

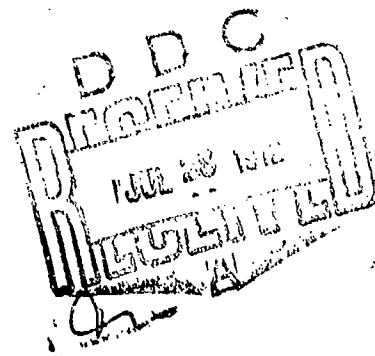
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MELIOIDOSIS: SEROLOGIC STUDIES ON US ARMY PERSONNEL
RETURNING FROM SOUTHEAST ASIA

BY

MAJ RICHARD A. KISHIMOTO, MSC, USA, LTC GEORGE L. BROWN, MSC, USA,
COL EUGENE B. BLAIR, MSC, USA, AND DOROTHY WENKHEIMER

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Melioidosis: Serologic Studies on US Army Personnel Returning from Southeast Asia

MAJ Richard A. Kishimoto, MSC, USA*

LTC George L. Brown, MSC, USA**

COL Eugene B. Blair, MSC, USA**

Dorothy Wenkheimer*

THE first documented fatal case of melioidosis was observed in 1912 by Whitmore.¹⁰ He noted numerous bacterial abscesses in various organs and attributed death to septicemia. *Pseudomonas pseudomallei* was subsequently established as the etiologic agent of melioidosis.¹⁰

Melioidosis is interesting from an ecologic viewpoint, in that primary infections have been found only in areas between the 20° north and south parallels of latitude. The disease is endemic in southeast Asia and northern Australia. A few cases have been reported from India, Panama, Turkey, Philippines, and Ecuador.^{2,8,15} Transmission of the etiologic agent has been proposed to occur through contamination of broken skin with infected soil or water, or inhalation of contaminated dust or water.^{15,16} Results of a serologic survey by Nigg¹² suggest that in Thailand subclinical or unrecognized forms of mild melioidosis are prevalent among the populace.

The clinical manifestations of melioidosis range from acute fulminating bacteremia to subclinical infection, which occasionally exacerbates to acute disease after months or years of dormancy.^{4,13} The purpose of this investigation was to evaluate US Army personnel returning from Vietnam for melioidosis antibodies. The study was extended to determine the immunologic response in rabbits sensitized intravenously with formalized *P. pseudomallei* cells.

Materials and Methods

Human sera

A single serum specimen was taken from each of the 500 subjects. The following groups of Vietnam returnees were studied: (I) Patients wounded by gunshot or shrapnel; (II) medically-ill patients (not wounded); (III) medically-asymptomatic personnel; and (IV) a control group of military personnel who had never been to Vietnam.

Measurement of antibody activity

Antigen preparation and serologic procedures were performed as described elsewhere.¹¹ Tannic acid-treated fresh rabbit erythrocytes and fresh untreated erythrocytes were used for the hemagglutination tests.

Absorption of sera

Sera with HA titers $\geq 1:80$ were adsorbed with suspensions of *Pseudomonas aeruginosa*, and with "O" and "H" antigens

From Fitzsimons General Hospital, Denver, Colo. 80240.

* Microbiology Service, Dept. of Pathology.

** Microbiology Division, US Army Medical Research and Nutrition Laboratory.

prepared from a serogroup B *Salmonella sp.* Bacterins containing 1×10^9 cells/ml of *P. aeruginosa* or *Salmonella sp.* were prepared in saline and preserved with formalin (0.5% v/v). Equal volumes of test serum and bacterin were mixed and incubated for one hour at 37°C. The mixture was centrifuged at 4,000 RPM for 10 min; the supernatant was tested for HA titer.

Preparation of bacterial filtrate antigen

P. pseudomallei smooth colonies from a 48 hr Trypticase Soy agar culture (TSA), were inoculated into Rice medium¹⁴ and incubated at 37°C for 14 days. The bacterial culture was centrifuged at 4,000 RPM for 15 min; the cellular sediment was discarded. The supernatant was filtered through a 0.45 μ Millipore filter; its protein component was recovered by precipitation³ with saturated $(\text{NH}_4)_2\text{SO}_4$. For the HA test, tanned sheep erythrocytes (SREC) were sensitized² with the extracted protein component from the pseudomallei-culture-filtrate.

Fluorescent antibody inhibition test

The specificity of HA reactions was evaluated by a modified one-step⁹ and the two-step⁶ fluorescent antibody inhibition tests. In the one-step procedure, unlabeled patient's serum was mixed with an equal volume of fluorescein isothiocyanate-conjugated anti-*P. pseudomallei* serum. The mixture was spread on a microscope slide previously coated with *P. pseudomallei* cells. Each preparation was incubated for 30 min at 37°C, followed by two 5-min washings with 0.15 M phosphate buffered saline, pH 7.3 (PBS). In the two-step method, slides coated with *P. pseudomallei* cells were exposed to unlabeled patient's serum for 30 min at 37°C and then were washed twice with PBS. Fluorescein tagged anti-*P. pseudomallei* serum was added and the preparation processed as described above. Test and control slides were examined with a Leitz Ortholux microscope: Osram HBO 200 mercury arc lamp, BG 22 heat, BG 12 exciter, and OG 1 barrier filters.

Vaccine preparation

P. pseudomallei was grown on TSA for 24 hr. at 37°C. The cells were harvested, washed with 0.5 per cent formalized saline, and were adjusted to a concentration of 1×10^9 cells/ml with formalized saline.

Animal inoculations

Randomly-bred New Zealand white rabbits weighing 1.8 to 2.4 kg were inoculated intravenously with 0.5 ml or 1.0 ml

TABLE I
HA* TITERS TO *P. PSEUDOMALLEI* IN FOUR GROUPS OF MILITARY PERSONNEL

Group	Specimens		Passive Hemagglutination Titer (Polysaccharide Antigen)							Total Reactive
	Subjects	No.	0	1:10	1:20	1:40	1:80	1:160	1:640	
I**	Wounded	200	173	9	11	1	5		1	27
II**	Medical	100	97	2				1		3
II***	Well	100	96	2	2					4
IV***	Control	100	97		2		1			3
	Total	500	463	13	15	1	6	1	1	37

* Sheep RBC sensitized with pseudomallei-polysaccharide antigen.

** Vietnam returnee.

*** Never been to Vietnam.

of the bacterial antigen; animals were again injected after 18 days. A final immunization series was initiated 21 days post secondary inoculation as follows: Animals initially sensitized with 0.5 ml antigen received 0.7, 0.9, and 1.1 ml antigen administered at 1, 4, and 8 days respectively; those sensitized with 1.0 ml antigen received 1.2, 1.4, and 1.6 ml antigen. All animals were bled 13 days after the last injection.

2-Mercaptoethanol (2-ME) treatment of serum

Human or rabbit serum, diluted 1:5 with PBS, was mixed with an equal volume of 2-ME (final concentration, 0.1 M) and incubated for 60 min at 37°C. Within 30 min after the incubation, 2-ME-treated samples were tested for antibody activity (3).

Anion exchange column chromatography

Anion-exchange cellulose adsorbent, DEAE, was prepared as a single batch in 0.02 M potassium phosphate buffer, pH 8.0, and used throughout these studies. Stepwise elution was performed with 0.02 M potassium phosphate, pH 8.0, as the initial buffer and 0.25 M potassium phosphate buffer, pH 4.5, as the final buffer. Protein content of each fraction was estimated by measuring its optical density³ at 280 m μ .

Results

Results of the serologic study on military personnel are summarized in Table I. RBC for the HA test were sensitized with the pseudomallei-extracted polysaccharide antigen. Titers $\geq 1:80$ (4.2 log₂) were recorded as diagnostic. In the wounded patient group, 6 (3.0 per cent) of 200 sera gave diagnostic titers, whereas only one specimen each from the medically ill and the control groups had titers $\geq 1:80$ (4.2 log₂).

The eight sera with titers $\geq 1:80$ were treated with 2-ME and also were absorbed with *P. aeruginosa* and *Serratia* sp. antigens. Serologic testing of the absorbed or ME-treated sera showed no changes from the previously observed titers. From this group, DEAE cellulose fractionation of two randomly selected serum samples showed two protein peaks. The first

peak, eluted with the initial buffer, contained ME-resistant HA hemagglutinating antibodies for *P. pseudomallei*. The second peak, eluted with the final buffer had no anti-pseudomallei globulin activity. Using antihuman IgG and IgM in radial immuno-diffusion studies on the two DEAE protein fractions showed the first peak to contain IgG and the second peak IgM.

The eight serum samples with diagnostic HA anti-pseudomallei titers were re-evaluated with the fluorescent antibody inhibition tests. In the one-step fluorescence inhibition procedure, each serum sample inhibited fluorescence (Table II); similar results were recorded with the two-step test. The same sera were further evaluated with the complement fixation test. Results of this study showed no evidence for complement fixing antibodies.

The primary and secondary immune responses in rabbits sensitized with 0.5 ml or 1.0 ml killed *P. pseudomallei* were quantitatively alike; Fig. 1 illustrates results in animals receiving 0.5 ml vaccine. In the animal study, 2-ME sensitive antibodies were observed on days 3, 6, 9, and 12; 2-ME resistant antibodies were observed on the 15th day. These results are in agreement with reported findings for primary and secondary responses to soluble and particulate antigens.¹

Discussion

Serum titers before treatment with 2-ME were recorded as the total functional antibody concentrations. Previous studies³ have shown that IgG activity is 2-ME resistant, whereas IgM is sensitive to 2-ME. In the present study (i.e., human or animal sera studies), absence of 2-ME resistant antibody suggested that the immune globulin activity was almost likely that of IgM.

The incidence (3 per cent) of subclinical or unrecognized melioidosis found in wounded soldiers was considerably higher than in the other groups tested ($p = 0.1$), if an HA titer $\geq 1:80$ was arbitrarily regarded as diagnostic. The 29 subjects with anti-pseudomallei titers $< 1:80$ (Table I) pose an interesting epidemiologic question as to what constitutes serologic

TABLE II
 RESULT OF ONE-STEP FLUORESCENT ANTIBODY INHIBITION TEST USING PATIENT SERA, *P. PSEUDOMALLEI* AND FLUORESCIN-CONJUGATED ANTI-*P. PSEUDOMALLEI* SERUM

Patient	HA Titer	Final Dilution of Unlabeled Patient's Serum with Corresponding Observed Fluorescence*						
		1:4	1:8	1:16	1:32	1:64	1:78	1:256
R.B.	1:80	1+	2+	3+	4+	4+	4+	4+
K.P.	1:80	—	1+	2+	3+	4+	4+	4+
J.W.	1:80	—	1+	2+	3+	4+	4+	4+
W.H.	1:80	1+	1+	2+	2+	3+	4+	4+
A.H.	1:80	1+	1+	2+	2+	3+	4+	4+
D.C.	1:160	—	1+	2+	2+	3+	4+	4+
J.R.	1:640	—	—	1+	1+	2+	3+	4+
K.M.	1:80	—	1+	1+	2+	3+	4+	4+
Negative Control		4+	4+	4+	4+	4+	4+	4+
Positive Control		—	—	—	1+	2+	3+	4+

* Intensity of fluorescence: (— to 4+ i.e., no fluorescence to maximum fluorescence, respectively).

evidence of clinical or subclinical infection by this organism. In this study, 21 (11.5 per cent) wounded subjects had titers < 1:80 which far exceeded the number found in the medically ill (2 per cent), asymptomatic (4 per cent), or control (2 per cent) groups (Chi square, Yates' correction; $p = 0.015$). If any titer of serum HA activity is accepted as constituting serologic evidence for infection, the increased incidence of contact with *P. pseudomallei* by wounded patients over the other test groups (Table I) reaches a high level of significance (p (corrected) < 0.001). These findings are not surprising when one considers that the etiologic agent is a common inhabitant of soil and water in Vietnam. The medical patient with a 1:80 titer had been evacuated for psychiatric observation; the "diagnostic" titer in the control group was from a nurse who had never been to Vietnam, but had worked on wards with melioidosis patients for several months. All subjects with titers $\geq 1:80$ were evaluated bacteriologically and radiologically with negative findings.

HA-reactive sera were evaluated for anti-*P. pseudomallei* antibodies, using the one- and the two-step fluorescence inhibition tests. Partial or complete inhibition of fluorescence was recorded as evidence of specificity of the HA test. The hemagglutinin activity in patient sera with diagnostic titers was further evaluated with mercaptoethanol treatment, radial immunodiffusion studies, and anion exchange chromatography. Results of these studies suggested an immune globulin with IgG characteristics, thus indicating infections from frequent contacts with the organism.

Numerous workers are not in agreement as to the serologic interpretation of anti-*pseudomallei* titers; serum reactivity

from 1:80 to 1:3000 have been suggested as diagnostic for clinical or subclinical melioidosis.^{4,9,10} Malizia *et al*⁷ and Nigg¹² showed that the HA test, using a polysaccharide antigen, detected *P. pseudomallei* agglutinins; Malizia⁷ established 1:80 as the diagnostic titer. Nigg¹² using a crude aqueous extract protein antigen, (CAE), found that the HA test was not specific for the diagnosis of subclinical infection in man. In related studies, however, Nigg¹² showed that the CAE antigen was sensitive and specific for melioidosis in experimentally infected animals.

The present investigation showed the polysaccharide antigen, used in the HA test, to be serologically specific for the detection of *pseudomallei* antibodies among the Vietnam returnees tested (Tables I and II). However, it was not sero-specific for the experimentally immunized animals; physical differences in the antigen stimulus used for sensitizing animals perhaps accounts for these results.

Serum samples showing positive HA titers were negative for complement fixing antibodies to *pseudomallei*. Nigg¹² stated that the antibodies for complement fixation and HA reactions are not identical.

The present data clearly established that three per cent of Vietnam returnees (wounded personnel) had significant titers to *P. pseudomallei*. Therefore, an assumption must be made that these individuals had contact(s) with the organism during their tour in southeast Asia, and anti-*P. pseudomallei* activity being associated with only IgG suggests frequent exposure to melioidosis. The question arises as to whether these individuals experienced subclinical or clinical melioidosis. Another question which must await elucidation

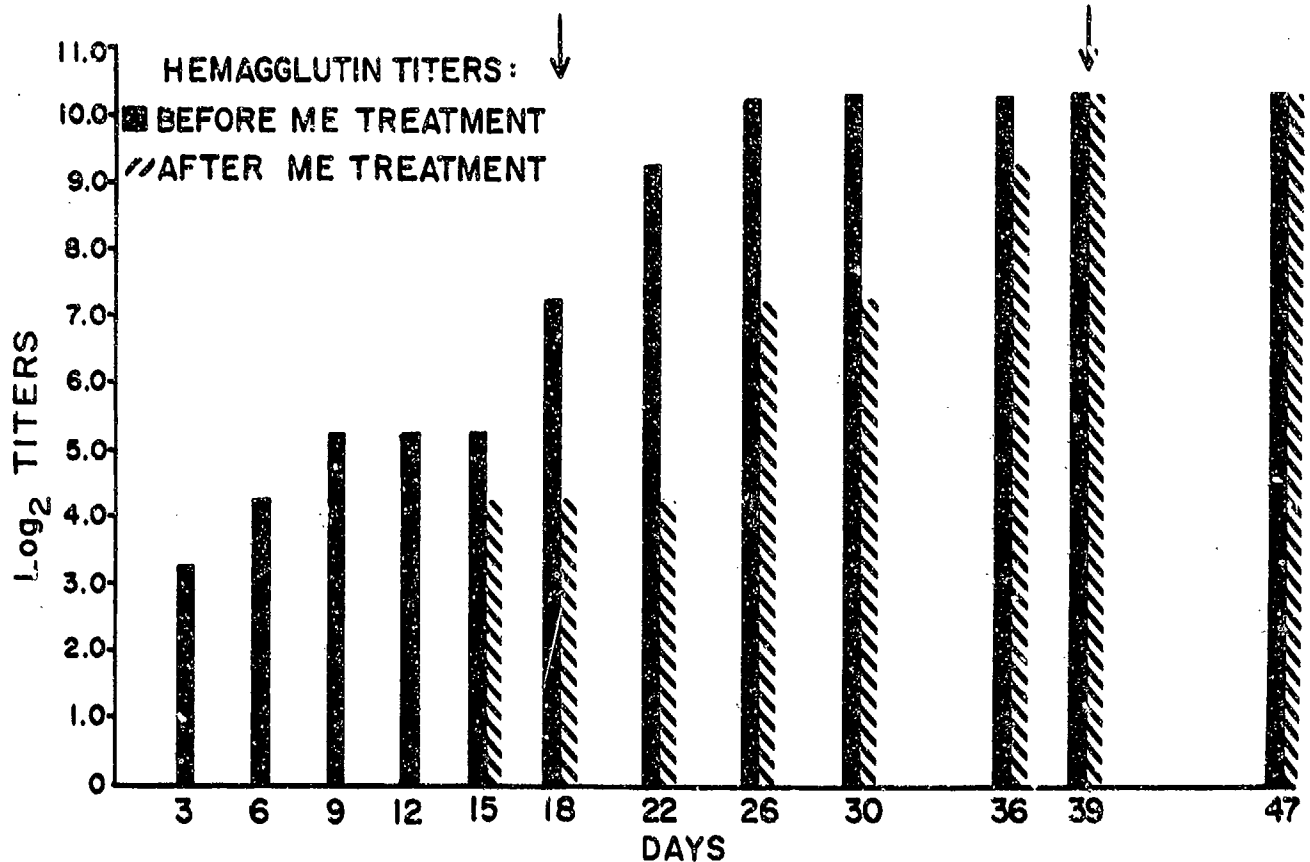


Fig. 1. Primary and secondary responses to *Ps. pseudomallei* (formalized preparation) in rabbits; * each column represents mean values of four rabbits. Arrows indicate repeated bacterin injections.

* HA test, tanned erythrocytes sensitized with protein component extracted from *pseudomallei*-culture-filtrate.

is whether or not personnel exhibiting "diagnostic" serological evidence for this organism will later experience exacerbations of acute disease. Studies should be undertaken to evaluate additional Vietnam returnees, wounded and non-wounded, for prevalence of subclinical melioidosis. This investigation has established that the HA test, using a polysaccharide antigen, is a valuable serologic tool for the evaluation of clinical or subclinical melioidosis.

Summary

Military personnel returning from Vietnam were evaluated serologically for melioidosis, using the hemagglutination test (HA). A total of 500 serum samples were studied. Titers $\geq 1:80$, observed when test erythrocytes were sensitized with polysaccharide antigen extracted from *Pseudomonas pseudomallei*, were recorded as diagnostic. In the wounded patient group, six (three per cent) had diagnostic titers. One serum specimen from the medically-ill group and one from the US control group had diagnostic titers.

Antibody activity in all positive serum samples (i.e. diagnostic titer) was mercapto-ethanol (2-ME) resistant. The primary and the secondary immune responses of rabbits immunized with killed *P. pseudomallei* cells were serologically evaluated with the hemagglutination test; appropriate erythrocyte suspensions were sensitized with polysaccharide or

protein component extracted from *pseudomallei* cells. Results of this study are discussed.

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