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Laboratory Diagnosis of Pulmonary Toxoplasmosis in Patients with Acquired Immunodeficiency Syndrome

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In four cases of pulmonary toxoplasmosis occurring in patients with acquired immunodeficiency syndrome, *Toxoplasma* sp. was discovered in bronchoalveolar-lavage fluid (three cases) and in a lung biopsy specimen (one case) by using the following methods: direct examination of smears stained with eosine-methylene blue fast stain, indirect immunofluorescence assay, and inoculation of MRC5 fibroblast cell line in tissue culture.

Encephalitis is the most common complication of *Toxoplasma* infection in patients with acquired immunodeficiency syndrome and is usually related to reactivation of a previously acquired infection (6). Other organs may be affected, but only a few cases of pneumonitis entirely due to *Toxoplasma gondii* have been reported (7, 8, 11, 12). In such cases, the clinical and radiological patterns are nonspecific, and the diagnosis relies on the detection of *Toxoplasma* organisms by bronchoalveolar lavage (BAL) or lung biopsy (1).

The recent observation of four cases of pulmonary toxoplasmosis occurring in patients with acquired immunodeficiency syndrome gave us the opportunity to comparatively evaluate several diagnostic methods for demonstration of the parasite in different specimens. Four patients infected with human immunodeficiency virus type 1 were admitted to the hospital for pneumonitis. Their chest roentgenographs revealed diffuse interstitial involvement of both lungs. None had clinical symptoms indicating *Toxoplasma* encephalitis; however, in one patient, the computed tomography of the head showed a small parietal lesion which further resolved under pyrimethamine-sulfadiazine therapy. *Pneumocystis* pneumonia was suspected, and BAL was performed in three patients. Induced sputum was also obtained from one patient. The fourth patient died early after admission, and lung biopsy was performed only postmortem. Immediately after definitive diagnosis of pulmonary toxoplasmosis, a search for *Toxoplasma* species in bone marrow smears (one case) and in blood buffy coats (three cases) was made. Heparinized blood (10 ml) was collected and allowed to sediment in a tube for 2 h at 37°C; the supernatant plasma was then discarded, and the buffy coat, consisting of the upper fraction of the sediment, was collected. Serum *Toxoplasma* antibody titers were determined for each of the four patients.

A portion of the BAL fluid and induced sputum liquefied by dithiothreitol (5) was cytocentrifuged on microscope slides (600 × *g*, 10 min), and touch preparations were prepared from the lung biopsy specimen. For each specimen, three smears were prepared. One smear was stained with eosine-methylene blue fast stain (RAL 555; Société Chimique Pointet-Girard, Villeneuve la Garenne, France), and one was stained by the rapid silver methenamine method (10). Smears prepared from blood buffy coats were stained by RAL 555. For each specimen, one smear was fixed with cold acetone and tested by an indirect immunofluorescence (IFI) assay, with a monoclonal antibody anti-P30 membrane antigen of *T. gondii* as the first antibody and a fluorescein-labeled anti-mouse immunoglobulin as the second antibody (Monofluo Kit Toxo; Diagnostics Pasteur, Marne, France). The smears were examined for *Toxoplasma* sp. with a fluorescent-light microscope. In a preliminary experiment, positive controls consisting of smears of trophozoites of *T. gondii* obtained from infected mice were tested; by IFI, bright fluorescence was observed in the membrane of the parasites, with a very low background due to nonparasitic cells.

Isolation of *T. gondii* by tissue culture was attempted by using the human embryonic fibroblast cell line MRC5 (Bio-Mérieux, Lyon, France). Cover slip cultures were prepared in each well of 24-well plates (Nunc, Roskilde, Denmark), as previously described (2). Minimum essential medium (10 ml) containing ampicillin (100 μg/ml), kanamycin (200 μg/ml), and amphotericin B (5 μg/ml) was added to each blood buffy coat, each BAL fluid sample, each induced sputum sample liquefied with dithiothreitol, and each lung biopsy specimen previously homogenized in a Potter tissue grinder. After centrifugation (1,000 × *g* for 10 min), the pellet was resuspended in 4 ml of medium, and 1 ml was inoculated onto each coverslip cell culture (i.e., four replicate cultures for each specimen). Plates were incubated at 37°C in a moist atmosphere of 5% CO_2–95% air, and the medium was changed after 24 h. After 2 days of culture, monolayers were fixed with cold methanol and examined by immunofluorescence, as previously described (2), with a rabbit anti-*T. gondii* antibody (30 min at 37°C) and a fluorescein-labeled anti-rabbit immunoglobulin G (IgG) conjugate (30 min at 37°C). After two washings of 5 min each with phosphate-buffered saline, the cover slips were mounted onto slides and examined by fluorescent-light microscopy.

Serum anti-*Toxoplasma* antibody titers were determined by an IFI assay (Bio-Mérieux), and results were expressed in international units per milliliter (9). IgM antibodies were determined by an immunosorbent agglutination assay (Bio-Mérieux) (4) (Table 1).

In the smears stained with eosine-methylene blue fast

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TABLE 1. Diagnosis of pulmonary toxoplasmosis[a]

Case no. and sample	Antibody titer for IgG (IU)[b]	Presence of *Toxoplasma* sp. determined by:		
		Direct examination		Tissue culture (2-day culture)
		Eosine-methylene blue	IFI	
1				
Serum	20			
Lung biopsy		+++	+++	+++
2				
Serum	200			
BAL		+++	++	+++
Blood		−	+	+++
Bone marrow		+	+	ND[c]
3				
Serum	200			
BAL		+	+	++
Induced sputum		−	−	−
Blood		−	−	−
4				
Serum	200			
BAL		+	+	++
Blood		−	−	−

[a] Results are presented according to the number of *Toxoplasma* organisms or parasitized cells in each smear or tissue culture: absence (−), 1 to 5 (+), 5 to 100 (++), and >100 (+++).

[b] Positive serology for titers is ≥2 IU/ml. IgM titers were 0 for all samples.

[c] ND, Not done.

stain, numerous parasites were observed in the BAL from one patient and in the lung biopsy preparation; in two cases, only a few parasites were observed in the BAL smears. Most of the parasites were extracellular, although some intracellular trophozoites were observed in the cytoplasm of monocytes and polymorphonuclear leukocytes. Very few parasites were seen in the bone marrow smear of patient 2. In preparations stained with silver methamine, *Pneumocystis* sp. was not visible and *T. gondii* trophozoites were not stained.

By IFI, the parasites appeared brightly fluorescent on a dark background, with no nonspecific staining of other cells. In the smears prepared by cytocentrifugation, the thickness of the preparation was not found to interfere with the detection of the parasites, which could be easily recognized; however, in the touch preparations, the morphology of *Toxoplasma* organisms was not fully preserved and their identification was more difficult because of excessive cell debris.

Two days after inoculation, all the replicate cultures showed numerous foci of parasitized cells. Each cover slip culture inoculated with a BAL or lung biopsy specimen contained at least 100 foci; all the cultures inoculated with the blood buffy coat of patient 2 were positive. Antibody determinations showed that the four patients had a positive serology for *Toxoplasma* (IgG titers, ≥2 IU/ml), which is consistent with a chronic infection; i.e., IgG antibody titers were below 400 IU/ml and there were no IgM antibodies (3) (Table 1). From two patients, samples of serum which had been taken several months earlier were available; no significant change in the antibody titers greater than a twofold increase or decrease was observed. These serological results strongly suggest that pulmonary toxoplasmosis as well as toxoplasmic encephalitis results from a reactivation of chronic infection.

It is usually admitted that reactivation most often takes place in the brain, since this organ is the most common site of parasite encystation after an acquired infection; however, little is known about the frequency of *T. gondii* cysts in other organs, such as the lung. Our observations of four cases of pneumonitis due to *Toxoplasma* suggest that reactivation may also initially take place in the lungs, and as a result, this site may be the source of secondary dissemination. Because of the severity of the clinical symptoms and the potential risk of dissemination, rapid diagnosis of pulmonary toxoplasmosis is imperative. Direct examination of BAL smears stained with eosine-methylene blue fast stain is very efficient for diagnosis, but examination of the slides is time-consuming; moreover, in cases of mild infection, the parasites may be scarce and unidentifiable by this method. IFI appears to be a good additional technique for rapid systematic screening.

Tissue culture provided evidence of infection within 2 days after inoculation. In all cases, identification of *Toxoplasma* sp. in these cultures was easy, since the parasites replicated actively and could be readily identified by immunofluorescence. The sensitivity of this method, which has been demonstrated in a previous experimental study (2), was assessed in one of our cases (patient 2); parasitemia was demonstrable in tissue culture, whereas parasites were not visible in the stained smears and were scarce in the IFI preparations. We propose that this method of tissue culture be used in addition to direct examination methods since it may be helpful in determining the incidence of pulmonary involvement, which is likely to be underestimated in patients with acquired immunodeficiency syndrome.

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