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A Trial of Identification of Tissue Parasites by Using Unlabeled Antibody Enzyme Method 1. *Toxoplasma gondii*

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When the tissue preparations from an autopsy case demonstrated in the present study (Case 1) were brought to our laboratory by a pathologist we were unable to identify the invading organisms which were observed in most organs examined, except in the brain. Accordingly, we have sent a hematoxylin and eosin stained lung section to a colleague for identification of the organism. However, on account of our limited experience with *Toxoplasma gondii* as a crescent-shaped parasite, we have first felt a difficulty to accept his identification that the ovoid organisms observed in the lung tissue were probably *Toxoplasma* tachyzoites. In an attempt to confirm the etiologic diagnosis, staining specifically with fluorescent antibody technique was tried, according to his suggestion. No matter how stained, directly or indirectly, we have not been able to fluoresce the organism in the formalin-fixed tissue sections. Accordingly, we surmised that the antigenic activity of the organism may have been completely lost after prolonged formalin fixation.

The unlabeled antibody enzyme (peroxidase-anti-peroxidase) method has been recently developed as alternate to the labeled antibody method. Since its introduction by Mason *et al.* (1969) and Sternberger *et al.* (1970), there have been increasing number of reports on its success in formalin-fixed and paraffin-embedded sections (Palmer *et al.*, 1974; Garvin *et al.*, 1974; Klockars and Osserman, 1974; Afroundkis *et al.*, 1976). We made, thus, another attempt to stain the organism, and obtained a better result than was anticipated. Encouraged by this success, we screened additional stored tissues with this method, and could detect the organism in two additional cases.

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Materials and Methods

Human tissues. In the present study tissues from three autopsy cases were available. As shown in Table 1. all patients died of a disseminated infection with small organisms, possibly *Toxoplasma gondii*, as a complication to malignant diseases (opportunistic infection). Small pieces of tissue specimens were cut from the fixed organ which was kept in 10% formalin.

Animal tissues. Tissue specimens from mice and kitten experimentally infected with *T. gondii* were used to establish the validity of the present method. Inguinal lymph nodes were taken from acutely ill mice infected 4 days previously with a high virulent (RH) strain and brains were obtained from mice infected 6 months previ-

Table 1 Human tissues used in the present study

Case	Age	Sex	Underlying disease	Chemotherapy used	Main organ invaded	Tissue used	Preserved in (Duration)
1	60	F	Leukemia	VCR, EX, PR	Entire RES	Lung	Paraffin block (2 mo)
2	44	F	Breast cancer	5-FU, MMC, PR	Brain	Brain	Paraffin block (3 yr)
3	62	M	Stomach cancer	5-FU, CA, PR	Heart	Heart	10% Formalin (3 yr)

VCR: Vincristine; EX: cyclophosphamide; PR: predonisolone; 5-FU: fluorouracil; MMC: mitomycin C; CA: cytosine arabinoside

ously with a low virulent (Beverley) strain of *T. gondii*, respectively. Small intestine was extirpated from kitten infected one week previously by feeding of mouse brain containing *Toxoplasma* cysts. Tissues were fixed with 10% formalin for up to 10 days.

All tissues were dehydrated through graded alcohols and embedded in paraffin.

Antisera. All antisera used in the present study were prepared in our own laboratory. Two kinds of anti-*Toxoplasma* antisera were prepared: The one was obtained from 2 rabbits infected 3 months previously with Beverley strain. The other was produced in 2 rabbits by immunization with sonicated *Toxoplasma* tachyzoites harvested from mouse peritoneal cavities. Immunization was performed by injecting into multiple sites the antigen emulsified in Freund's complete adjuvant at biweekly intervals for 8 weeks. To absorb the antibodies against mouse peritoneal cell components, an appropriate amount of sonicated mouse peritoneal cells, mostly peritoneal macrophages, which were harvested from peritoneal cavities of mice injected peritoneally one week previously with 0.5 ml incomplete adjuvant, was added, and after keeping for two hours in room temperature the precipitate thus developed was removed by centrifugation. Goat antiserum to rabbit IgG was prepared 3 years ago for another study and stored in small aliquots at −80 C. Antisera to horseradish peroxidase were prepared by a series of subcutaneous injections of this enzyme into 2 rabbits following the description of Mason *et al.* (1969), and the soluble horseradish peroxidase-anti-horseradish peroxidase complex (PAP) were also prepared according to Mason and Sammons (1978).

Table 2 Antigens and antisera used for control study

a. Antigen (tissue preparations)

Mammal	Tissue	Protozoa
Man	Skin (biopsy)	*Leishmania tropica*
Man	Lung (autopsy)	*Pneumocystis carinii*
Mouse	Heart	*Trypanosoma cruzi*
Hamster	Spleen	*Leishmania donovani*
Cat	Intestine	*Isoapora felis*

b. Antisera

Animal	Antiserum against	Produced by
Rabbit	*Trypanosoma cruzi*	Immunization with epimastigotes
Rabbit	*Leishmania donovani*	

Control specimens. In order to ascertein its specificity, a number of control tissue specimens and antisera which were obtained from humans and animals infected or immunized with parasitic protozoa other than *T. gondii*, as presented in Table 2, was incorporated.

Tissue preparation. The tissue sections were prepared in the usual manner. The formalin-fixed paraffin blocks were sectioned 4 μ tick and floated on a water bath. The sections were picked up on the slide smeared thinly with a mixture of egg albumin and

glycerol. The excess water was drained and slides were placed at 60 C for at least 3 hr. The sections were deparaffinized through three changes of xylol, dehydrated through graded ethanols and washed thoroughly with distilled water.

Staining procedure. The enzymatic staining was performed through the following sequential steps. After each step the slides were rinsed with three changes of phosphate buffered saline (PBS) for 10 min each.

Step 1. 0.3% hydrogen peroxide in methanol (to block the endogenous peroxidase activity)..... 5 min

In the following steps 2 to 6, one to 2 drops of the reagents were applied to each slide which was incubated in a moist chamber.

Step 2. 2% normal goat serum in PBS (to inhibit the non-specific antibody binding)..... 30 min

Step 3. Rabbit anti-*Toxoplasma* serum (in 1:100 dilution)..... 30 min

Step 4. Goat anti-rabbit IgG serum (in 1:200 dilution)..... 30 min

Step 5. Rabbit PAP (in 1:200 dilution) ..... 30 min

Step 6. 0.05% 3-3′ diaminobenzidine tetrahydrochloride and 0.003% hydrogen peroxide (in 0.05 M trishydroxymethyl-aminomethane-HCl, pH 7.6)..... 20 sec

Following the enzymatic staining the conventional hematoxylin staining was added to demonstrate the nuclei of tissues. The slides were then dehydrated with graded alcohols, in xylol and mounted in Permount for examination by light microscopy.

Electron microscopy. The specimens used for the electron microscopic studies were obtained from both the formalin-fixed, paraffin blocks (Case 1 and 2) and the formalin-fixed tissue (Case 3). The paraffin blocks were deparaffinized through xylol and rehydrated through graded ethanols. All tissues were fixed for 4 hr in 3% glutaraldehyde, postfixed for one and half hours in 1% osmium tetroxide and embedded in Epon. Ultrathin sections were cut on a LKB microtome. Sections were stained with uranyl acetate and lead citrate and examined under a JMS 1,000 electron microscope.

Results

By means of the peroxidase marker, *Toxoplasma* was clearly seen as brown corpuscles isolated or forming clusters which stood out prominently against the background and the hematoxylin staining of the host cell nuclei. The necrotic tissue around *Toxoplasma* trophozoites stained light brown, presumably a specific reaction to the antigenic substances released from the organisms. With this method the organism was stained somewhat irregularly, probably due to the multilayer antibody binding system. Therefore, it could not be used to ascertain the antibody binding site or the fine structure of the organism. Since the discrimination between brown colored organisms and blue colored cell nuclei, especially nuclear debris were often difficult in the black and white pictures photographs illustrating this paper were taken on preparations stained with the immunoenzyme method alone, without counterstain with hematoxylin.

In the lung from Case 1, there was diffuse interstitial pneumonia with extensive necrosis. The organisms were found lying either in the alveolar lining cells or in proliferating interstitial macrophages. Free organisms were also seen within the necrotic tissue (Fig. 1). By the immunoenzymatic staining the organisms could be seen in striking contrast against the background (Fig. 2). This case may be an acute infection with tachyzoites.

A 10×7 mm-sized necrotic focus located in the right occipital cortex was seen in the brain from Case 2. It was composed of irregular areas of necrosis with slight micro-

glial and lymphocytic reaction. Within the zone of necrosis there were abounant free organisms and cysts at the periphery (Fig. 3). The organisms were stained well by the present method (Fig. 5). It is likely that this case is an exacerbation of an occult bradyzoite infection.

Within the myocardial fibers of Case 3, clusters of the organisms were observed. However, no free organisms could be found. In the perinuclear position of myocardial cells fine granules, presumably lipofuscin, were also observed (Fig. 5). By the immunoenzymatic staining the organisms were stained somewhat weaker than those of the above 2 cases, probably due to inadequate preservation of the specimen, as discussed later. Nevertheless, the perinuclear granules were denser-staining than *Toxoplasma* (Fig. 6).

The tachyzoites in lymph node and the cyst in brain from mice (Figs. 7, 8), and schizonts and the gametocytes in the mucosal epithelial cells of a feline intestine (Fig. 9) could be stained with the present method.

However, no cross-reactions either between *Toxoplasma* and the heterospecific antisera anti-*Trypanosoma cruzi* and anti-*Leishmania donovani*) or between the control organisms (*L. tropica, Pneumocystis carinii, T. cruzi, L. donovani and Isospora felis*) and anti-*Toxoplasma* antibody were observed.

Discussion

The demonstration of *Toxoplasma gondii* in tissue is the most reliable diagnosis of toxoplasmosis in autopsy or biopsy cases suspected of being caused by this organism. However, this organism is ordinarily difficult to identify in the tissues stained by conventional methods. Because of lack of prominent morphological features and its small size it may be confused with other organisms, such as *Leishmania, Trypanosoma, Sarcocystis, Besnoitia,* fungi and others (Frenkel, 1956). Several investigators (Goldman, 1962; Kalderon *et al.*, 1964; Callaway *et al.*, 1968; Daneshbod, 1977) have indicated that the use of electron microscopy can facilitate the differentiation of *Toxoplasma* from other organisms. However, this is difficult to apply, unless the organism is present in large numbers. Moreover, the formalin-fixed specimen is not suitable for the electron microscopic study, especially after prolonged fixation. We tried in our three cases and demonstrated organisms in Case 1 (Fig. 10) and 2, but failed in Case 3 in which only a few myocardial fibers were parasitized. Staining with labeled antibodies, fluorescein or enzymes, is widely used as a tool for demonstration or identification of the parasites in tissue section. These methods also have limitation to the use as routine diagnostic aid, since these are generally preferred to be used unfixed and rapid frozen tissue to preserve antigenic activity of the organism.

The method used in the present study has potential advantages over the conventional labeled antibody methods, as have emphasized by several investigators (referred above): (1) The major advantage of the PAP technique is that it does not call for equipment or techniques other those normally used in histology laboratories. (2) By counterstaining with routinely used dyes, such as hematoxylin or methylgreen the localization of the staining object can be easily identified. (3) Permanent slides are produced, permitting re-studying and review of the evidence.

Probably the main shortcoming of this method relates to non-specific staining of the residual pigments, such as lipofuscin occurring in various tissues and hemoglobin residures appearing occasionally in the necrotic tissues. Since these granules may be brown in color and may have also the peroxidase activity, they could be confused with the reaction products. In generally, however, these granules can be readily

distinguished from the organism with their irregular distribution pattern and their densely staining property.

In an effort to obtain highly immunoreactive and specific antibodies to *Toxoplasma*, two kinds of antisera obtained from rabbits chronically infected and immunized were employed. However, they showed essentially similar reactivity in our specimens. From a practical view point, therefore, it is likely that any potent antiserum would be suitable for identify the organism.

Although it was demonstrated that the antigenic activity of *Toxoplasma* can be sufficiently preserved in the formalin-fixed, paraffin block it appeared to be less resistant in formalin, since the organisms from Case 3 which were preserved in 10% formalin for 3 years stained weaker than those from Case 2 which was preserved in paraffin block for the same duration. Further assessments will be required to determine the effects of the fixatives.

In conclusion, the successful demonstration of *Toxoplasma* in formalin-fixed and paraffin section illustrated the usefulness of the method for diagnosis.

Further studies for applying this system to the histological diagnosis of other parasitic diseases are now in progress in our laboratory, and the results will be present in a following paper.

Summary

The unlabeled antibody enzyme (peroxidase-anti-peroxidase) method for demonstrating *Toxoplasma gondii* in formalin-fixed and paraffin-embedded tissue section is described. The organisms are clearly seen in the tissue as brown corpuscles standing out in sharp contrast to non-stained background. By counter-stain with conventionally utilized dyes the localization of the organisms can be easily identified. This method was proved to be of great value for diagnosis of toxoplasmosis.

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非標識抗体酵素法による組織内寄生虫同定の試み
1. トキソプラズマ

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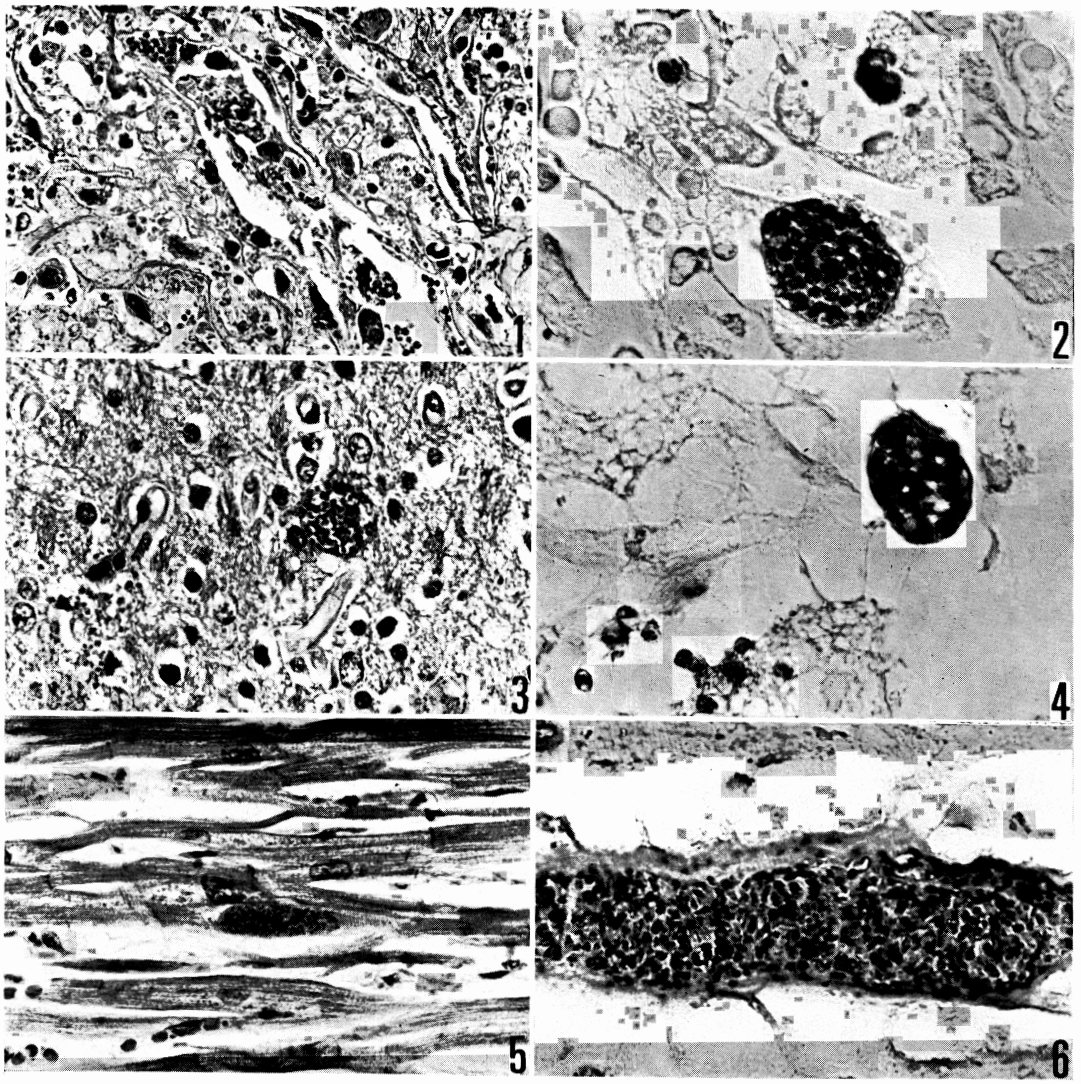
トキソプラズマ症の疑われる症例の剖検または生検組織中にトキソプラズマ（Tp）が見出されれば診断はほぼ確実であるが，通常の染色を施した組織標本中に見付け出すというのは重症感染例でもない限りかなり難かしい．新鮮な未固定の組織が得られれば凍結切片を作製して標識抗体（螢光色素または酵素）による染色で Tp を検出することができるが，ホルマリンで固定された標本では成功しない．

非標識抗体酵素法の抗原検出感度はきわめてすぐれ，通常のホルマリン固定・パラフィン包埋組織切片中の抗原も検出可能であるとした免疫組織学領域からの報告に着目し，組織標本中の Tp の染色を試みた．

Tp 感染が直接の死因となったとみられる悪性腫瘍患者3例からの剖検組織では，Tp は抗体によく反応し鮮明な黄褐色に染色され，ヘマトキシリンの後染色により Tp の組織中での局在も明確に知ることができる．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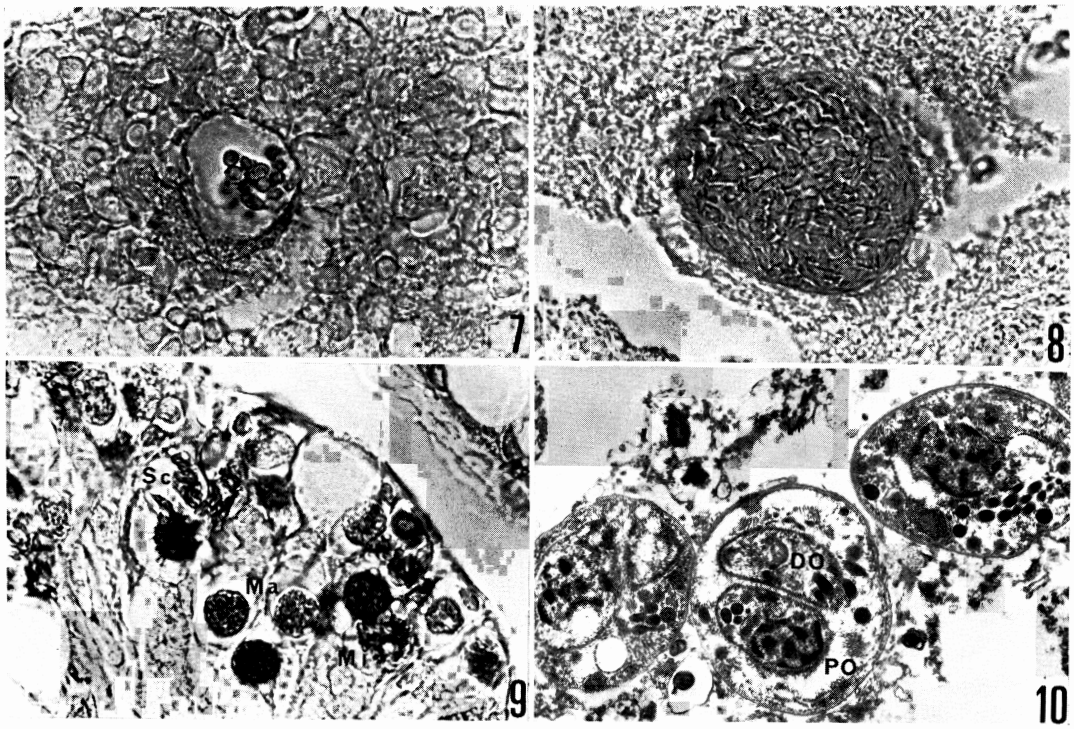

Explanation of Figures

Fig. 1 Lung section of Case 1. *Toxoplasma* tachyzoites are seen proliferating in alveolar lining. H. & E. stain, ×400.

Fig. 2 Cluster of the organisms in alveolar lining of Case 1. PAP stain, ×1000.

Fig. 3 Brain section of Case 2. Cyst and free organisms are seen at the edge of the necrosis focus. Microgrial and inflammatory reactions are slight. H. & E. stain, ×400.

Fig. 4 Cyst and free organisms in the necrotic brain tissue of Case 2. PAP stain, ×1000.

Fig. 5 Myocardium of Case 3. Cyst in the myocarial fiber is not accompanied by inflammation. Lipofusin granules are observed in each myocardial fiber. H. & E. stain, ×400.

Fig. 6 Cyst in myocardial fiber of Case 3. PAP stain, ×1000.

Fig. 7 Tachyzoites in the mouse lymph node. PAP stain, ×1000.

Fig. 8 Cyst in the mouse brain. PAP stain, ×1000.

Fig. 9 Macrogametocyte (Ma), microgametocyte (Mi) and Schizont (Sc) in the gut epithelium of cat. PAP stain, ×1000.

Fig. 10 Electron micrograph of the organisms of Case 1. In middle, it is seen a dividing parent organism (PO) containing two daughter organisms (DO).