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Secondary Infections of AIDS Autopsy Cases in Japan with Special Emphasis on *Mycobacterium Avium-Intracellulare* Complex Infection

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Ohtomo, K., Sumin, W., Masunaga, A., Iwamoto, A. and Sugawara, I. *Secondary infection of AIDS Autopsy Cases in Japan with Special Emphasis on Mycobacterium Avium-Intracellulare Complex Infection.* Tohoku J. Exp. Med., 2000, **192** (2), 99-109 —— In order to study the frequency of secondary infections of AIDS autopsy cases in Japan, especially the frequency of *Mycobacterium aviumintracellulare* complex (MAC) infection, retrospective autopsy study was conducted between 1986 and 1997 at the affiliated hospital of Institute of Medical Sciences, University of Tokyo. Secondary infections of various organs from 43 AIDS autopsy cases were examined using histopathology, genetic diagnosis of tuberculosis, Ziehl-Neelsen stain for acid-fast bacilli and immunohistochemistry. Nontuberculous mycobacterial infection (*Mycobacterium avium*) was observed in 17 cases (40%) out of 43 using polymerase chain reaction (PCR), but *M. tuberculosis* infection was not observed. Ziehl-Neelsen staining showed a positive reaction in lung and spleen tissues of 7 AIDS autopsy cases. Immunohistochemistry using anti-BCG antibody revealed positivity in 7 AIDS autopsy cases. CD4 counts of 17 AIDS patients with mycobacterial infection were less than 18.7/μl. Other opportunistic infections were also examined by histopathology. Secondary infections were present in every case, and these included cytomegalovirus infection (32 cases), *Pneumocystis carinii* (15 cases), *Candida* (16 cases), *Aspergillus* (12 cases), *Cryptococcus* (6 cases), *Toxoplasma* (6 cases), methicillin-resistant *Staphylococcus aureus* (3 cases), herpes virus (1 case) and *Entamoeba histolytica* (1 case). Malignant lymphoma was recognized in 14 cases and Kaposi's sarcoma in 6. This is the systemic report on secondary infections of AIDS autopsy cases in Japan. In diagnosis of mycobacterial infections, PCR was more useful than staining for acid-fast bacilli and immunohistochemistry. Secondary infections (especially

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mycobacterial infection) were closely associated with the low CD4 count.
AIDS; mycobacterial infection; autopsy; secondary infection

Severity of human immunodeficiency virus (HIV) infections correlates with emergence of secondary infections. The effects of HIV and of the agents of opportunistic infections can interact in a complex fashion on the immune system. In Africa the risk of seroconversion after exposure is greater and the rate of disease progression is faster. This phenomenon may be due in part to the increased state of immune activation associated with higher rate of parasite infestation and other infections.

On the other hand, tuberculosis among patients infected with human immunodeficiency virus has become an epidemic. In developed countries, the overall proportion of patients with AIDS who have tuberculosis is less than 10%, while in developing countries, particularly Africa, where more than two-thirds of all HIV-positive adults and 90% of all HIV-positive children reside (De Cock et al. 1992; Mann et al. 1992), tuberculosis presents an overwhelming public health problem. In those countries, the 1.0% to 2.5% annual risk of tuberculous infection means that by the time of infection with HIV, half of the adults or more have already had a primary infection with tubercle bacilli.

As HIV infection is the strongest known risk factor for the development of tuberculosis, it is meaningful to examine the relationship between tuberculous and nontuberculous mycobacterial infections and AIDS. Autopsy studies have been carried out in order to examine the frequency of tuberculosis in AIDS autopsy cases in Africa (Abidjan and Kinshasa), France, Portugal, China and Latin America (Pangon et al. 1988; Lucas et al. 1993; Nelson et al. 1993; Liu and Lin 1996; Politi et al. 1996; Drut et al. 1997). Here in Japan, there are few pathologic studies of the frequency of tuberculous and nontuberculous mycobacterial infection and in AIDS patients mainly because there are not many AIDS cases (Maeda et al. 1989; Koike et al. 1992; Koike and Maeda 1993; Hagiwara et al. 1995; Moriyama et al. 1998). Here we present details of retrospective secondary infections of 43 AIDS autopsy cases conducted between 1986 and 1997 with special emphasis on nontuberculous mycobacterial infection.

Materials and Methods

Patient materials

Major tissues including lung, liver, spleen, lymph nodes, brains and intestines from 43 AIDS autopsy cases (40 Japanese and 3 foreigners) were obtained from the Department of Laboratory Medicine and Pathology, The Institute of Medical Sciences, University of Tokyo. The patients (mean age; 38.5 years old, sex ratio; 40 : 3) comprised 17 hemophiliacs, 12 homosexuals, 7 heterosexuals, 2 bisexuals, one drug abuser, two persons with a blood transfusion history and 2 persons with

TABLE 1. *Risk factors in AIDS*

Risk factor	Male	Female	Total
Hemophilia	17	0	17
Homosexual contact	12	0	12
Heterosexual contact	5	2	7
Bisexual contact	2	0	2
Blood transfusion	1	1	2
Drug abuse	1	0	1
Unknown cause	2	0	2
Total	40	3	43

unknown causes (Table 1).

Histological analysis

Sections (4 μm thick) from paraffin blocks containing brain, lung, spleen, lymph nodes and interstines were stained with hematoxylin and eosin for routine histopathology, and by the Ziehl-Neelsen method for acid-fast bacilli (AFB). Periodic acid-Schiff (PAS) stain and Grocott's stain were also used for examining other secondary infections. Most of secondary infections were not detected before autopsies are carried out. Diagnoses of malignant lymphoma and Kaposi's sarcoma were made utilizing lymph node and skin biopsies.

DNA preparation and PCR

DNAs from the spleens and lungs were purified using a Nucleon II DNA extraction kit (Scotlab., Coatbridge, Scotland), as described previously (Wilson 1995). Primers corresponding to portions of the *M. tuberculosis* IS6110 and 19-kDa antigen DNA sequences were synthesized on a 381A DNA synthesizer (Perkin-Elmer Cetus, Norwalk, CT, USA) (Sugawara et al. 1998). The primer set for *M. avium* was as follows: 5′ ^{163}AAGGACTTCACCTTCGTCT^{182}G 3′ and 5′ ^{437}AACTGGATCTCGTTGTTGTTCG^{456}G 3′. The amplification reaction mixture (50 μl total volume) contained 5 μl of template DNA, as recommended by the Taq polymerase supplier (AmpliTaq™ Gold kit, Perkin-Elmer Cetus). The samples were amplified through 30 cylces in a programmable thermal cycler (Perkin-Elmer Cetus) with a three-step cycle of denaturation for 1.5 minutes at 94°C, annealing for 1.75 minutes at 60°C and extension for 2.5 minutes at 72°C. The amplification products (541, 320 and 275 bp) were analyzed by electrophoresis through an agarose 2.0% gel with a Tris-borate-EDTA buffer system, and visualized by ethidium bromide fluorescence (Young et al. 1988; Sugawara et al. 1998).

Immunohistochemistry

Immunohistochemistry was performed using avidin-biotin complex (ABC)

TABLE 2. *Clinical profiles and experimental results*

Case No.	Age	Sex	Risk factor	PCR			Stainin(g)		CD4/μl	Infection											Others
				AVI	19kDa	IS6110	Z-N	BCG		ASP	CAN	CMV	CRY	EH	HER	HPV	MAC	PC	MRSA	TOX	
1	26	M	Hemophilia A	−	−	−	−	−	16		+								+		
2	46	F	Transfusion	−	−	−	−	−	60						+						
3	39	M	Hemophilia A	−	−	−	−	−	2		+	+									ML
4	30	M	Hemophilia B	−	−	−	−	−	63	+		+									
5	41	M	Hemophilia B	+	−	−	+	+	1	+	+	+					+	+			ML
6	49	M	Homosexual	−	−	−	−	−		+								+			
7	37	M	Homosexual	−	−	−	−	−	19			+						+		+	
8	34	M	Hemophilia A	+	−	−	+	+				+					+				
9	39	M	Homosexual	−	−	−	−	−		+	+	+									ML
10	28	M	Hemophilia A	−	−	−	−	−	40			+						+			
11	23	M	Hemophilia A	+	−	−	−	−	25		+	+	+				+				
12	34	M	Hemophilia A	+	−	−	−	−	2								+	+			
13	30	M	Unknown	−	−	−	−	−													
14	42	M	Hemophilia A	+	−	−	+	+	0			+					+	+			
15	33	M	Heterosexual	−	−	−	−	−	150			+	+					+		+	ML
16	29	M	Unknown	−	−	−	−	−				+					+			+	
17	12	M	Hemophilia B	−	−	−	−	−	2	+		+			+		+				ML
18	46	M	Heterosexual	−	−	−	−	−	302												
19	40	M	Homosexual	+	−	−	−	−	24		+	+					+	+			
20	29	M	Homosexual	−	−	−	−	−	5		+	+	+								ML
21	37	M	Hemophilia A	+	−	−	+	+	1		+	+					+		+		
22	45	F	Heterosexual	−	−	−	−	−	106	+	+	+								+	
23	49	M	Homosexual	+	−	−	+	+	19	+	+	+					+			+	ML, KS
24	43	F	Heterosexual	+	−	−	−	−	32			+	+				+	+			ML

25	54	M	Homosexual	−	−	−	−	−	227	+		+								
26	36	M	Heterosexual	−	−	−	−	−				+	+							
27	35	M	Hemophilia A	−	−	−	−	−	1		+	+						+		
28	56	M	Homosexual	−	−	−	−	−	58			+					+		+	KS
29	50	M	Homosexual	−	−	−	−	−	0				+				+			ML
30	19	M	Hemophilia A	−	−	−	−	−	2			+								
31	47	M	Drug user	+	−	−	+	+	25		+	+			+	+				
32	35	M	Heterosexual	−	−	−	−	−	92			+								
33	26	M	Hemophilia A	−	−	−	−	−	26											
34	46	M	Bisexual	+	−	−	−	−				+				+				ML, KS
35	26	M	Hemophilia A	+	−	−	+	+	50	+	+					+				
36	47	M	Hemophilia A	+	−	−	−	−	42	+		+				+				
37	67	M	Homosexual	−	−	−	−	−	195		+									ML, KS
38	62	M	Bisexual	−	−	−	−	−	15		+						+			ML, KS
39	28	M	Hemophilia A	+	−	−	−	−	3			+				+	+			
40	46	M	Heterosexual	+	−	−	−	−	5	+		+				+				ML
41	47	M	Homosexual	+	−	−	−	−	8	+		+				+	+			ML
42	60	M	Transfusion	+	−	−	−	−	44			+		+		+				KS
43	54	M	Heterosexual	−	−	−	−	−	41	+		+					+	+		

Z-N, Ziehl-Neelsen; BCG, anti-BCG antibody; ASP, Aspergillus; CAN, Candida; CMV, Cytomegalovirus; CRY, Cryptococcus; EH, Entamoeba histolytica; Her, Herpes virus; HPV, Human papilloma virus; MAC, Mycobacterium avium-intracellualre complex; PC, Pneumocystis carinii; MRSA, Methicillin-resistant Staphylococcus aureus; TOX, Toxoplasma; KS, Kaposi's sarcoma; ML, Malignant lymphoma; AVI, *M. avium*.

peroxidase (PO) as described in detail previously (Hsu et al. 1986; Sugawara et al. 1995). Anti-*Mycobacterium bovis* (BCG) antibody (Dakopatts, Copenhagen, Denmark) was used at a final concentration of 0.1 μg/ml. For negative control slides, all these steps were repeated, but with an irrelevant, isotype-matched, polyclonal antibody (anti-keratin) or non-immune serum substituted for the primary antibody (Sugawara et al. 1995).

Results

Clinical profiles and results of PCR and immunohistochemistry

Table 2 shows the clinical profiles. Most of secondary infections were not detected before the AIDS patients died. The detail of secondary infections using histopathology, the PCR, acid-fast bacilli stain and immunohistochemistry is also described. Nontuberculous mycobacterial infection (*M. avium*) was detected in 17 out of 43 cases (40%), but *M. kansasii* and *M. tuberculosis* infection were not recognized by PCR and Ziehl-Neelsen stain. Fig. 2 shows positive cases of *M. avium* infection demonstrated by PCR. The mean CD 4 count of these 43 patients just before death was 47.3/μl. The CD 4 counts in these *M. avium*-positive cases were less than 18.7/μl.

Secondary infections other than mycobacterial infection were observed by histopathology of various organs: cytomegalovirus (32 cases), *Candida* (16 cases), *Pneumocystis carinii* (15 cases), *Aspergillus* (12 cases), *Cryptococcus* (6 cases), *Toxoplasma* (6 cases), Methicillin-resistant *Staphylococcus aureus* (MRSA) (3 cases), herpes virus (one case), papilloma virus (one case) and *Entamoeba histolytica* (one case) (Figs. 1A-E). They resided at sites different from those in MAC infection.

Acid-fast bacilli staining results

Ziehl-Neelsen staining for acid-fast bacilli revealed 7 positive cases (16%). Immunohistochemistry using anti-*M. bovis* (BCG) antibody showed that 7 cases (16%) were positive for BCG antigens (Fig. 3), which overlapped with those of Ziehl-Neelsen staining in anti-BCG antibody positivity. At the same time, malignant lymphoma (B cell type) and Kaposi's sarcoma were detected in 14 and 6 cases out of 43, respectively.

Discussion

This is the systemic report on the frequency of secondary infections of 43 AIDS autopsy cases in Japan. Since there are not many cases of AIDS in Japan (4583 cases in June, 1999) and autopsy was not carried out fully at medical institutions, the frequency of secondary infections in terminal AIDS patients remains unknown.

In this report, the frequency of nontuberculous mycobacterial infections in AIDS patients was 40%. The incidence of disseminated MAC disease in HIV-

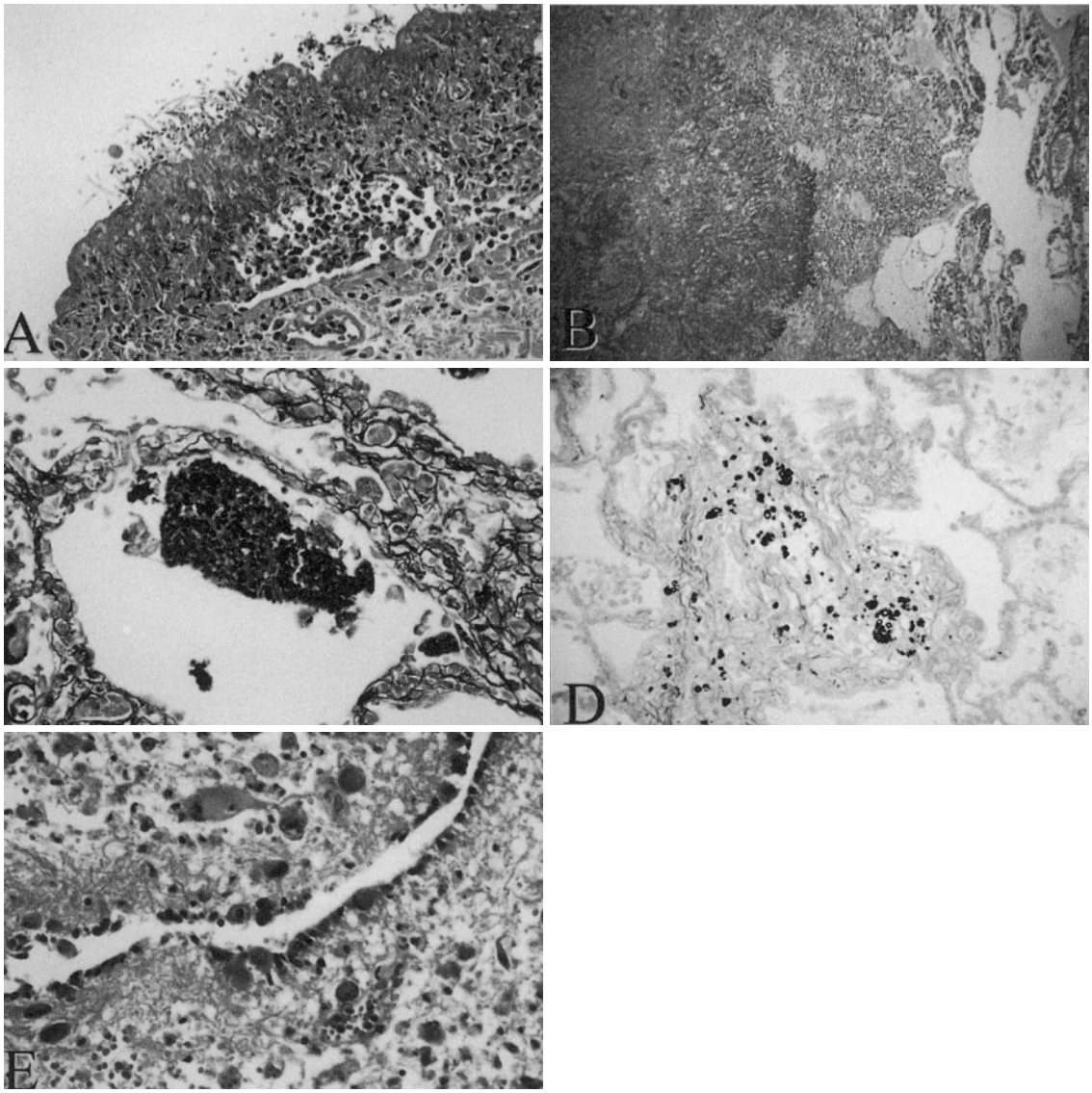

Fig. 1. Typical histologies showing candidiasis (A, Case 3), aspergillosis (B, Case 9), *Pneumocystis carinii* infection (C, Case 19), cryptococcosis (D, Case 24) and cytomegalovirus infection (E, Case 40). A, B and E: H & E; Magnification, ×200. C and D: (Grocott's stain; Magnification, ×100).

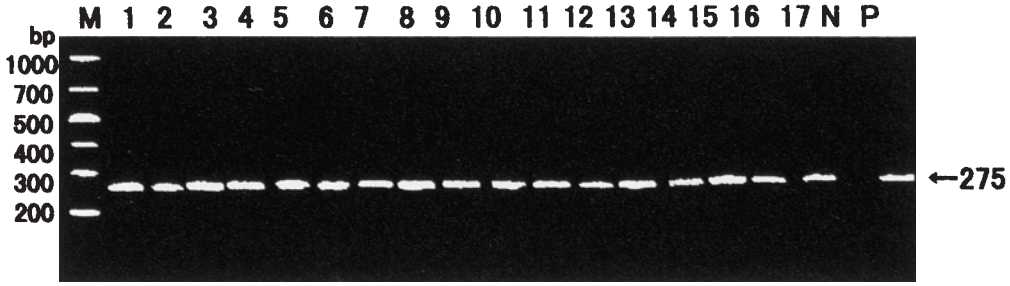

Fig. 2. PCR analysis shows 17 MAC-infected cases (275 bp). M, size marker; N, negative control (in the absence of mycobacterial DNA); P, positive control (DNA from *M. avium*).

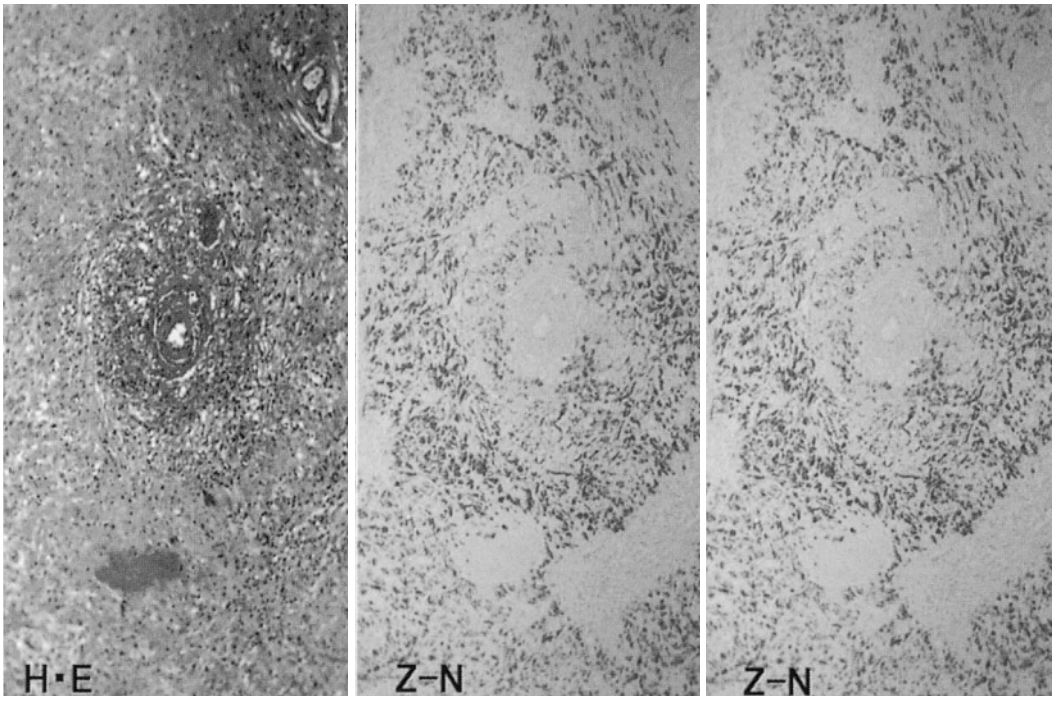

Fig. 3. Microscopy of spleen shows scattering macrophages lacking granuloma formation around blood vessel (Case No. 8) (A). H & E; magnification, ×200) (B) Ziehl-Neelsen staining shows Mycobacteria and macrophages phagocytosing them. Magnification, ×200. (C) Immunohistochemistry using anti-BCG antibody shows BCG antigen-positive macrophages. ABC-PO method. Magnification, ×200.

infected patients ranges from 20% to 40% in the absence of chemoprophylaxis or potent antiretroviral therapy. Horsburgh (1991) has reported that the incidence of *M. avium* infection is 50% in autopsy cases. There are a few reports on nontuberculous mycobacteriosis in a patient with AIDS (Maeda et al. 1989; Koike et al. 1992; Hagiwara et al. 1995). A 28-year-old hemophilia A AIDS patient presented progressive hepatosplenomegaly caused by disseminated nontuberculous mycobacteriosis. It is also reported that five out of 24 cases (20.8%) were positive for MAC (Koike et al. 1992). Although *M. kansasii* infection was observed in AIDS patients, we could not detect it in our study (Koike et al. 1992).

On the other hand, we could not detect *M. tuberculosis* in our autopsy cases. Koike and coraborates have also reported no detection of *M. tuberculosis* in their autopsy cases (Moriyama et al. 1998). Twenty five out of 649 HIV patients (3.9%) at Komagome Metropolitan Hospital were positive for *M. tuberculosis* (Moriyama et al. 1998). Therefore, it is safe to say that the incidence of tuberculosis is very low in Japan. On the contrary, the incidence of tuberculosis in foreign countries is high (Barnes et al. 1991). There are several reports on the incidence of tuberculosis including nontuberculous mycobacterial infection. In Africa, 38% of all HIV-positive cadavers and 43-54% of those dying with AIDS-defining pathology have active tuberculosis (Lucas et al. 1993; Nelson et al. 1993). In the heart tissues of 73 Portuguese necropsies, involvement of the heart

was recognized in 66 cases. Mycobacterial infection of the heart was seen in 4 cases (6%) (Politi et al. 1996). Thirty-four cases (23%) of mycobacteriosis including *M. tuberculosis* and *M. avium* were found among 151 autopsied Chinese AIDS patients. *M. avium* infection involved mostly the lymph nodes and spleen (Liu and Lin 1996). The main species of mycobacteria were isolated in 62 (20%) of 316 AIDS patients at the Claude Bernard Hospital, Paris (Pangon et al. 1989). Postmortem specimens obtained from 110 AIDS patients were cultivated during the period 1983-1986. In 20 patients, at least one specimen was positive for mycobacterium: *M. tuberculosis* in 2 cases and *M. avium* in 18 cases (Pangon et al. 1988). Although the methods for detection of nontuberculous mycobacterial infection are different, our frequency ratio (40%) was intermediate. However, the ratio of mycobacterial infection in pediatric HIV/AIDS patients is low. Fungal infections were the most common infection in 53 cases of 74 pediatric autopsies (Drut et al. 1997). Further study will be required to clarify the cause.

Because of the known incidence of infection by *M. tuberculosis* in developing countries, it is usually assumed that the postprimary tuberculous disease follows from reactivation of latent infection. Autopsy of HIV-positive tuberculosis patients frequently reveals old fibrous or calcified lesions of tuberculosis in the thorax adjacent to recent active lesions with tubercle bacilli. Studies in the preAIDS era found that 26% of primary complex lesions (lung and hilar nodes) contained cultivable bacilli (Opie and Aronson 1997). However, this does not prove the reactivation hypothesis because new lesions may also represent reinfection. Recent observations in Kenya suggest that at least some HIV-associated tuberculosis in adults is reinfection; relapse has been observed with a different genotype of *M. tuberculosis* (Hawken et al. 1993). Primary infection with *M. tuberculosis* in previously HIV-infected adult patients is observed in countries of low tuberculosis endemicity. In our patients it was not possible to determine whether infection was due to reactivation or reinfection, although IS6110-specific probes were utilized to trace the fingerprinting of individual patients.

CD 4 counts are used as a useful marker of the state of host cellular immunocompetence (normal range, 500 to 2000×10^6/liter) (Nightingale et al. 1992). An autopsy study in Abidjan found that the median CD 4 counts for patients who had disseminated tuberculosis with tuberculous meningitis were significantly higher than those for tuberculosis patients without meningitis(De Cock et al. 1992; Lucas et al. 1993). This suggests that patients presenting with tuberculous meningitis are arriving at health centers earlier. CD 4 counts in the present patients ranged from 1 to 302/μl. CD 4 counts in AIDS patients with tuberculosis ranged from 1 to 50/μl. Thus, mycobacterial infection may be closely associated with low CD 4 counts.

The incidence of secondary infections other than mycobacterial infection were remarkable. Closer examination revealed various infections including viral, fungal, protozoal infections. Interestingly, the sites where these infectious agents

reside differed from place to place. They never resided in the same sites and colonized different places. In spleen tissue as a common target of disseminated inflammation (Lucas et al. 1993), macrophages contained several to numerous tubercle bacilli, but no granuloma formation was observed (Lucas et al. 1993). It is relatively rare for AIDS patients to suffer from *Aspergillus* infection (Hasleton 1996; Moriyama et al. 1998). However, the incidennce of Aspergillosis is 28% in this study. Furrther study will be required to elucidate this discrepancy.

Suppuration and coagulative necrosis had replaced the typical caseating granulomatous response. The large number of acid-fast bacilli within macrophages is reminiscent of proliferation in the naive host. This disease state may reflect advanced HIV disease with severe immunosuppression.

In conclusion, the secondary infections found in AIDS patients were mixed ones. The highest frequency was cytomegalovirus infection (32 cases) (Moriyama et al. 1998) and the second highest was nontuberculous mycobacterial infection (18 cases). PCR using *Mycobacterium*-specific primer sets is useful for detecting tuberculous and nontuberculous mycobacterial infections. Although we compared PCR with immunohistochemistry and Ziehl-Neelsen staining for acid-fast bacilli, PCR was the most sensitive and reproducible method. However, at least two sets of primers should be used in order to increase reliability (in our case, IS6110- and 19 kDa antigen gene-specific primer sets).

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