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# OCCULT DIFFUSE INVOLVEMENT OF SUPRATENTORIAL WHITE MATTER DETECTED BY MAGNETIC RESONANCE IMAGING IN HTLV-I CARRIERS

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**Abstract:** We describe three human T lymphotropic virus type I (HTLV-I) carriers, who are characterized by a dissociation between the clinical features including scarce cognitive impairments and peculiar hyperintensities in the deep and subcortical white matter detected by T2-weighted magnetic resonance imaging (MRI) scans. Unlike spotty MRI findings which are encountered in clinically asymptomatic elderly individuals and are previously described in some HTLV-I carriers, the MRI changes in our patients were diffuse and extensive. Known central nervous system disorders accompanying a cerebral white matter involvement were failed to be diagnosed. These findings support a possibility that extensive lesions in the supratentorial white matter are associated with HTLV-I infection with minimal symptoms, and suggest that MRI scans may detect occult diffuse inflammatory changes associated with the virus infection in these patients.

**Keywords:** Human T lymphotropic virus type I (HTLV-I); Cerebral white matter; T2-weighted image; High intensity.

## INTRODUCTION

Sustained prevalence of human T lymphotropic virus type I (HTLV-I), a member of the oncoviridae subfamily, is common in many parts of the tropics and in southern Japan (Osame and McArthur, 1992). The virus causes two well defined diseases: adult T cell leukemia/lymphoma, which is a neoplastic disease characterized by clonal expansion of HTLV-I-transformed T cells, and HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP). The latter is a non-fatal neurological syndrome characterized by predominant involvement of the spinal cord and manifested by chronic spastic paraparesis. Although cognitive impairment is included in less common neurological findings in this disease (Osame and McArthur, 1992), several necropsy studies have revealed that the critical pathological findings including mononuclear infiltration or perivascular cuffing, which is prominent in the thoracic spinal cord, are commonly seen in the cerebral white

matter of HAM/TSP patients (Izumo *et al.*, 1989). Cerebral white matter involvement detected by magnetic resonance imaging (MRI) has been reported in an HTLV-I infected symptomless individual (Mattson *et al.*, 1987) and the incidence of cerebral MRI abnormalities is significantly higher in patients with HAM/TSP than in the controls (Kira *et al.*, 1988; Furukawa *et al.*, 1989). These observations suggest that occult involvement of the cerebral white matter may be associated with HTLV-I infection. In previous cerebral MRI studies on HTLV-I infected individuals, common abnormal findings were multiple spotty high intensities in deep and subcortical areas on T2-weighted images (Kira *et al.*, 1988; Furukawa *et al.*, 1989). Moreover, diffuse white matter involvement detected by MRI was also described in connection with pyramidal tract signs and cognitive dysfunctions in patients with HAM/TSP (Natori, 1989; Valderrama *et al.*, 1989; Uyama *et al.*, 1991; Konagaya and Iida, 1991). Here we report three non-demented HTLV-I carriers, whose cerebral MRI

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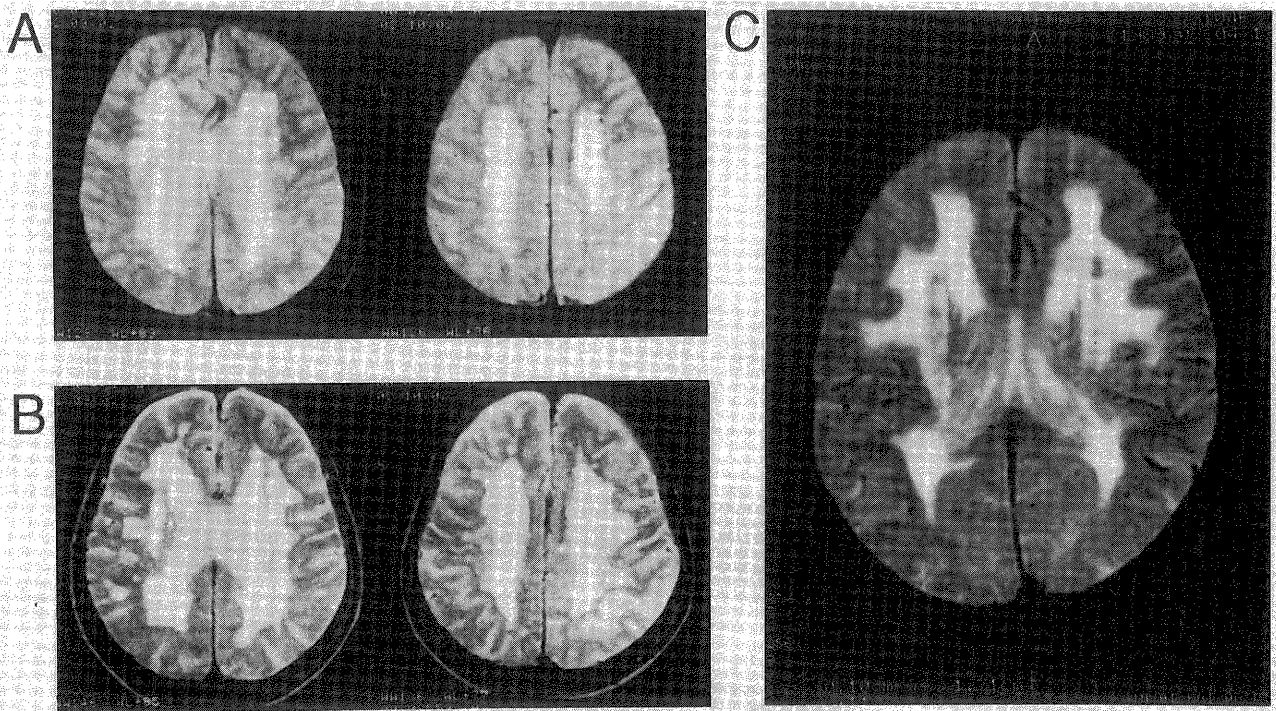


Figure 1. T2-weighted axial magnetic resonance images (spin echo relaxation time 2000, echo delay 100) of the patients demonstrate abnormal high intensity diffusely distributed in the supratentorial white matter. A; patient 1, B; patient 2, and C; patient 3, respectively.

revealed diffuse and widely distributed white matter high intensity on T2-weighted scans, and two of whom were free from apparent features of pyramidal tract impairment.

#### CASE REPORTS

**Patient 1.** A 74-year old man presented with a 34-year history of slowly progressive gait disturbance. Neurological examinations revealed spastic paraparesis with brisk tendon reflexes and extensor plantar responses. Vibration sensation was decreased on both legs. The score of Mini-Mental Examination was 26/30. Routine laboratory examinations including coagulation studies were all within normal limits. Electroencephalogram (EEG) showed a poor organization and modulation. Myelography and myelo-computed tomography (CT) scans demonstrated the cervical ossification of posterior longitudinal ligament (OPLL) with canal stenosis. MRI scans revealed diffuse high intensity signals in the deep and subcortical white matter on T2-weighted images (Figure 1A).

**Patients 2.** A 86-year old woman with a history of atrial fibrillation and episodes of angina pectoris presented with a transient weakness of her right arm.

Routine neurological examinations disclosed no abnormality. Her intelligence quotient (IQ) was 102 on the Wechsler adult Intelligence Scale (WAIS). Routine laboratory examinations including coagulation studies were within normal limits. The EEG record showed normal results. Cerebral CT scans revealed diffuse hypodensities in the deep white matter, and MRI scans demonstrated high intensity signals diffusely distributed in the white matter on T2-weighted images (Figure 1B).

**Patients 3.** A 73-year-old woman with a history of hypertension presented with a hand tremor. Neurological examinations revealed mild bradykinesia, mild hyperreflexia without extensor plantar response, and orthostatic hypotension. Her IQ was 100 on the WAIS. Routine laboratory examinations including coagulation studies were within normal limits. Cerebral CT scans disclosed diffuse hypodensity in the deep white matter, and MRI scans revealed diffuse high intensity signals in the white matter on T2-weighted images (Figure 1C).

These 3 patients showed positive anti-HTLV-I antibody titers in serum (1: 512, 1: 2048, and 1: 65536, respectively), and in patient 3 the antibody was also detected in cerebrospinal fluid (CSF) (1: 2028) by particle agglutination (PA) method (Fujirebio). The CSFs obtained from patients 1 and 2 were negative for the

antibody (PA). Western blot analysis confirmed the presence of antibodies to HTLV-I antigens derived from MT-2 cell line in sera both as IgG and IgM in these three patients (data not shown). Oligo clonal IgG was detected in CSF from patient 3, but not in patients 1 and 2. The level of myelin basic protein in CSF and circulating adrenocorticotrophic hormone was normal in these three patients. Anti-cardiolipin antibody (ACLA) was not detected in patient 1 and 2, but the patient 3 showed the increased serum ACLA level both as IgG and IgM (ELISA). The concentration of serum cholestanol was not increased in patient 1 and 3 (not tested in patient 2).

#### DISCUSSION

Cerebral MRI changes in the present cases were diffuse and extensive, unlike periventricular leukoaraiosis (caps and lining) and increased T2 signal spots in the deep white matter which are encountered in clinically asymptomatic elderly individuals (Shmidt *et al.*, 1992). Two of these three patients have cerebrovascular risk factors including cardiac disease in patient 2 and hypertension and serum anticardiolipin antibody, which would enhance coagulation conditions, in patient 3. However, the occurrence of ischemia or expanded arteriosclerosis (i. e. Binswanger's disease) distributed to all MRI-evident lesions can not be predicted from the clinical features in these cases. The diagnoses of central nervous system disorders including leukodystrophy, storage diseases, multiple sclerosis, systemic effect of metabolic abnormalities, and toxins, which show a cerebral white matter involvement and may be asymptomatic in early stages, can be excluded on the basis of laboratory examinations and clinical features. Although patient 1 has spastic paraparesis, the diagnosis of HAM/TSP is equivocal because of his possible manifestation of cervical OPLL.

The seropositivity for HTLV-I together with a dissociation between the minimal cognitive impairment and remarkable MRI abnormality in the cerebral hemisphere white matter is the common characteristic in our three patients. Similar cases exhibiting unpredictable white matter MRI lesions have previously described in patients with HAM/TSP (Natori, 1989; Valderrama *et al.*, 1989; Uyama *et al.*, 1991; Koyanagi and Iida, 1991), and a possible correlation between the lesions and cognitive impairments was discussed (Uyama *et al.*, 1991). Therefore, the significance of this report involves not only a confirmation of the dissociation between cognitive functions and MRI findings in HTLV-I infected individuals but also a suggestion that the diffuse

MRI abnormalities are not related to apparent pyramidal tract signs at least in some cases. These observations indicate that less symptomatic extensive lesions in the cerebral white matter may be associated with HTLV-I infection, and suggest that MRI scans may detect occult diffuse inflammatory change associated with the virus infection in these patients. However, it still remains to be elucidated that possible processes, which result in cerebral white matter abnormal findings on MRI scans with less correlation with cognitive impairment (Mirsen *et al.*, 1991; Almkvist *et al.*, 1992; Tupler *et al.*, 1992), are facilitated by HTLV-I infection.

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# EPIDEMIOLOGICAL FACTORS AFFECTING PROGNOSIS OF NEONATAL TETANUS IN DAKAR, SENEGAL.

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**ABSTRACT:** To investigate factors affecting prognosis of neonatal tetanus (NNT), the present study was conducted in the infectious diseases ward of the Dakar University Teaching Hospital from January 1992 to December 1993. One hundred ninety one cases of NNT were collected. According to the questionnaire, the main important epidemiological factors affecting prognosis of NNT are: home delivery, age less than 7 days, cutting and dressing the umbilical cord with nonsterile instruments and lack of tetanus immunization during pregnancy.

**KEYWORDS:** NEONATAL, TETANUS, EPIDEMIOLOGY, DAKAR.

## INTRODUCTION

Neonatal tetanus (NNT) is a major cause of morbidity and mortality in developing countries. The portal of entry is usually the umbilical cord. According to WHO(1992), 1 million children contract NNT each year, 800,000 of which die. Mortality rates of NNT varied markedly from country to country, ranging from 0 to 70 per 1000 live births. Despite implementation of the Expanded Programme on Immunization (EPI), NNT remains a great public health problem in the Third World.

Previously, the incidence rate of NNT in Dakar was evaluated at 85 cases per 1000 live births (Sow, 1982). Therefore, we evaluated the epidemiological factors affecting prognosis of NNT at the infectious diseases ward of the Dakar University Teaching hospital by questionnaire.

## PATIENTS AND METHODS

*1. Patients:* All patients at the age of 0 to 28 days who had clinical findings compatible with NNT (spasticity, impossibility to feed, risus sardonicus), were enrolled in this study at the infectious diseases ward of Dakar University Teaching Hospital from January 1992 to December 1993.

*2. Methods:* A questionnaire was used for each case. The assessment included: age of the neonate, sex, place of delivery, instruments used for cutting and dressing the umbilical cord.

The number of visiting health care center and tetanus vaccine doses during pregnancy were also obtained from each mother.

*3. Statistical analysis:* Epidemiological data were analysed on microcomputers using software for data management and epidemiological analyses (EPIINFO. 5, CDC/WHO). Comparison of frequency of epidemiological features in the patients was performed using the chi-square test. A p value of less than 5% was considered to be statistically significant. The interval confidence of the relative risk (RR) was calculated with an interval limit of 95%.

## RESULTS

During the study period, 191 cases of NNT were observed. One hundred and fifteen (60.2%) were male and 76 female (39.8%). The lethality rate was high in both females and males: 56.5% and 52.1% respectively. However there was no statistical significance in the incidence of death between the two sexes, ( $p=0.57$  Table 1).

Eighty eight cases (46%) were at an age of less than

7 days, 91 cases from 8-14 days and 12 cases more than 15 days. The lethality rate was higher in neonates aged between 0 and 7 days (75%) compared with those aged between 8 and 14 days (40.6%) and aged more than 15 days (0%), ( $p < 0.0001$  Table 2).

One hundred and fifty cases (79%) were delivered at home and 21% at maternity clinics. The lethality rate was 59.3% in the cases of home delivery versus 34.1% at clinics,  $p = 0.0002$  with a relative risk (RR) = 1.77 (1.12 < RR < 2.44). (Table 3).

In all cases, portal of entry of *Clostridium tetani* was assumed to be through the umbilical cord cut with nonsterile instruments: razor (34%), kitchen knife (5%), stem (26%). Sterile scissors were used in only 35%. The lethality rate was 59% when nonsterile instruments were used versus 44% for sterile scissors [ $p = 0.03$  and relative risk (RR) = 1.34 (1.02 < RR < 1.77)].

The dressing of the wound stump was made with various material and nonsterile materials were used in 77% of all cases: application of coconut oil, traditional butter, clay, talcum powder. Multiple and various methods of tie were used on the umbilical cord: woven morsel from the cloth of the mother (50%) and thread pulled from the traditional birth attendant or the mother (43%). The lethality rate was high when nonsterile ties were used (62%) compared with that 42% when sterile ties were used [ $p = 0.004$  with a relative risk (RR) = 1.48 (1.12 < RR < 1.95)].

More than one half of the mothers of these neonates (62%) had never been to a health care center (HCC) during pregnancy. Only 4.2% had 3 prenatal consultations (PNC) recommended. The lethality rate was 60% when mothers did not visit HCC versus 0% for those attending HCC during pregnancy, ( $p = 0.00000$ .. Table 4).

Lack of adequate tetanus immunization during pregnancy is frequently seen (73%) in our study. When mothers had not been immunized, it was associated with a higher rate of lethality of the neonate due to tetanus (65%). Only 8% had received the 2 doses recommended by WHO. The incidence of NNT death was influenced by the vaccinal status of the mother, ( $p = 0.0001$  Table 5).

Seventy five per cent of the delivery in our study was done by traditional birth attendants (TBAs) and in 25% by registered midwives. The lethality rate was 61.2% for TBAs and 31% for registered midwives, ( $p = 0.0002$ ).

Table 1. Sex and neonatal tetanus lethality rate.

Sex	Total	Number of deaths (%)	p value
Female	76	43 (56.5%)	0.57
Male	115	60 (52.1%)	
Total	191	103 (53.9%)	

Table 2. Age and neonatal tetanus lethality rate.

Age (days)	Total	Number of deaths (%)	p value
0-7	88	66 (75%)	<0.0001*
8-14	91	37 (40.6%)	
>=15	12	0 (0%)	
Total	191	103	

\* Legend: The rate of lethality of the newborn, aged from 0-7 days, is higher than the other two group aged more than 7 days.

Table 3. Place of delivery and neonatal tetanus lethality rate.

Place	Total	Number of deaths (%)	p value
Home	150	89 (59.3%)	0.0002*
Clinic	41	14 (34.1%)	
Total	191	103	

\* Legend: The lethality rate is higher in cases delivered at home than that at maternity clinic.

Table 4. Prenatal consultation (PNC) and neonatal tetanus lethality rate.

PNC	Total	Number of deaths (%)	p value
0 PNC	118	78 (66.1%)	<0.0001*
1 PNC	36	14 (38.8%)	
2 PNC	29	11 (37.9%)	
3 PNC	8	0 (0%)	
Total	191	103	

\* Legend: The lethality rate is higher when mothers did not visit the health care center during pregnancy.

Table 5. Tetanus toxoid immunization during pregnancy and neonatal tetanus lethality rate.

Tetanus toxoid	Total	Number of deaths (%)	p value
0 dose	140	85 (60.1%)	0.0001*
1 dose	36	13 (36.1%)	
2 doses	15	5 (33.3%)	
Total	191	103	

\* Legend: The lethality rate is higher when mothers had not received tetanus vaccine; no difference is found by frequency of immunization.



Photo 1: Case with neonatal tetanus showing spasticity and risus sardonicus.

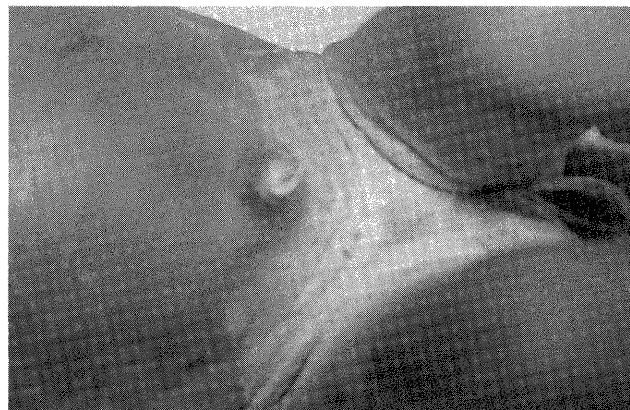


Photo 2: The infected umbilical cord by the way of cutting and dressing is usually the portal of entry of *Clostridium tetani*.

#### DISCUSSION

NNT is a common disease in the infectious diseases ward of the Dakar University Teaching hospital. Previously, the predominance of NNT among neonatal infections in Dakar had been evaluated at 85% (Coll et al., 1986). It also had an important place among neonatal infections during the study period. NNT formed 96% of all serious neonatal infections, including bacterial meningitis (3 cases), pneumoniae (2 cases) and septicemia (1 case). According to WHO (1992), NNT represents from 40 to 80% of all admitted neonatal infections in developing countries, such as Africa and South-East Asia.

In our study, the lethality rate was 53.9%. High lethality due to NNT is also found in other African countries, in Togo with a rate of 71% (Grunitzky et al., (1991), in Nigeria with a rate of 68% (Ergie, 1992) and in Ethiopia with a rate of 40% (Alemu, 1993). Commonly, the high lethality due to NNT in Africa can be explained by multiple factors, such as, severity of tetanus in neonates, consultation-delay and lack of adequate intensive care units.

In the present study, we found that the majority of our patients were at age of less than two weeks with a mean age of 7 days, at admission. The lethality rate is very high during this period. According to the literature, a short incubation period is a bad prognosis factor (Ferron, 1969; Feil, 1987; Whiteman et al., 1992) in tetanus.

The majority of our NNT cases (71%) were transferred from the suburban and rural areas. Previously, the high incidence of NNT in these locations has been emphasized by several authors (Huault, 1964; Alihonou, 1969; Sow, 1982; Galazka, 1984; Cliff, 1985). In suburban

and rural areas of developing countries, lack of health education, availability of health services, environmental and sociocultural behaviour are factors contributing to the morbidity of NNT. A case - control study conducted in a rural area in Senegal, showed that contamination of the hands and careless dressing by TBAs were significantly associated with a high incidence of NNT (Leroy and Garenne, 1991).

Although we did not try to isolate the bacteria, the portal of entry of *Clostridium tetani* was probably the umbilical cord for all cases, because these babies did not have any other lesion where the pathogen could enter. It was demonstrated that 95% of NNT had an infected umbilical cord in Dakar (Alihonou, 1969; Sow, 1982; Feil, 1987). In developing countries, the portal of entry of *Clostridium tetani* is usually the umbilical cord, in Burkina faso (Tall, 1991), Togo (Grunitzky, 1991), Bangladesh (Hladly et al., 1992) and in Ethiopia (Alemu, 1993).

We clearly revealed that home delivery by TBAs induced a higher mortality in the present study, because hygienic principles are not executed. From data analysis, it is suggested that the main source of *Clostridium tetani* may be the hands of the TBAs. The use of a nonsterile instrument for cutting the umbilical cord and the unsanitary method of dressing the wound stump might make for heavy contamination by bacteria resulting in development of serious infection causing higher lethality.

Lack of immunization of tetanus toxoid during pregnancy is also a very important risk factor for death due to NNT in our study. Only 11% of the women in Africa had received the recommended two doses of tetanus vaccine (Gasse, 1987). Eight per cent of the NNT cases in the present study were babies delivered



by the mothers who had received two doses of tetanus toxoid immunization during pregnancy. A case-control study conducted in rural Bangladesh found that the risk of NNT was not reduced by receiving two doses of tetanus toxoid (TT2) (Hlady et al., 1992). They estimated the efficacy of TT2 at 45% and emphasized the need for improved quality of tetanus vaccine in developing countries.

The present study reveals that these multiple epidemiological factors during pregnancy and at delivery contribute to a high lethality rate of NNT.

Health facilities and health education must be improved in the Third World. Acceleration and promotion of tetanus toxoid immunization of all women of child-bearing age are very important priorities. Training of TBAs for proper obstetric care, coupled with continuous supportive supervision, is recommended in order to successfully execute NNT elimination in developing countries.

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# STOOL ANALYSIS OF PEDIATRIC DIARRHEA IN SURABAYA, INDONESIA

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**Abstract:** Forty seven stool specimens of pediatric diarrhea in Surabaya, Indonesia during the period from July to August 1993 were studied. The isolation rates of enteropathogens were as follows: 47% for enteropathogenic *Escherichia coli* (EPEC), 11% for *Vibrio cholerae*, 9% for *Shigella* spp, 21% for Rota virus and so on. The mixed infection found in 8 cases out of 35. A pH of the diarrheal stools was variable from case to case. The concentration of lysozyme and protease in diarrheal stools were higher than those in normal control stools, although the total protein content in diarrheal stools was lower than that in control stools.

## INTRODUCTION

The oral rehydration solution (ORS) therapy promoted by Primary Health Care activities has lead to decreasing the mortality rate of diarrheal diseases in the past decade. However, a microbiological study for diarrheal disease should be continued, because the isolation frequency of the enteropathogens differs from place to place, from year to year, and the patterns of their drug sensitivity are changing. In this communication, the enteropathogens and biochemical analysis in the stools of pediatric diarrhea in Surabaya, Indonesia are described.

## MATERIALS AND METHODS

**Patients and stools:** A total of 47 patients aged from 5 to 24 months at Dr. Soetomo Hospital (Surabaya, Indonesia) were examined during the period from July to August in 1993. The stool samples were collected in plastic containers, and subjected to examination within 2 hours after sampling. The stools from 38 healthy children under 2 years of age were used as controls.

**Microbiological examinations:** The isolation of *Escherichia* spp., *Salmonella* spp., *Shigella* spp., *Vibrio* spp. and *Aeromonas* spp. were carried out from all diarrheal stools as previously reported (5). Heat-labile enterotoxin (LT) of *E. coli* was tested by using the

Biken method for LT(4), and heat-stable enterotoxin (ST) of *E. coli* by using the ST detection Kit (COLIST EIA: Denka Seiken Co., Tokyo, Japan). Rotavirus was examined by using the Rotavirus detection kit (Denka Seiken Co., Tokyo, Japan).

**Biochemical examination:** The stool samples were centrifuged at 10,000 rpm for 10 min in Eppendorf tubes. Each supernatant was tested for pH, total protein contents, and the activities of lysozyme and protease. The control stools were diluted roughly ten times in normal saline solution and centrifuged as described above, and the supernatants being used for the biochemical analyses. Protein concentrations were determined by using the Bio-Rad protein assay kit (Bio-Rad Lab., Calif., U. S. A.) with bovine serum albumin as the standard. Lysozyme activity was bioassayed using *Micrococcus luteus* NCTC2665 as a substrate. Crystallized hen-egg white lysozyme was used as the standard. Total proteolytic activity was assayed on skim milk agar plates as described by Honda *et al.* (3) with protease K as the standard.

## RESULTS

**Isolation frequency:** Enteropathogenic bacteria were isolated from 35 cases out of 47 examined (74%). Enteropathogenic *E. coli* (EPEC) predominated pathogens (Table 1). Enterotoxigenic *E. coli* (ETEC) was isolated from two cases. They produced ST only. Rota

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Table 1 Isolation Frequency of Enteropathogens

No. examined	47
Pathogen detected	35(74%)
EPEC	22(47%)
ETEC	2( 4%)
Shigella	4( 9%)
V. cholerae	5(11%)
Aeromonas	1( 2%)
Rota virus	10(21%)
Pathogen not detected	12(26%)

Table 2 Biochemical analysis of the stools

	Group			
	I	II	III	IV
pH	5.2±1.0	5.9±1.5	5.7±1.4	5.1±0.7
Total protein (mcg/gr)	193±90	523±430	437±399	928±805
Lysozyme (mcg/gr)	3.3±4.3	3.1±6.5	3.1±5.6	0.2±0.2
Protease (mcg/gr)	4.1±2.8	45.5±182	33.7±154.4	1.2±1.4

I : diarrhea, pathogen detected  
 II : diarrhea, pathogen not detected  
 III : diarrhea, ( I + II)  
 IV : control stools  
 Numerals indicate the mean value±standard deviation.

virus ranked second in the isolated pathogens. The isolation rates of *Shigella* spp. and *V. cholerae* 01 were 9% (4/47) and 11% (5/47), respectively. From 8 cases, more than 2 pathogens were isolated.

Biochemical analysis of stools: Since biochemical data were so variable from case to case, the patients were classified into 3 groups as follows (Table 2); Group I includes diarrheal patients with enteropathogens detected, group II includes diarrheal patients without enteropathogen, and group III includes all cases with or without detected pathogens. Healthy control children were classified as group IV. The data were expressed as the mean values of each group and their standard deviations (Table 2). The pH of the stools revealed a great variety. The activities of lysozyme and protease were higher in diarrheal stools than in normal stools, although the total protein contents in normal stools were higher than those in diarrheal stools.

## DISCUSSION

The enteropathogens were detected in 74% of 47 diarrheal cases. This detection rate seems to be about the same with the results of other studies reported (1, 5, 6, 8). In agreement with another report (5), we found that EPEC was the bacterial agent most frequently isolated from children with diarrhea in this area. The recovery rate of ETEC(4%) was lower than reported in China(20%) (6) and Philippines (17.8%) (1). The rate of isolation of *Shigella* spp. (9%) are similar to that reported in India (6%) (8) and Philippines (11.6%) (1), but higher than that reported in China (3%) (6). Diarrhea in 12 cases without detection of enteropathogens may be due to other pathogens, such as *Campylobacter* spp., *Bacteroides* spp., *Clostridia* spp.,

Protozoa, etc., or may be due to other reasons including use of antibiotics.

Eight (17%) out of 47 cases excreted two or three pathogens in the same stool sample. The mixed infection in children with diarrhea is commonly seen elsewhere (1, 5, 6, 8). In such cases, it is difficult to decide which pathogen is responsible for the illness.

Rota virus was detected at about 20%. The high isolation rate of this virus in Surabaya was previously reported by Iwanaga et al. (5), and also in the other countries in Asia (1, 6, 8). Further investigations are needed to collect more detailed information on it.

The relationship between enteropathogens isolated and biochemical compositions of the stool was not determined in the present study because of the small number of samples. However, its relation must be very important to understand pathophysiology of the diarrheal diseases. The inversion of total protein contents and enzyme activities between diarrheal and normal stools may be explained in part by following speculations; 1) A high fluid contents in diarrheal stools may reduce the protein contents, 2) An inflammation caused by enteropathogens may lead to a high enzyme activities. However, further investigations will be need to correct the speculations. Comparative studies of the chemical composition of stools between diarrheas with a single pathogen and normal stools, or between diarrheas caused by each pathogen will elucidate the specific response of the host against each specific pathogen.

*Vibrio cholerae* 0139 which had caused an epidemic in India and Bangladesh (2, 7) was not isolated in this research. However, since the organisms are spreading to the neighboring countries, we should carefully monitor the enteropathogenic bacteria causing diarrhea in this areas.

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## A COMPARATIVE STUDY ON *STAPHYLOCOCCUS AUREUS* ISOLATED IN LAO PDR AND IN JAPAN

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During the past decade in Japan, methicillin resistant *Staphylococcus aureus* (MRSA) has been recognized as one of the major pathogens for nosocomial infection<sup>5, 6</sup>, and the lethal cases due to MRSA were occasionally seen<sup>4</sup>. Increasing of its isolation frequency was coincident with increasing use of third-generation cephem-antibiotics. Therefore, the careful use of these antibiotics is now recommended. MRSA may rarely be isolated in the area such as Lao PDR where the third generation cepheims are not used. In this communication, the strains of *S. aureus* isolated in Lao PDR are described with reference to their drug sensitivities and coagulase types comparing to those in Japan.

*Staphylococcus aureus* isolated at Mahosoto Hospital, Vientiane, Laos and at the Ryukyu University Hospital, Okinawa, Japan were used. The 54 strains of Laos were isolated in 1993, and 95 strains of Japan were isolated in 1992.

Minimum inhibitory concentrations (MICs) of Erythromycin (EM), Tetracycline (TC), Ampicillin (ABPC), Cefdinir (CFDN), and Methicillin (DMPPC) were determined by plate dilution method. Two fold dilution series of each drug in heart infusion agar (HIA) was prepared with the drug concentration ranging from 100 to 0.2  $\mu\text{g/ml}$ . The concentration of NaCl in methicillin containing HIA plates was adjusted at 4%. Cultures of the isolates in heart infusion broth at 37°C for 6hr were diluted 1 to 10 with normal saline solution (ca. 10<sup>7</sup>/ml), and were inoculated by using Microplanter (Sakuma Co. model MITP #00257) on the drug containing HIA plates including a control plates without drug. MICs of each drug were evaluated after 24hr incubation at 37°C.

Antigenic types of coagulase produced by the isolates were examined by neutralization test using "Coagulase Typing Immune Sera Kit" (Denka Seiken Co., Tokyo). Coagulation inhibition by adding anti-sera was examined using plastic microdilution plates<sup>7</sup>.

Drug sensitivity patterns of the isolates from Laos and Japan were clearly different from each other. MIC<sub>90</sub> of EM was 0.4  $\mu\text{g/ml}$  in Lao isolates but 100  $\mu\text{g/ml}$  or more in Japanese isolates. Actually Japanese isolates revealed 2 peaks of susceptibility against EM (Fig. 1-A). TC was less effective to Lao isolates. MICs of TC against 58% of Lao isolates and 25% of Japanese isolates were 12.5  $\mu\text{g/ml}$  or more. (Fig. 1-B). MICs of ABPC showed a pattern of normal distribution with the frequent MIC of 3.13  $\mu\text{g/ml}$  in Lao strains and 25  $\mu\text{g/ml}$  in Japanese strains (Fig. 1-C). All Lao isolates were inhibited at 0.8  $\mu\text{g/ml}$  or less of CFDN, whereas only 37% of Japanese isolates. Thirty-eight per cent of Japanese isolates were resistant to 100  $\mu\text{g/ml}$  of CFDN (Fig. 1-D). All Lao isolates were inhibited at 6.25  $\mu\text{g/ml}$  or less of DMPPC, but 63% of Japanese isolates were not inhibited at 100  $\mu\text{g/ml}$  of DMPPC (Fig. 1-E).

The most frequent antigen type of coagulase in Lao isolates was type 5 (54%), and in Japanese isolates, it was type 2 (53%). There were 11% of type 2 in Lao isolates and there were only 4% of type 5 in Japanese isolates (Table 1).

Table 1. Coagulase typing

type	Laos		Japan	
	number	percentage	number	percentage
1	0	0%	1	1%
2	6	11%	50	53%
3	6	11%	18	19%
4	2	4%	2	2%
5	29	54%	4	4%
6	0	0%	1	1%
7	8	15%	13	14%
8	1	2%	0	0%
NT	2	4%	6	6%
total	54	100%	95	100%

In Lao PDR, Mahosoto Hospital is the greatest hospital in the viewpoints of its size and function. Antibiotics being used over there are penicillin-G, amox-

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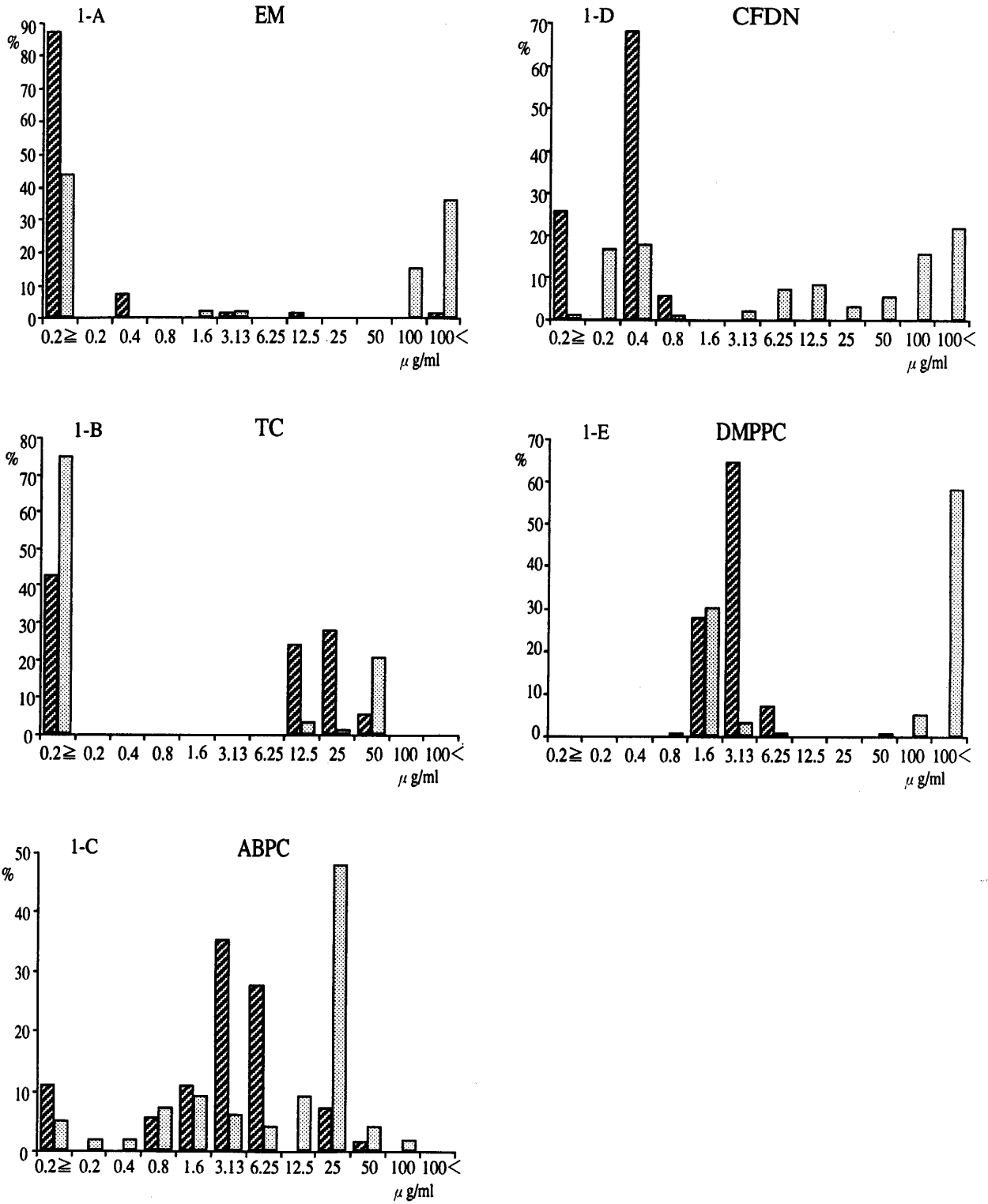


Fig. 1 MIC of A: Erythromycin, B: Tetracycline, C: Ampicillin, D: Cefdinir, E: Methicillin  
 ▨ Laos   ▩ Japan

icillin, tetracycline, chloramphenicol, gentamicin, nalidixic acid, and erythromycin. Among them, ampicillin and tetracycline are most frequently used. Reflecting this background, considerable number of *S. aureus* resistant to these two drugs were isolated. Erythromycin is less frequently used in Laos because of its price, and there were very few resistant isolates. It is noteworthy that there was no MRSA in the examined isolates from Lao. However, it is no wonder because of the antibiotics consumption pattern in Laos. The third-generation cepheims, which are closely related to the development of MRSA<sup>1, 3)</sup>, might have never been used over there. The frequency of MRSA at Ryukyu University Hospital is in an average level of all Japan<sup>2)</sup>. It is well recognized that nosocomial infection due to MRSA is a serious problem in the present Japan, however, there is no problem of this resistant organism in Laos. Reviewing the drug sensitivity pattern in Laos and Japan, we have to consider the proper use of antibiotics.

Coagulase type of *S. aureus* can be used for an epidemiological study. The most frequent coagulase type in Laos and Japan was type 5 (54%) and type 2 (53%), respectively. While, the frequency of type 5 in Japan was only 4% and type 2 in Laos was 11%. It is not known what the type 5 means, but type 2 is closely related to MRSA. The relation between coagulase type and disease specificity should be considered.

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# THE ROLE OF TUBULAR PROJECTIONS OF *PNEUMOCYSTIS CARINII* IN PATHOGENICITY OF *P. CARINII* PNEUMONIA

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**Abstract:** The aim of this research is to clarify the role of tubular projections of *Pneumocystis carinii* in *P. carinii* pneumonia. Rats, mice and cats with *P. carinii* infections were investigated by electron microscopy and semithin section light-microscopy. We found that the tubular projections adhered closely to the type I alveolar epithelial cells in the same manner as the body of trophozoites attached to the type I epithelium. Numerous tubular projections were observed between the type I epithelium and small clusters of trophozoites, and some of these projections adhered closely to the type I epithelium. In the heavily infected alveoli, many tubular projections were found around the trophozoites. These projections are likely to link the organisms, creating a big mass of organisms too big to be discharged from the lung by the surfactant alveolar clearance mechanism. We think that the tubular projections may play a role in the adherence of *P. carinii* to the type I epithelium as well as in formation of large masses of these organisms that cannot be cleared from alveoli. This can then give rise to an increasingly severe respiratory failure.

## INTRODUCTION

The development of *Pneumocystis carinii* pneumonia is significantly affected by the host defense mechanisms. It is generally considered that impaired cellular immunity is more important than impaired humoral immunity in predisposing to *P. carinii* infection. On the other hand, the parasitic behavior of *P. carinii* is also thought to be critical for the manifestation of its pathogenicity. *P. carinii* has been reported to be adherent to type I alveolar epithelial cells and form a significantly large mass in the alveoli. This parasitic behavior seems to be related to interference with gas diffusion and failure of ventilation. The pellicle of *P. carinii* trophozoite carries tubular projections, called tubular expansions, that show the same structure as the pellicle from which they arise (Vavra and Kucera, 1970; Ham *et al.*, 1971; Vossen *et al.*, 1978). Freeze-fracture replication studies of *P. carinii* revealed that the projection could be classified morphologically into four types as follows: tubular, lobopodia, branching and beaded structures (Yoshikawa *et al.*, 1987). However, the role of tubular projections in the pathogenicity of *P. carinii* pneumonia is not clear.

In this study, we show that the tubular projections

are one of the specialized structures which allow *P. carinii* to fix themselves to type I epithelial cells of alveoli that expand and contract during respiration. Furthermore, the projections around *P. carinii* may also adhere to each other giving rise to a big mass of *P. carinii* that hinders respiratory air exchange.

## MATERIALS AND METHODS

The methods of provoking pulmonary alveolus filling with *P. carinii* in cortisone-treated rats, conventionally raised nude mice and corticosteroid-treated cats have been described (Frenkel *et al.*, 1966; Shiota *et al.*, 1986, 1990). For the electron microscopic investigations the lungs were removed and cut into small pieces. These tissue fragments were then put in a specially designed plastic syringe containing 2.5% glutaraldehyde in cacodylate buffer (0.05M, pH 7.4), and air was removed from the alveoli by negative pressure produced by withdrawal of the plunger (Ebe *et al.*, 1968; Takeuchi, 1980). The tissues were immersed in the same fixative for 3 hours at 4°C and washed with the same buffer containing 8.2% sucrose. Then the tissues were dehydrated through a series of acetone and embedded in epoxy resin. Ultrathin sections were cut with a diamond



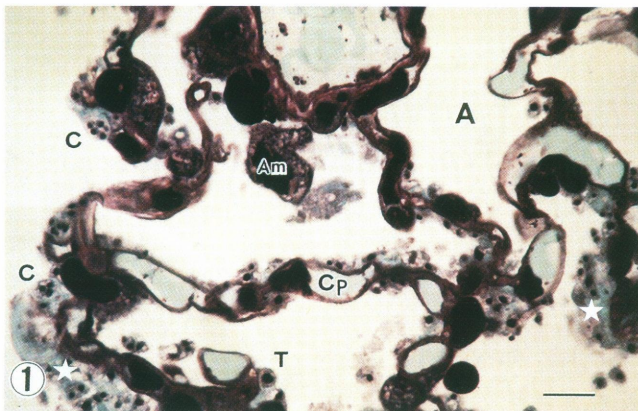


Figure 1 JB-4 plastic-embedded  $1\ \mu\text{m}$  thick section of lightly infected alveoli from the lung of a rat (after five weeks of corticosteroid treatment). In this figure, about 80 trophozoites (T) and 2 cysts (C) are observed along the surface of epithelial cell. A, air space of alveoli; Cp, capillary; Am, alveolar macrophage; \*, a small cluster of *P. carinii*; Scale bar,  $10\ \mu\text{m}$ . (Giemsa).

knife and placed on uncoated copper grids. The ultrathin sections were stained with uranyl acetate and lead citrate, and observed under a JEOL 100S electron microscope. The procedures for light microscopic investigations of Giemsa stained  $1\ \mu\text{m}$  sections embedded in JB-4 plastic were briefly as follows. The sections were stained with Giemsa (diluted 20 times with pH 7.2 PBS). More than three hours staining in an incubator at  $37^\circ\text{C}$  was required to obtain satisfactory results because the sections were so thin (Shiota, 1984).

## RESULTS

### Lightly infected alveoli:

Lightly infected alveoli were examined in  $1\ \mu\text{m}$  section embedded in JB-4 plastic and stained with Giemsa. As shown in Figure 1, the alveolar septa were almost normal, and contained much air. The alveolar capillary membrane (respiratory membrane) and blood capillaries were well preserved. Trophozoites and cysts were found to be closely attached to the type I epithelium. Measurements of the respiratory membrane in Figure 1 showed that about 20% of the entire membrane was covered with the organisms.

In TEM sections, fine structures of *P. carinii* were similar to those reported previously (Vavra and Kucera, 1970). A membrane to membrane attachment was observed between the trophozoite of *P. carinii* and type I epithelium (Figure 2). A few tubular projections

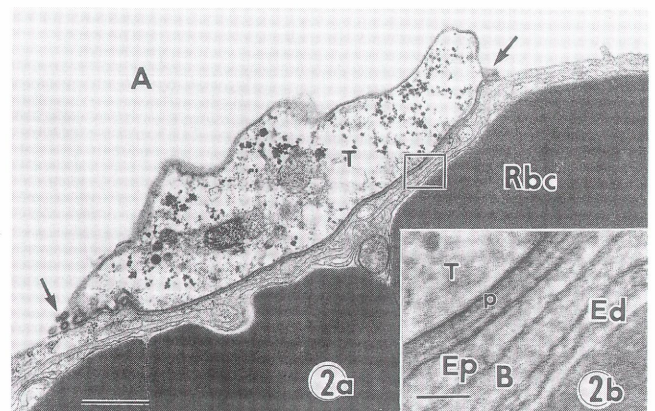


Figure 2a-b Cat, corticosteroid treated for five months. 2a: A few tubular projections (arrows) are found at the peripheral attachment surface of a small trophozoite (T) adhering to the respiratory membrane. 2b: Higher magnification of the area in the square in Figure 2a shows that the trophozoite is attached to the respiratory membrane. A, air space of alveolus; P, pellicle of the trophozoite; Ep, cytoplasm of type I epithelium; B, basement membrane; Ed, endothelial cell; Rbc, red blood cell. Scale bar,  $100\ \text{nm}$ .



Figure 3a-b Nude mouse, five months old, conventionally raised. 3a: Many tubular projections developing between a small cluster of trophozoites (T) and the respiratory membrane. 3b: Higher magnification of the area in the square of Figure 3a showing some of the projections (arrows) adhered closely to the type I epithelium. A, air space of alveolus; Rbc, red blood cell; Cp, capillary; Ep, cytoplasm of type I epithelium; B, basement membrane; Ed, endothelial cell. Scale bar,  $100\ \text{nm}$ .

were found peripheral to the attachment surface of trophozoites (Figure 2). Small clusters of trophozoites frequently showed much better developed tubular projections than the solitary single trophozoite. Tangles of

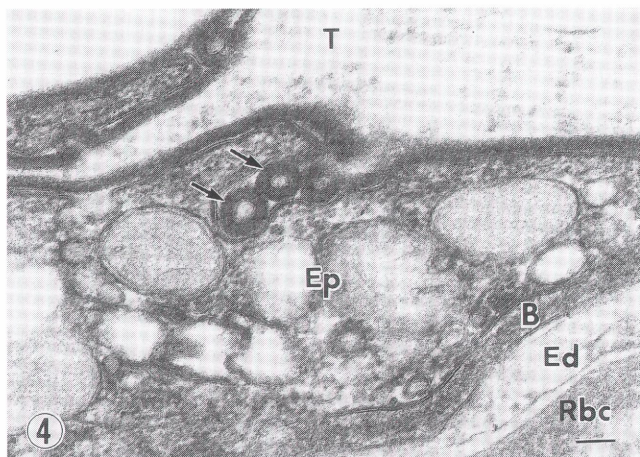


Figure 4 Cat, corticosteroid treated for five months. Some tubular projections (arrows) invaginated in the type I epithelium. T, trophozoite; Ep, cytoplasm of type I epithelium; B, basement membrane; Ed, endothelial cell; Rbc, red blood cell. Scale bar, 100 nm.

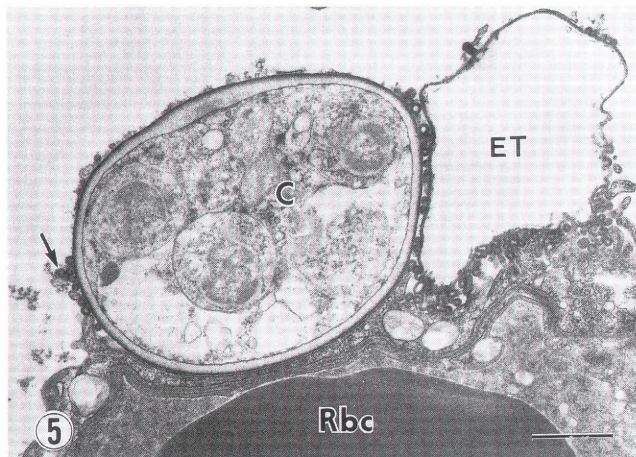


Figure 5 Cat, corticosteroid treated for five months. A few tubular projections (arrow) are found on the side of the cyst (C) adhering to the respiratory membrane. An empty trophozoite (ET) with many tubular projections is seen next to the cyst. Rbc, red blood cell. Scale bar, 1  $\mu$ m.

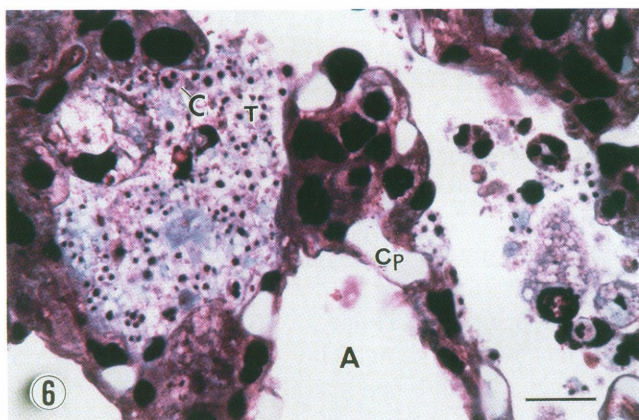


Figure 6 Rat, corticosteroid treated for five weeks. Many trophozoites (T) and cysts (C) are forming a big mass of organisms in heavily infected alveoli. The alveolar air space (A) was almost completely occupied with the clumped trophozoites and cysts. Cp, capillary. Scale bar, 15  $\mu$ m. (Giemsa).

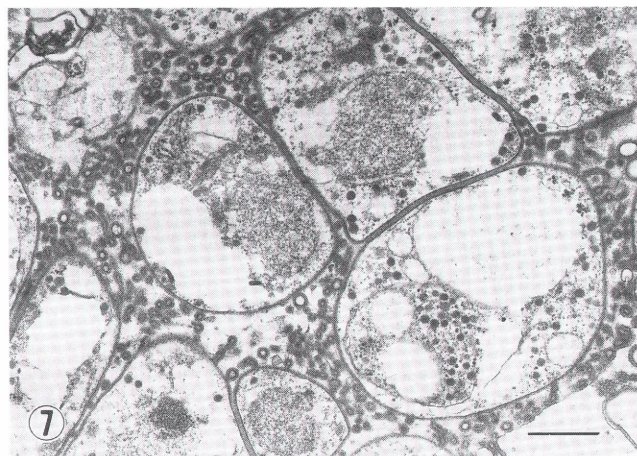


Figure 7 Nude mouse, five months old, conventionally raised. Many tubular projections are found around the trophozoites filling the heavily infected alveolus. Scale bar, 1  $\mu$ m.

numerous projections were observed between the trophozoites and type I epithelium (Figure 3a). Some of the projections adhered closely to the type I epithelium in the same manner as the trophozoites attached to its surface. That is, the surfaces of the organism and the host cells were closely opposed without fusion of the cell membranes (Figure 3b). Some tubular projections invaginated into the type I epithelium so that the epithelial cells became wider (Figure 4). A few tubular projections were also found peripheral to cysts adhering to the type I epithelium (Figure 5). The cyst adhered

closely to the type I epithelium in the same manner as the single trophozoite shown in Figure 2a. An empty trophozoite with many tubular projections was often seen beside such cysts (Figure 5).

#### Heavily infected alveoli:

In the JB-4 plastic embedded sections, many alveoli were filled with trophozoites, cysts, debris of the host cells and occasionally with foamy macrophage (Figure 6). In TEM sections, many tubular projections were found around the many trophozoites filling the alveolus

(Figure 7). These tubules showed some variation in diameter and were often filled with dense granular materials. However, they contained no microtubules, intermediate filaments or microfilaments. The tubular projections were not found on the surface of intracystic bodies (daughter cells) in the mature cysts. The structure of tubular projections and the mode of adhesion to the type I epithelium was not different among rats, nude mice and cats.

## DISCUSSION

The pellicle of *P. carinii* trophozoites consists of a 25-30 nm thick layer in which strata can be distinguished: at the inner side a unit membrane as the boundary of the cytoplasm and on the outside a 20-25 nm thick layer of moderately electron-dense material. This electron-dense outer layer contains a considerable amount of carbohydrates which bind to Concanavalin A and a lectin from *Macura ponrifera* (Yoshikawa *et al.*, 1988). These carbohydrates are known to play many roles in cell to cell adhesion. In *P. carinii* cyst walls, glucose, mannose and galactose were found and these sugars may mediate *P. carinii* host interaction (De Stefano *et al.*, 1990). A major surface glycoprotein (gp 120), which was of high-mannose type, was detected in *P. carinii*, and a possible role for the adherence of *P. carinii* through this glycoprotein to host mucin components in lung tissue was indicated by Radding *et al.* (1989).

Numerous reports have demonstrated that *P. carinii* attach themselves to a specific alveolar lining cell, the type I epithelium (Barton and Campbell, 1969; Vavra and Kucera, 1970; Ham *et al.*, 1971; Lanken *et al.*, 1980; Yoneda and Walzer, 1980, 1981; Yoshida *et al.*, 1984; Henshaw *et al.*, 1985). *P. carinii* is tightly attached to the type I epithelium, as judged by freeze-fracture electron microscopy (Yoneda and Walzer, 1983). The interaction of *P. carinii* with this cell plays a central role in the host-parasite relationship in the infection (Walzer, 1986). Tubular projections have been known for a long time (Yoneda and Walzer, 1980; Yoshida *et al.*, 1984; Henshaw *et al.*, 1985). *P. carinii* can alter its morphology to fit the contour of its immediate surroundings. Indeed, the analogy of a rock climber tenaciously grasping the side of a steep incline may well reflect the situation with *P. carinii* (Long *et al.*, 1986). Some authors clearly show these structures, but do not explain the process.

It seemed that *P. carinii* depends on the adherence of the pellicles to the type I epithelium for its par-

asitism in the alveoli. The type I epithelium extends and contracts when alveoli expand and contract with breathing. Thus, it seems that the tubular projections may help to fix the organisms to the motile type I epithelium, especially when the number of organisms had increased to fill the alveoli and many organisms had contact with type I epithelium.

In the lightly infected lungs, gas diffusion through the respiratory membrane may be interfered with by the membrane to membrane attachment of *P. carinii* and by limiting the functional area of the respiratory membrane. Furthermore, the large tangles of tubular projections, which were often found between the clusters of trophozoites and respiratory membrane, may also interfere with efficient ventilation locally.

In heavily infected lungs almost all alveoli are filled with trophozoites, cysts, debris of the host cells and occasionally with foamy macrophages (Shiota, 1984). In the present study, we found that many tubular projections developed around the trophozoites and filled the alveoli. These projections seem to link the organisms together, creating a large mass of these organisms in the alveoli anchored to the type I epithelial cells. Because clearance of alveoli normally depends on macrophages which are inhibited by immunosuppression, these masses cannot be cleared. The mixture of organisms and macrophages would need to advance into the bronchioles before becoming subject to removal by ciliary action and coughing.

In conclusion, tubular projections may play an important role in the adhesion of *P. carinii* to the type I epithelium and to one another preventing their being cleared.

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