential for development of immunodiagnostic methods for melioidosis.

INTRODUCTION

Melioidosis is an infectious disease caused by a gram negative bacillus, *Pseudomonas pseudomallei.* The disease is endemic in Southeast Asia and northern Australia (Ashdown and Guard, 1984; Howe *et al,* 1971; Patamasucon *et al,* 1982). However, sporadic cases have also been reported recently from Korea, Hong Kong, Sri Lanka, India, Iran, Turkey, England, France, Africa, the former USSR and the United States (Ashdown and Guard, 1984; Leelarasamee, 1986; Patamasucon *et al,* 1982). The causative organisms are natural residents of soil, stagnant water and rice paddies (Ellison *et al,* 1969; Strauss *et al,* 1969). Infection is usually acquired from contamination through contact of pre-existing skin abrasions or ulcers with infectious soil or water or by inhalation of infectious dust particles. The clinical features of this disease are protean and can mimic other diseases, such as plague, tuberculosis, leptospirosis, malaria, typhoid fever and several common bacterial infections (Poe *et al,* 1971). The definitive diagnosis is made after isolation of the organism from clinical specimens, which requires a minimum of 48 hours even in experienced hands. Since the acute septicemic form has a mortality rate of 80-90% with death occuring 24-28 hours after onset, an early diagnosis on admission is critical (Chaowagul *et al,* 1989). Attempts have been made in the past to develop sensitive and specific immunodiagnostic methods but considerable cross-reactivity with other bacterial infections remains to be eliminated (Alexander *et al,* 1970; Appassakij *et al,* 1990; Ashdown, 1981a, b; Ashdown *et al,* 1989; Khupulsup and Petchclai, 1986; Kunakorn *et al,* 1990; Leelarasamee, 1985; Nigg, 1963) In addition, the interpretation of the tests is hampered by elevated titers of antibodies in normal individuals and is sometimes negative in patients with acute septicemic melioidosis (Ashdown, 1981b; Ashdown *et al,* 1989; Chaowagul *et al,* 1989; Khupulsup and Petchclai, 1986; Leelarasamee, 1985).

The development of reliable immunodiagnostic methods for any disease requires a systematic analysis of the antigens and a full understanding of the host-parasite relationship. Although different kinds of *P. pseudomallei* antigens, such as soluble crude extract, culture-filtrated and lipopolysaccharide antigens were used in immunodiagnostic tests, the complexity of these antigens has never been characterized (Appassakij *et al,* 1990;

Ashdown *et al*, 1989; Khupulsup and Petchclai, 1986; Kunakorn *et al*, 1990). The purpose of the present study was to analyse and identify various *P. pseudomallei* components from somatic, partially purified and culture-filtrated antigens that may have potential for immunodiagnosis of this disease.

MATERIALS AND METHODS

Bacterial strain and human sera

Since our preliminary data demonstrated that different *P. pseudomallei* isolated from patients with various clinical profiles had similar somatic, partially purified cell extract and culture-filtrated antigen patterns on SDS-PAGE, the *P. pseudomallei* strain used was thus selected from a patient with disseminated septicemic melioidosis admitted to Srinagarind Hospital (Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand). The organism was identified by colonial morphology, gram staining and biochemical reactions (Gilardi, 1985).

Positive sera used in this study were collected from proven melioidosis patients. The disease was identified by signs and symptoms consistent with melioidosis and also by the recovery of *P. pseudomallei* from clinical specimens. Sera from negative controls were obtained from blood donors at the Central Blood Bank, Srinagarind Hospital. Sera from individual patients whose hemocultures were positive for either *Escherichia coli, Enterobacter* spp., *Klebsiella pneumoniae, Proteus mirabilis, Salmonella* spp., *Staphylococcus aureus, Streptococcus* spp., *P. aeruginosa* or *P. stutzeri* were also included in the study in order to examine for cross-reactivity with *P. pseudomallei* antigens.

Antigen preparations

Somatic antigens (SOM): Soluble crude extract antigen was prepared as described by Bocter *et al* (1989), with some modification. *P. pseudomallei* was cultured on trypticase soy agar (TSA, Difco, Detroit, Michigan, USA) for 24 hours at 37°C. The confluent surface growth was harvested and finally resuspended in sterile 0.85% NaCl. The suspension was then sonicated in the presence of 0.1 mM phenyl-methyl sulfonylfluoride (PMSF, Sigma Chemical Co, St Louis, MO, USA) and 0.1 mM L-1 tosylamide 2-phenylethyl chloromethylketone (TPCK, Sigma) in an ice-bath by an ultrasonic disintegrator (Soniprep 150, MSE Scientific Inc, Sussex, England) at an amplitude of 18 microns for 10 minutes. The disrupted cells were removed by centrifugation at 12,800*g* for 30 minutes at 4°C (J2-21 centrifuge, Beckman, Palo Alto, CA, USA). After filtration through a 0.45 μm membrane filter, the supernatant fluid was then stored in aliquots at-20°C until used.

Partially purified cell extract antigens (PCE): The method to be used was carried out in a similar manner as described by Barber *et al* (1966). In brief, a single identified colony of *P. pseudomallei* was inoculated into trypticase soy bean (TSB, Difco) and incubated at 37°C with continuous shaking. After 6 hours, a subculture was made overnight at 37°C to TSA slant. The bacterial cells were harvested with sterile 0.85% NaCl and then washed and dried with acetone. Acetone-dried cells were extracted with 0.15 M Veronal buffer, pH 8.4, with a ratio of 3.5 g dried cells per 50 ml of buffer. The supernatant obtained was dialyzed and further purified by precipitation with 10% trichloroacetic acid. The pellet was then resuspended and dialyzed extensively against 0.01 M phosphate buffer saline (PBS), pH 7.2, at 4°C. After filtering sterilization, the antigen was stored in small aliquots at -20°C until used.

Culture-filtrated antigens (CF): The culture-filtrated antigens were prepared separately in 3 different types of synthetic media: glycine broth medium (GB), modified Proskauer and Beck medium (MPB) and Rice, Kohst and Duthie medium (RKD) (Alexander *et al*, 1970; Rice *et al*, 1951; Youmans, 1979). The cultures were incubated for 3 weeks at 37°C. In order to obtain the optimal time for culturing, the bacterial growth rate and the antigen production were also determined at time intervals by bacterial colony counts and protein and carbohydrate assays (Lowry *et al*, 1951; Scott and Melvin, 1953) in the supernatant culture. The culture-filtrated antigens were collected by centrifugation at 12,800g, 4°C for 20 minutes (Beckman) and filtered through a 0.45 μm membrane. The antigens obtained were then dialyzed thoroughly against three changes of PBS and kept frozen at -20°C until used.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Slab gel electrophoresis was performed in the

presence of 1% (wt/vol) sodium dodecyl sulphate (SDS) as described by Laemmli (1970). The protein bands were separated using SDS- PAGE (12% wt/vol acrylamide in the running gel and 4% in the stacking gel) under reducing conditions at 30 mA per gel for 3 hours and stained with silver nitrate (Merril *et al,* 1981). The relative molecular weights of electrophoretically separated components were determined by comparison with standard markers (Segrest and Jackson, 1972; Weber and Osborn, 1969).

Western blot immunoassay

After the antigen samples were fractionated by SDS-PAGE, the resolved proteins were electrotransferred to nitrocellulose membrane (Hoefer Scientific Instruments, San Francisco, CA, USA) using the semi-dry blotter (TE 70 Apparatus, Hoefer) as described by Kyhse-Andersen (1984) for 1.5 hours using 1 mA/cm^2. The non-specific binding sites were then blocked by incubating the membrane with 5% skimmed milk in 0.02 M Tris-HCl and 0.5 M NaCl pH 7.5 (TBS) overnight at 4°C. Individual strips (5 mm wide) were excised vertically and incubated with tested sera (at a dilution of 1:100 in TBS containing 1% skimmed milk) for 2 hours at 37°C. The strips were then washed five times with 0.85% NaCl. Horseradish peroxidase conjugated rabbit anti-human IgM, IgA, IgG, kappa and lambda (Dakopatts a/s, Glostrup, Denmark), diluted 1:1000 in TBS containing 1% skimmed milk, was allowed to react with the strips for 2 hours at 37°C. The strips were then washed and dried prior to being developed by addition of chromogenic substrate solution containing 35 mg of 4-chloro-1-naphthol (Sigma) in 7 ml of absolute methanol mixed with 20 µl of 30% H_2O_2 in 63 ml of TBS.

RESULTS

Effects of different synthetic media on bacterial growth and amount of antigen production

To identify the suitable synthetic media for *P. pseudomallei* growth and antigen production, the viable bacterial counts and protein and carbohydrate contents in the culture-filtrate were determined at time intervals. The results demonstrated that RKD (Rice, Kohst and Duthie medium) provided for the maximum and most rapid growth of bacteria when compared with others (data not shown). However, the bacteria will grow up to the stationary phase within 2-3 days regardless of the media used. When protein and carbohydrate contents in culture-filtrate were determined, RKD was still the medium that resulted in maximum bacterial antigen production (data not shown). The protein and carbohydrate peaks in RKD were reached during the third week whereas the protein and carbohydrate peaks of those in MPB (Modified Proskauer and Beck medium) were reached during the second. The GB (glycine broth medium) which contained only glycine, 0.2% of dextrose and low concentrations of NaCl and Na_2HPO_4 salts, provided very low protein and carbohydrate contents (data not shown).

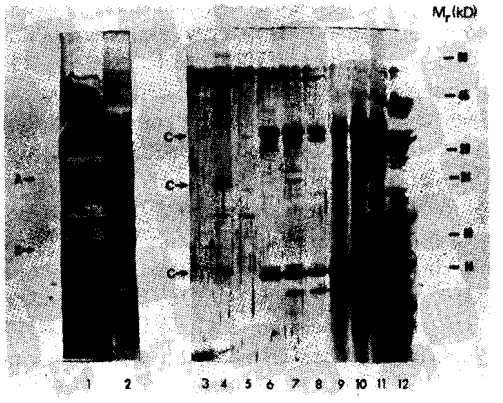

Fig 1—Silver nitrate stained SDS-PAGE profiles of partially purified cell extract (PCE, lanes 1 and 12), somatic antigen (SOM, lane 2) and culture-filtrated antigens (CF) which were prepared from glycine broth medium (GB, lanes 3, 4 and 5), modified Proskauer and Beck medium (MPB, lanes 6, 7 and 8) and Rice, Kohst and Duthie medium (RKD, lanes 9, 10 and 11) for 7 days (lanes 3, 6 and 9), 14 days (lanes 4, 7 and 10) and 21 days (lanes 5, 8 and 11). Arrows A and B indicate the major components of PCE and SOM respectively whereas arrows C indicate the common components of CF.

Antigenic profiles of *P. pseudomallei*

The protein components in various *P. pseudomallei* preparations were analyzed by SDS-PAGE and stained with silver nitrate. The results are shown in Fig 1. The molecular weights of SOM and PCE ranged from 86 to 12.7 and 48 to 10

kDa, respectively. The SOM consisted of a major component with an Mr of 21.8 kDa, whereas that of PCE was 34 kDa (Fig 1, lanes 1, 2 and 12). The staining pattern of the soluble somatic extract was shown to be considerably more complicated than that of PCE. It consisted of proteins with high molecular weight when compared to PCE. Unlike the somatic extract and partially purified cell extract, the culture-filtrated antigens prepared from different synthetic media exhibited simple SDS-PAGE patterns (Fig 1). Although the numbers of polypeptides detected in GB, MPB and RKD were different, with the molecular weight ranging from 67 to 14, 61 to 14 and 47 to 14 kDa, respectively (Fig 1, lanes 3-11), all of them contained common components with Mr of 40, 26 and 16 kDa (Fig 1, arrows C). The SDS-PAGE profile of antigens prepared in MPB for 14 days was clearly separated and contained most of the proteins appearing in other media. Because of the low protein concentration in antigens from GB and the high carbohydrate content in that from RKD, the characterization of culture-filtrated antigens was carried out by using antigens prepared from MPB medium which had been cultured for 14 days.

Antigenic characterization of *P. pseudomallei*

Our preliminary data demonstrated that SOM of *P. pseudomallei* from 25 isolates gave similiar SDS-PAGE patterns. These results were also observed when those of PCE and CF were compared (data not shown). Therefore, the antigenicity of these antigens was further analyzed using only one *P. pseudomallei* isolate against sera from patients with proven melioidosis; the results are shown in Fig 2-4. The polypeptide components in SOM shown to react with antibodies from pooled positive sera had Mr of 70, 55.5, 47, 41, 37.5, 36, 31.5, 30, 25.3, 23, 19.5, 13.5 and 12.7 kDa (Fig 2, lane 1). However, when this antigen preparation was allowed to react with normal human sera, some degree of non-specific reaction with peptides having Mr of 70, 37.5, 31.5 and 30 kDa occurred (Fig 2, lane 2). Results obtained with a series of human sera from individuals known to be infected with *P. pseudomallei* showed some variation in the existence and the intensity of various bands, but the most consistent finding was the presence of antibodies reactive with the 19.5 kDa component (Fig 2, arrow). Unfortunately, this component was considerably cross-reactive with serum from a patient with *Streptococcus* spp. (Fig 2, lane 17).

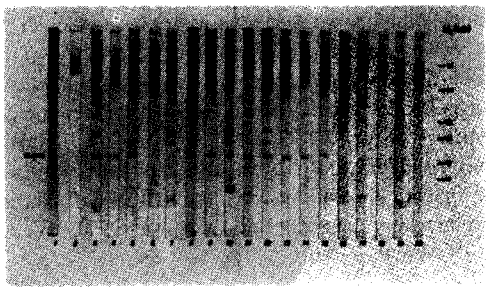

Fig 2—Western blot patterns of *Pseudomonas pseudomallei* somatic antigens (SOM) reacted with pooled *P. pseudomallei* infected human sera (lane 1), pooled normal human sera (lane 2), individual sera from patients with proven melioidosis (lanes 3-15) and individual sera from patients with other bacterial infections caused by *Klebsiella pneumoniae* (lane 16), *Streptococcus* spp (lane 17) and *Staphylococcus aureus* (lanes 18-20). Arrow indicates that the 19.5 kDa component reacted with almost all infected sera tested. Relative molecular weights (Mr) of standard markers are shown.

The antigenic patterns reactive with positive and negative human sera when PCE were analyzed again showed some non-specific interactions with the negative controls (Fig 3). The specific components of PCE reacting with almost all positive sera from individual patients had Mr of 38, 34, 20.5 and 19 kDa (Fig 3). However, the bands with Mr of 38 and 34 kDa still had some weak reactions with sera from other bacterial infections. The 20.5 and 19 kDa antigens therefore appeared to be specific for *P. pseudomallei,* as they reacted only faintly or totally failed to react with sera from other bacterial infections (Fig 3).

As with the protein-staining patterns, a less complicated reactive antigenic pattern was noted with CF when reacting with sera from melioidosis patients. The specific antibodies reacted with the bands with Mr of 40, 39, 30, 29 and 19 kDa (Fig 4). The most consistent band appearing in the CF which reacted with both pooled and individual patient sera was the 40 kDa polypeptide (Fig 4).

DISCUSSION

Different immunological methods, such as complement fixation, indirect hemagglutination

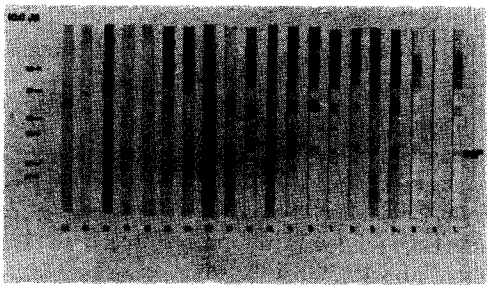

Fig 3—Western blot pattern of *Pseudomonas pseudomallei* partialy purified cell extract (PCE) reacted with pooled *P. pseudomallei* infected human sera (lane 1), pooled normal human sera (lane 2), individual sera from patients with proven melioidosis (lanes 3-15) and individual sera from patients with other bacterial infections caused by *Staphylococcus aureus* (lanes 16-18), *Klebsiella pneumoniae* (lane 19) and *Streptoccocus* spp (lane 20). Arrows indicate the 19 and 20.5 kDa components which reacted consistently and specifically with *P. pseudomallei* positive sera. Relative molecular weights (Mr) of standard markers are shown.

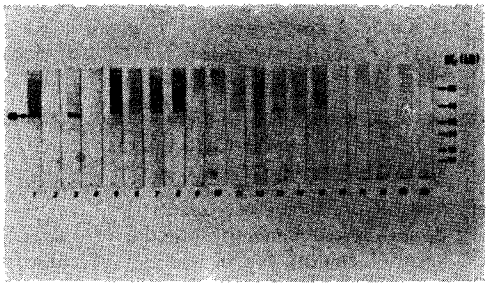

Fig 4—Western blot pattern of *Pseudomonas pseudomallei* modified Proskauer and Beck medium (MPB) antigens reacted with pooled *P. pseudomallei* infected human sera (lane 1), pooled normal human sera (lane 2), individual sera from patients with proven melioidosis (lanes 3-15) and individual sera from patients with other bacterial infections caused by *Staphylococcus aureus* (lanes 16-18), *Klebsiella pneumoniae* (lane 19) and *Streptoccocus* spp (lane 20). Arrow indicates the 40 kDa component reacted specifically with *P. pseudomallei* positive sera. Relative molecular weights (Mr) of standard markers are shown.

(IHA), indirect fluorescent antibody and enzyme-linked immunosorbent assay (ELISA), have been employed in attempts to develop a method for the diagnosis of *P. pseudomallei* infection (Alexander *et al,* 1970; Appassakij *et al,* 1990; Ashdown, 1981a, b, Ashdown *et al,* 1989; Khupulsup and Petchclai, 1986; Kunakorn *et al,* 1990). Recently, the combined use of IHA and IgM-ELISA was reported to give the most sensitive and most specific serodiagnosis (Kunakorn *et al,* 1990). Since the antigens used in various methods varied, the interpretation of the results was not comparable. However, there are no data concerning the specificity of the antigen. In order to improve specificity of the reaction for immunodiagnostic purposes, one must have a more purified antigen for antibody detection because the crude antigens used in previous studies cross-reacted with some other bacterial infections (Ashdown *et al,* 1989; Khupulsup and Petchclai, 1986; Kunakorn *et al,* 1990). To study the protein components in each of several *P. pseudomallei* antigen preparations, a systematic analysis of *P. pseudomallei* antigens was carried out by SDS-PAGE and Western blot. The results of this study showed that all of the antigens used could provoke an immune response in melioidosis patients. The SOM contained more than 40 polypeptides, with few components shown to be immunogenic. However, some of these components also reacted with normal sera and sera from individuals infected with unrelated bacteria (Fig 2). The presence of non-specific or cross-reactive antigens may explain why immunological tests developed in the past, using this kind of antigen, gave high titers of antibodies in healthy blood donors or in other bacterial infections (Ashdown, 1981b; Ashdown *et al,* 1989). Therefore, the findings of very high antibody titers to this antigen in some healthy controls from endemic areas, who had been interpreted as having subclinical infections, may be misinterpretations (Appassakij *et al,* 1990; Ashdown, 1981b; Ashdown *et al,* 1989; Khupulsup and Petchclai, 1986; Kunakorn *et al,* 1990; Nigg, 1963). Moreover, these findings affect the variation in the cut-off level of the titer for seropositivity in various epidemiological surveys. The variations have to be considered before the results are interpreted. From the above data, it is clear that investigators should be aware of cross-reactions before reaching any conclusion concerning the significance of the serological test results using this kind of antigen.

Unlike the somatic antigen, partially purified cell extract antigen was found to be considerably more specific. It appeared that bands with Mr of 20.5 and 19 kDa reacted with almost all patient sera tested and were shown to be *P. pseudomallei* specific antigens. These two bands did not react with serum from a patient with *Streptococcus* spp whereas the 19.5 kDa of SOM did, so that the 20.5/19 kDa of PCE and the 19.5 kDa of SOM appear not to be the same molecule although their molecular weights on SDS-PAGE were similar. However, the 20.5 and 19 kDa bands were found to be present in small quantities in PCE stained by silver nitrate. This makes it more difficult to purify them for use as specific antigens for the development of immunodiagnostic methods.

Analysis and interpretation of the data obtained with the culture-filtrated antigens was fairly simple. The major components were the 26 kDa for GB and 40, 16 kDa for both MPB and RKD. The immunogenic component in MPB that reacted strongly with all immune sera employed was 40 kDa. Since this peptide may have a potential to be used as a specific antigen for immunodiagnosis, the specificity of this component was further clarified. It failed to react with sera from patients infected with *K. pneumoniae, S. aureus, Streptococcus* spp, *E. coli, Enterobacter* spp, *Proteus mirabilis, Salmonella* spp, *P. aeruginosa* and *P. stutzeri* (data not shown). Although the immunological assay was done using the antigens produced only from MPB, whether the 40 kDa component that appeared in RKD and MPB are antigenically identical remains to be investigated. However, MPB is still a suitable medium for *P. pseudomallei* antigen production because it provides a less complicated protein profile, thus making it easier to be purified.

Altogether, the results presented in this study showed that PCE and CF contained proteins that reacted specifically with *P. pseudomallei.* The 40 kDa protein of CF is a potent immunogen and could be used for immunodiagnosis, although its antigenic specificity has not been fully characterized. The above data clearly demonstrate that it reacted with sera from *P. pseudomallei* infections but not with sera from individuals with other bacterial infections tested. We are now in the process of isolating this component as a possible candidate for development of specific immunodiagnostic assays. In addition, cloning of the corresponding gene in *E. coli* has also been performed.

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