INFECTION AND IMMUNITY, Oct. 1997, p. 4319–4321 Vol. 65, No. 10
0019-9567/97/$04.00+0

Arabinose Assimilation Defines a Nonvirulent Biotype of *Burkholderia pseudomallei*

M. D. SMITH,[1,2]† B. J. ANGUS,[1,2] V. WUTHIEKANUN,[1] AND N. J. WHITE[1,2]*

Faculty of Tropical Medicine, Mahidol University, Bangkok 10400, Thailand,[1] and Centre for Tropical Medicine, Nuffield Department of Clinical Medicine, John Radcliffe Hospital, Oxford University, Oxford OX3 9DU, United Kingdom[2]

Received 31 January 1997/Returned for modification 20 March 1997/Accepted 28 July 1997

Two distinct types of *Burkholderia pseudomallei*, differentiated by the ability to assimilate L-arabinose but with similar morphologies and antigenicities, can be isolated from soil in Thailand. Approximately 25% of soil isolates from northeast Thailand were arabinose assimilators (Ara^+), but in 1,200 sequentially studied patients, only arabinose "nonassimilators" (Ara^-) caused melioidosis ($P < 0.0001$). In a murine model, there was a striking difference in virulence between Ara^- and Ara^+ *B. pseudomallei*. The mean (standard deviation) 50% lethal dose (LD_{50}) inoculum for Ara^- isolates was 182 (111) CFU/mouse compared with approximately 10^9 CFU/mouse for Ara^+ soil isolates. There was no significant difference between the LD_{50}s for clinical and soil Ara^- isolates. All attempts to convert the biochemical phenotype by selective culture failed, which suggests that the biotype is stable.

Burkholderia (formerly *Pseudomonas*) *pseudomallei*, an environmental saprophyte, causes melioidosis in mammals and birds. *B. pseudomallei* has been isolated from the environment throughout the tropics, but infection in humans occurs only in eastern Asia and northern Australia (3), where *B. pseudomallei* is endemic. In northeastern Thailand, *B. pseudomallei* is a major source of morbidity and mortality, causing one-fifth of all community-acquired septicemias (2). However, *B. pseudomallei* is also found in soil from other regions of Thailand, where clinical disease is rare (9, 10). In northeast Thailand, two types of soil isolates which react similarly in the indirect hemagglutination assays used for melioidosis serology have been identified, but they have significant phenotypic and genotypic differences and are readily distinguishable by the ability to assimilate the pentose sugar L-arabinose (15). In northeast Thailand, 75% of soil isolates cannot assimilate L-arabinose (Ara^-), whereas the other 25% can (Ara^+). However, in central Thailand, where clinical infection is very rare, only Ara^+ strains have been isolated from the soil (15). There also appear to be two distinct groups of *B. pseudomallei*, based on ribotype patterns, and these correspond closely to the arabinose assimilation phenotypes (12a). Ribotype group II isolates and those with DNA that is not digested by *Bam*HI are found only in the environment. All clinical isolates and half of the environmental isolates have group I ribotypes. Thus, ribotype group and the ability to assimilate L-arabinose appear to differentiate virulent from nonvirulent biotypes of bacteria currently speciated as *B. pseudomallei*. We have tested this hypothesis with a murine model of infection.

MATERIALS AND METHODS

Clinical isolates were obtained from patients admitted with melioidosis to Sappasitprasong Hospital, Ubon Ratchatani, northeast Thailand, as part of clinical studies which will be described elsewhere. These isolates were obtained from cultures of blood, sputum, urine, or pus. Soil isolates were obtained from samples from the surrounding area. They were identified by characteristic colonial morphology on a differential agar (1), positive oxidase reaction, resistance to colistin and gentamicin (4), and a biochemical profile based on the results of API 20NE (bio-Merieux, Basingstoke, United Kingdom), which includes tests for arabinose utilization and positive latex agglutination (11). Nine representative isolates of *B. pseudomallei* were selected for use in this animal study. Further biochemical characteristics of these isolates were determined with API 50CH (bio-Merieux) as described previously (15). The isolates were also characterized by ribotyping. Strains 576a, 924a, and 1021a are Ara^- clinical isolates from patients. Strains E8, E25, and E281 are Ara^- soil isolates, and E27, E32, and E276 are Ara^+ soil isolates. Biochemical characteristics and ribotypes of these isolates are provided in Table 1.

Inocula of *B. pseudomallei* were prepared as follows. The isolates were cultured overnight in Trypticase soy broth and diluted in sterile distilled water to approximately 10^6 CFU/ml. A viable count was performed by plating 100-μl aliquots of a series of 10-fold dilutions onto whole plates of Columbia agar (Unipath, Basingstoke, United Kingdom). The initial dilution was kept overnight at 4°C, and when the viable count was known, a further series of dilutions were made in distilled water to give inocula ranging from 100 to 10,000 CFU/ml. Mice were inoculated intraperitoneally with 100 μl of the dilutions, with a goal of giving inocula of 10, 50, 100, 500, and 1,000 CFU to each mouse. The actual inocula given were calculated by further viable counts of the residual inoculum after injection.

These studies were carried out in accordance with guidelines for humane practice given in the United Kingdom Animals (scientific procedures) Act of 1986.

Animal experiments. BALB/c mice, approximately 6 weeks old, were used for the study, which was performed in an isolation area. There were three rounds of experiments. Each round used one Ara^- clinical isolate, one Ara^- soil isolate, and one Ara^+ soil isolate of *B. pseudomallei* at the five different inoculum concentrations. Each of these five inoculum groups contained 8 mice, i.e., a total of 40 mice for each isolate and a total of 360 mice overall. Following inoculation, the mice were observed daily for a period of 28 days. The mice had free access to food and water. Mice showing signs of clinical illness were marked, and if the illness was obviously progressing rapidly, the mice were sacrificed. Postmortem examinations were performed on mice dying early in the experiment or unexpectedly to look for abscess formation and take samples for culture. Blood from the heart, liver, and spleen was cultured for *B. pseudomallei* with a selective agar and broth as described previously (14). The end point of the experiment was either natural death or sacrifice because of progressive illness within the 28-day period, regardless of whether *B. pseudomallei* was cultured subsequently (mice showing signs of illness but surviving 28 days were not included in the mortality numerator).

Stability of the biochemical phenotypes. Approximately 10^6 organisms from five different Ara^+ and three Ara^- isolates were maintained continuously in triple-distilled water and 0.9% saline for 9 months at ambient temperature (28 to 30°C), with three monthly assessments of biochemical phenotype. Inocula of 10^7 Ara^- organisms and 10^7 Ara^+ organisms from each of three different isolates were also maintained in 0.2 and 0.4% L-arabinose and 0.2 and 0.4% D-glucose solutions for 8 days. Arabinose utilization was assessed as described previously. A selective minimal salts agar containing 0.2% L-arabinose, which preferentially

* Corresponding author. Mailing address: Faculty of Tropical Medicine, Mahidol University, 420/6 Rajvithi Rd., Bangkok 10400, Thailand. Phone: 66 2 246 0832. Fax: 66 2 246 7795.

† Present address: Public Health Laboratory, Musgrove Park Hospital, Taunton, Somerset, TA1 5DB, United Kingdom.

TABLE 1. Characteristics of *B. pseudomallei* isolates used to inoculate mice[a]

Strain	Origin	LD_{50} (CFU)	Assimilation of:										Ribotype
			Ado	L-Ara	D-Ara	Dul	Ery	Gly	5-Ke	Suc	Tre	D-Xy	
576a	Human	228	−	−	−	+	+	−	−	−	+	−	I
924a	Human	65	−	−	+	+	+	−	−	−	+	−	I
1021a	Human	148	−	−	−	+	+	+	−	−	−	−	I
E8	Soil	14	−	−	−	+	+	−	−	−	+	−	I
E25	Soil	30	−	−	−	+	+	−	−	−	+	−	I
E281	Soil	138	−	−	−	+	−	−	−	−	+	−	I
E27	Soil	$>2 \times 10^{3}$[b]	+	+	−	−	−	+	+	−	−	+	ND
E32	Soil	$>3 \times 10^{3}$[b]	+	+	−	−	−	+	+	+	−	+	ND
E276	Soil	$>6 \times 10^{8}$[b]	+	+	−	−	−	+	+	+	−	+	I

[a] Ado, adonitol; L-Ara, L-arabinose; D-Ara, D-arabinose; Dul, dulcitol; Ery, erythritol; Gly, glycerol; 5-Ke, 5-keto-gluconate; Suc, sucrose; Tre, trehalose; D-Xy, D-xylose. ND, not digested.

[b] No deaths occurred with this dose in this group.

supports the growth of Ara^+ organisms, was also used for screening multiple colonies from isolates.

Statistical analysis. Proportions were compared by the chi-square test or Fisher's exact test. Normally distributed continuous data were compared by using Student's *t* test. Continuous data not conforming to a normal distribution were compared by the Wilcoxon rank sum test. Cumulative survival tables were plotted with Statview 4.1 (Abacus Concepts, Berkley, Calif.). The 50% lethal dose (LD_{50}) was calculated by using WinNolin 1.1 (SCI Inc., 1996 version) with the inhibitory sigmoid E_{max} model and a general equation as follows: $E = E_{max}*\{1 - [C^{**}\gamma/(C^{**}\gamma + LD_{50}{}^{**}\gamma)]\}$, where E and E_{max} are the effect and the maximum effect, respectively; C is the inoculum (dose); and γ is a constant.

RESULTS

Clinical isolates. Since September 1986, 1,200 patients with melioidosis have been studied prospectively in Sappasitprasong Hospital. Every clinical *B. pseudomallei* isolate has had a biochemical phenotype characterized by the failure to utilize arabinose (Ara^-), and over the 11-year period there has never been a change in phenotype in routine subculture and storage. In the soil of surrounding areas, *B. pseudomallei* can be recovered from 50% of soil samples from rice farms, and 25% of soil isolates utilize arabinose ($P < 0.0001$). A more detailed description of the biochemical differences between these two biotypes has been published recently (15).

Animal experiments. The calculated LD_{50} data for each group of mice are shown in Table 1. The first two rounds of experiments showed a striking difference in virulence between Ara^- and Ara^+. *B. pseudomallei* Ara^+ isolates at the standard inocula were not lethal for mice. Therefore, in the third round of experiments, Ara^- clinical and soil isolates were each used at the standard inocula, but the inocula for the Ara^+ isolate (strain E32) were increased in 10-fold steps from 6×10^5 to 6×10^9 CFU/mouse.

Among the 80 mice that received the standard inocula of Ara^+ soil isolates, only 1 died. This mouse, inoculated with 230 CFU, died suddenly on day 23. At postmortem examination, there was no sign of abscess formation and *B. pseudomallei* was not isolated from any of the sites cultured, so it is unlikely that this death resulted from melioidosis. In comparison, 117 of 240 (49%) mice inoculated with Ara^- isolates died or became ill and were sacrificed. There was no difference in virulence between clinical and Ara^- soil isolates (58 of 120 [48% deaths] and 59 of 120 [49% deaths], respectively). Only two deaths occurred before day 7. The median (range) day of death was 20 (3 to 28) for clinical isolates and 19 (7 to 28) for Ara^- soil isolates. Postmortem examination usually revealed massive hepatosplenomegaly with widespread visceral abscess formation. The mean (standard deviation) LD_{50} inoculum for all Ara^- isolates was 111 (88) CFU/mouse compared with $>10^8$ CFU/mouse for the Ara^+ isolates ($P < 0.001$). Muscle wasting was a prominent feature in the mice developing clinical illness. Characteristic neurological signs were also seen after the first week in the majority of sick mice. These were manifest initially as paresis of one hind leg, followed by paresis of the other hind leg, which usually progressed rapidly to complete paraplegia. Two mice developed hemiparesis. Neurological signs were evident with all Ara^- isolates of *B. pseudomallei* but were more common in mice inoculated with soil Ara^- isolates than in mice inoculated with clinical isolates (47 of 120 and 29 of 120, respectively; $P = 0.01$). Seventy of these mice died or were sacrificed; only six survived 28 days.

Stability of the biochemical phenotype. The biochemical profiles of the Ara^+ and Ara^- isolates maintained in distilled water for 9 months did not change. The organisms died after 6 months' culture in 0.9% saline, but before this the biochemical phenotype was unchanged. In all the experiments conducted with glucose and arabinose solutions, the biochemical phenotype of the original and subsequent isolates also remained the same. In all screening evaluations of large inocula with the minimal salts-arabinose selective agar, only Ara^+ organisms grew well, and we never observed change in the biochemical phenotype (i.e., growth of colonies of Ara^+ organisms following plating of an Ara^- isolate). Thus, inducing a change in the biochemical characteristics of these organisms was not possible despite the use of different forms of selective pressure.

DISCUSSION

We have recently identified an organism closely resembling the pathogen *B. pseudomallei* in soil samples from northeast Thailand. Although the morphology, antigenicity, and antimicrobial susceptibility of this organism are similar to those of the bacteria isolated from patients with melioidosis, this organism has a number of biochemical differences, exemplified by the ability to utilize L-arabinose (Ara^+). In northeast Thailand, where melioidosis is common, 75% of soil isolates cannot utilize L-arabinose (Ara^-), whereas in the central region, where melioidosis is uncommon, nearly all soil isolates have the Ara^+ phenotype. Since 1986, we have studied more than 1,200 patients with melioidosis, and in every case the organism isolated had the Ara^- biotype. Furthermore, in routine culture and subculture of these isolates, conversion to the Ara^+ biotype was never observed, suggesting that the Ara^- biotype is stably linked to the virulence of *B. pseudomallei.*

The results of our study of experimental melioidosis show a striking difference between the virulence of the Ara^+ and Ara^- strains of *B. pseudomallei* for mice. Ara^- strains are highly

virulent, whereas Ara^+ strains are essentially nonvirulent. Both biochemical types have antigenic similarities; both give positive reactions in the latex agglutination test, in which a polyclonal antibody raised to an Ara^- clinical strain of *B. pseudomallei* is used (11), and both give similar results in indirect hemagglutination assays for melioidosis. Thus, despite the similarity of their morphologies, culture characteristics, antibiotic susceptibility profiles, and antigenicities, one biotype causes melioidosis and the other does not. The LD_{50} inoculum for Ara^- isolates of *B. pseudomallei* reported here is higher than that reported previously by Dannenberg and Scott (5). These researchers used a single strain of *B. pseudomallei* that had been passaged in mice; the LD_{50} inoculum was 35 CFU given intraperitoneally and 16 CFU given by the respiratory route. However, Dannenberg and Scott's LD_{50} values were derived from the presence of pathological lesions at the time of death or sacrifice rather than from death alone and are therefore not strictly comparable to ours. Miller et al. (8) studied the virulence of a strain of *B. pseudomallei* of unknown origin in various animals. Although very low inocula were lethal for hamsters and guinea pigs (animals known to be highly susceptible), mice were much more resistant, with an inoculum of $>4.5 \times 10^5$ CFU given intraperitoneally needed to produce death. The strain of mouse used may account for this difference, as mice vary considerably in their susceptibility to *B. pseudomallei*. BALB/c mice appear to be highly susceptible. In their initial experiments with hamsters, Miller et al. used a different strain of *B. pseudomallei*, of unknown origin, which was found to be relatively avirulent. Dannenberg and Scott (6) also described two relatively avirulent strains of *B. pseudomallei* which each had an intraperitoneal LD_{50} of approximately 10^8 CFU/mouse. The origin of these strains was not given. Sublethal doses of these organisms produced transient or nonprogressive lesions, and low numbers of *B. pseudomallei* could still be recovered from liver, spleen, and lymph nodes 11 weeks after inoculation. These authors also describe subsequent partial immunity to challenge by virulent strains of *B. pseudomallei*. Subcutaneous inoculation of an avirulent strain increased the amount of the respiratory inoculum of a virulent strain needed to produce visible lesions in the lung (7). It is possible that these avirulent strains were Ara^+ soil isolates.

In the present series, rapidly progressive paraparesis was a prominent feature in approximately two-thirds of mice developing clinical illness. This neurological presentation has been described previously in natural and experimental infection of animals, particularly sheep and goats (12). In northern Australia, Woods et al. (13) described seven human cases of neurological melioidosis, accounting for 13% of patients seen over a period of 7 years. The syndrome was characterized by peripheral motor weakness, brain stem encephalitis, aseptic meningitis, and respiratory failure. There was no direct infection of the central nervous system, which suggests that this syndrome could have been induced by an exotoxin. Neurological manifestations without direct nervous system infection do occur in Thailand, but the incidence in patients with confirmed melioidosis is over 50 times lower (unpublished data), suggesting that strain variation or differences in host susceptibility may be important in the pathogenesis of this syndrome.

The ability to assimilate L-arabinose was more strongly associated with virulence than was ribotype group. All six virulent (Ara^-) isolates used in this study were from ribotype group I. However, one of the avirulent (Ara^+) isolates was also from group I; the DNA of the other two isolates was not digested by *Bam*HI. Further research is needed on the phenotypic and genotypic differences between Ara^- and Ara^+ *B. pseudomallei* and the immunological responses resulting from exposure to the different types. It is likely that environmental Ara^+ *B. pseudomallei* contributes to serological reactions in current tests, and this may explain in part the discrepancies between seroprevalence and disease prevalence. Such studies will help researchers understand the mechanisms involved in the pathogenesis of melioidosis and possibly lead to the development of a vaccine. The avirulent Ara^+ biotype may differ enough from the Ara^- organism that causes melioidosis to warrant its inclusion in a new species within the genus *Burkholderia.*

Editor: P. E. Orndorff

ACKNOWLEDGMENTS

We are extremely grateful to T. L. Pitt (Central Public Health Laboratory, London, United Kingdom) for ribotyping the bacterial isolates and reviewing the manuscript. We also thank Sayan Langla, Varunee Desakorn, and Amanda L. Walsh for their assistance with this work.

This study was part of the Wellcome-Mahidol University Oxford Tropical Medicine Research Programme, funded by the Wellcome Trust of Great Britain.

REFERENCES

1. **Ashdown, L. R.** 1979. An improved screening technique for isolation of *Pseudomonas pseudomallei* from clinical specimens. Pathology **11:**293–297.
2. **Chaowagul, W., N. J. White, D. A. B. Dance, et al.** 1989. Melioidosis: a major cause of community-acquired septicemia in Northeastern Thailand. J. Infect. Dis. **159:**890–899.
3. **Dance, D. A. B.** 1991. Melioidosis: the tip of the iceberg? Clin. Microbiol. Rev. **4:**52–60.
4. **Dance, D. A. B., V. Wuthiekanun, P. Naigowit, and N. J. White.** 1989. Identification of *Pseudomonas pseudomallei* in clinical practice: use of simple screening tests and API 20NE. J. Clin. Pathol. **42:**645–648.
5. **Dannenberg, A. M., and E. M. Scott.** 1958. Melioidosis: pathogenesis and immunity in mice and hamsters. I. Studies with virulent strains of *Malleomyces pseudomallei*. J. Exp. Med. **107:**153–166.
6. **Dannenberg, A. M., and E. M. Scott.** 1958. Melioidosis: pathogenesis and immunity in mice and hamsters. II. Studies with avirulent strains of *Malleomyces pseudomallei*. Am. J. Pathol. **34:**1099–1121.
7. **Dannenberg, A. M., and E. M. Scott.** 1960. Melioidosis: pathogenesis and immunity in mice and hamsters. III. Effects of vaccination with avirulent strains of *Pseudomonas pseudomallei* on the resistance to the establishment and the resistance to the progress of respiratory melioidosis caused by virulent strains; all or none aspects of this disease. J. Immunol. **84:**233–246.
8. **Miller, W. R., L. Pannell, L. Cravitz, W. A. Tanner, and T. Rosebury.** 1948. Studies on certain biological characteristics of *Malleomyces mallei* and *Malleomyces pseudomallei*. II. Virulence and infectivity for animals. J Bacteriol. **55:**127–135.
9. **Nachiangmai, N., P. Patamasucon, B. Tipayamonthien, A. Kongpon, and S. Nakaviroj.** 1985. *Pseudomonas pseudomallei* in southern Thailand. Southeast Asian J. Trop. Med. Public Health **16:**83–87.
10. **Smith, M. D., V. Wuthiekanun, A. L. Walsh, and N. J. White.** 1995. Quantitative recovery of *Burkholderia pseudomallei* from soil in Thailand. Trans. R. Soc. Trop. Med. Hyg. **89:**488–490.
11. **Smith, M. D., V. Wuthiekanun, A. L. Walsh, and T. L. Pitt.** 1993. Latex agglutination test for the identification of *Pseudomonas pseudomallei*. J. Clin. Pathol. **46:**374–375.
12. **Thomas, A. D., J. C. Forbes-Faulkner, J. H. Norton, and K. F. Trueman.** 1988. Clinical and pathological observations on goats experimentally infected with *Pseudomonas pseudomallei*. Aust. Vet. J. **65:**43–46.

12a. **Trakulsomboon, S., and T. L. Pitt.** Personal communication.

13. **Woods, M. L., B. J. Currie, D. M. Howard, et al.** 1992. Neurological melioidosis: seven cases from the northern territory of Australia. Clin. Infect. Dis. **15:**163–169.
14. **Wuthiekanun, V., D. A. B. Dance, Y. Wattanagoon, Y. Supputtamongkol, W. Chaowagul, and N. J. White.** 1990. The use of selective media for the isolation of *Pseudomonas pseudomallei* in clinical practice. J. Med. Microbiol. **33:**121–126.
15. **Wuthiekanun, V., M. D. Smith, D. A. B. Dance, A. L. Walsh, T. L. Pitt, and N. J. White.** Biochemical characteristics of clinical and environmental strains of *Burkholderia pseudomallei*. J. Med. Microbiol., in press.