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Ultrastructural immunolabelling of *Penicillium marneffei*

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Summary

For the immunoelectron microscopy, difficulties are commonly encountered on the preservation of the ultrastructure and the antigenicity at the same time. The factors such as reagents, concentration, duration, and temperature for each of the fixation, dehydration, infiltration and embedding, and antigen detection, have to be varied in order to optimise the results. In this study, the subcellular localization of an abundant antigenic candidate cell wall protein MpIp of pathogenic dimorphic fungus *Penicillium marneffei* was determined with a specific antibody raised against this recombinant protein. Different fixatives were assessed while the duration of the fixation, the dehydration with series of ethanol, the embedding with LR White resin and the signalling with gold-conjugated secondary antibody were applied constantly. It is concluded that the best fixation method for the ultrastructural immunolocalization of *Penicillium marneffei* is by suspending in 4% paraformaldehyde plus 2% glutaraldehyde in 0.1M phosphate buffer pH 7.4 for 30 minutes at room temperature.

Key words: Ultrastructural immunolabelling, Penicillium marneffei.

Introduction

Ultrastructural immunolabelling techniques have been well established and their values in the diagnostic pathology are universally recognized[1-5]. However the fixatives routinely used in the electron microscopy often destroy the target antigenicity. On the contrary, the fixatives used in the immunohistochemistry cannot preserve the ultrastructure significantly. In order to compromise the preservation of both the fine structure of the cells and the molecules of the antigens, the techniques for electron microscopy have to be modified. This includes the alteration of the type and duration of fixation, choice of embedding medium, pre- or post-embedding immunolabelling, and choice of anitbody detection system[6-13]. Unfortunately, no matter how great effort the scientists had given, there is no single procedure applicable to all antigens. Whenever a new tissue constituent is to be immunolabelled, a pilot study has to be carried out to assess the variables of the procedures which give optimum preservation of ultrastructure as well as antigenicity.

Penicillium marneffei is a dimorphic pathogenic fungus endemic in Southeast Asia and southern parts of China[14-17]. It is the causative agent of a disseminated and progressive disease, penicilliosis marneffei, of both immunocompetent and immunocompromised patients. However, penicilliosis is particularly common for people infected with human immunodeficiency virus (HIV). In certain parts of Southeast Asia, disseminated infection of *P. marneffei* is the third most common opportunistic infection in HIV-positive patients, after extrapulmonary tuberculosis and cryptococcal meningitis[16]. In addition, infections by *P. marneffei* have been reported for visitors travelling to the endemic region[17].

It was shown previously that patients with penicilliosis developed elevated titers of antibodies against *P. marneffei* cells in immunofluorescence and immunodiffusion tests[18-20]. We cloned a *P. marneffei* gene MP1 that encodes a novel highly antigenic protein Mp1p[21]. The sequence analysis of this protein revealed some structural features of cell wall proteins of fungi *Saccharomyces cerevisiae* and *Candida albicans*. Recombinant Mp1p protein was then produced and used to immunize rabbits to generate a specific rabbit polyclonal antibody[21]. This antibody was then used to study the subcellular localization of Mp1p protein.

Materials and Methods

Penicillium marneffei yeast cells were freshly obtained by harvesting from the culture medium and spinning at 300 x g rpm for 10 minutes. Cells were washed twice in 1 x PBS (13.7 mM sodium chloride, 0.27 mM potassium chloride, 1 mM phosphate buffer, pH 7.4) and fixed in one of the following fixatives for 30 minutes at room temperature. (A pilot study of fixation in 4% paraformaldehyde for 10, 15, 30 and 60 minutes at room temperature concluded that acceptable ultrastructure was obtained by fixing for 30 minutes.) For routine fixative control, the cells were fixed in 2.5% cacodylate buffered glutaraldehyde and postfixed in 1% cacodylate buffered osmium tetroxide for 30 minutes at room temperature respectively, dehydrated and embedded in epoxy resin.

Fixatives:

Stock Solution (8% paraformaldehyde):
8 g Paraformaldehyde
100 mL Phosphate Buffer (0.1 M, pH 7.4)

The solution was heated to 60°C with stirring. If the solution is milky, 1 M sodium hydroxide was added until it was clear.

A. 4% Paraformaldehyde (PF)

   50 mL Stock Solution
   50 mL Phosphate Buffer (0.1 M, pH 7.4)

B. 4% Paraformaldehyde +0.5% Glutaraldehyde (PF0.5G)

   2 mL Glutaraldehyde (25%)
   50 mL Stock Solution
   48 mL Phosphate Buffer (0.1 M, pH 7.4).

C. 4% Paraformaldehyde + 1% Glutaraldehyde (PF1G)

   4 mL Glutaraldehyde (25%)
   50 mL Stock Solution
   46 mL Phosphate Buffer (0.1 M, pH 7.4).

D. 4% Paraformaldehyde + 2% Glutaraldehyde (PF2G)

   8 mL Glutaraldehyde (25%)
   50 mL Stock Solution
   42 mL Phosphate Buffer (0.1 M, pH 7.4).

E. 2.5% Glutaraldehyde (G)

   10 mL Glutaraldehyde (25%)
   50 mL Cacodylate Buffer (0.2 M, pH 7.4)
   40 mL distilled water.

F. 2.5% Glutaraldehyde and 1% Osmium Tetroxide (GO)

   2.5% Glutaraldehyde was prepared as for fixative E.

   1% Osmium Tetroxide was prepared as follows,

   1 g Osmium Tetroxide
   100 mL Cacodylate Buffer (0.1 M, pH 7.4)

   Fixed in 2.5% Glutaraldehyde and then in 1% Osmium Tetroxide for 30 minutes at room temperature with cacodylate buffer washes in between.

Processing Procedure:

1. After fixation, the cells were rinsed in the buffers used in the corresponding fixatives.

2. They were then dehydrated in graded series of ethanol at room temperature:
   50% alcohol 5 min.
   70% alcohol 5 min.
   90% alcohol 5 min.
   100% alcohol 5 min.

3. The cells were infiltrated in 1:1 mixture of 100% ethanol and LR White medium grade resin (Sigma, St Louis, USA) for 60 minutes at room temperature, followed by two changes of pure LR White resin at room temperature for 60 minutes each. They were then infiltrated in fresh LR White resin for overnight and another change of resin for 60 minutes at room temperature.

4. The cells were embedded in fresh resin using re-capped gelatin capsules.

5. The resin was polymerised at 37°C for 96 hours.

Immunogold labelling:

1. Ultrathin sections were cut at 100 nm and mounted on 200 mesh uncoated gold grids.
2. The grids with the sections facing downwards were floated on 30 μL of 3% bovine serum albumin (BSA) in PBS for 20 minutes at room temperature.
3. The sections were incubated with different dilution of rabbit immunised against PMAP serum in PBS containing 3% BSA for 2 hours at room temperature.
4. They were rinsed in PBS containing 0.1% Tween 20 for 3 changes, 5 minutes each and a final rinse in 1% TBSA (20 mM Tris, pH 8.2, containing 1% BSA) for another 5 minutes.
5. The sections were then treated with gold conjugated goat anti-rabbit IgG (Amersham, Buckinghamshire, 10 nm) diluted 1:20 in 1% TBSA for 1 hour at room temperature.
6. Washing in 1% TBSA was carried out for 3 changes, 5 minutes each and then washed in distilled water.
7. They were counterstained with uranyl acetate for 2 minutes and lead citrate for 1 minute at room temperature.

Negative controls were incubated with preimmunized rabbit serum at the primary antibody incubation step. Sections were examined under JEOL JEM-100SX transmission electron microscope at 80 kV.

Results

The summary of the results of various fixatives is shown in Table 1. The sections incubated with rabbit immunised against PMAP serum diluted 1:40 were used for comparison. The best ultrastructural preservation of the ultrastructure of *Penicillium marneffei* was obtained by fixation of the cells in 2.5% glutaraldehyde and 1% osmium tetroxide while the worst preservation was by 4% paraformaldehyde.

As from the observation, the trend of the preservation of the antigenicity was always opposite to that of the ultrastructure. The best result came from the cells fixed in 4% paraformaldehyde with clean background.

Table 1. Preservation of Ultrastructure and Antigenicity by Various Fixatives.

Fixatives	**Ultrastructure#**	**Immunoreactivity#**
4% Paraformaldehyde	+	++++
4% Paraformaldehyde + 0.5% Glutaraldehyde	+	+++
4% Paraformaldehyde + 1% Glutaraldehyde	++	+++
4% Paraformaldehyde + 2% Glutaraldehyde	+++	+++
2.5% Glutaraldehyde	+++	++
2.5% Glutaraldehyde And 1% Osmium Tetroxide	++++	+

+ acceptable preservation

++ moderate preservation

+++ good preservation

++++ excellent preservation

With the addition of glutaraldehyde, the ultrastructure was enhanced while the preservation of antigenicity began to deteriorate. The fixation in 2.5% glutaraldehyde and 1% osmium tetroxide, the fixatives used in routine electron microscopy, gave the best ultrastructure but the number of gold labels decreased a lot.

The promising result for both ultrastructure and antigenicity was acquired by the fixation in 4% paraformaldehyde plus 2% glutaraldehyde in 0.1M phosphate buffer pH 7.4 for 30 minutes at room temperature. The electron micrograph showed that the immunogold particles were specifically distributed throughout the entire thickness of the *Penicillium marneffei* yeast cell wall (Fig. 1). The control section negatively stained with preimmunized serum was clear from gold labels (Fig. 2). These indicate that Mp1p is an abundant protein antigen located specifically on the outer layer of the cell wall without direct association with cytoplasmic membrane.

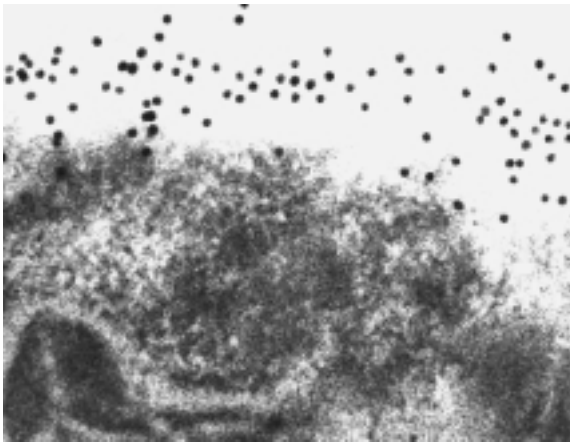

Fig. 1. Immunogold labelling of *Penicillium marneffei* fixed in 4% paraformaldehyde plus 2% glutaraldehyde in 0.1 M phosphate buffer pH 7.4, X 210,000.

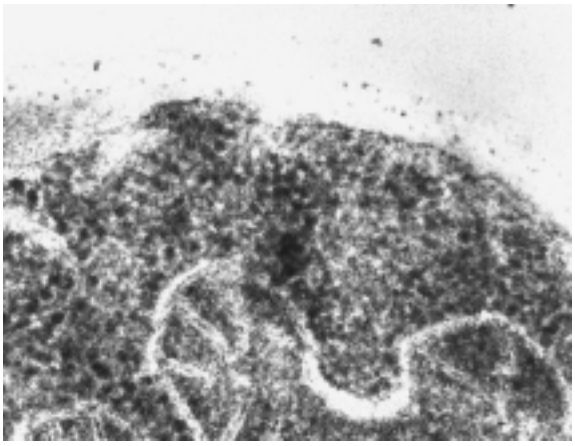

Fig. 2. Negative control showing no background and non-specific labelling, X 210,000.

Discussion

The trend of the present study conformed with the past findings[22]. The degree of antigen masking is directly proportional to the concentration of the glutaraldehyde used. It is due to the cross-linking properties with the protein. However it also explains why glutaraldehyde is the best of choice for conventional electron microscopy[23].

Formaldehyde is commonly used instead because of its minimal cross-linking properties and the reversibility of the formed cross-link[23]. Therefore the preservation of antigenicity is enhanced. However its use will lead to poor ultrastructural preservation. This limitation can be counterbalanced by the addition of glutaraldehyde. With the balance of the concentration of the two chemicals, a good compromised result of both ultrastructure and antigenic preservation can be obtained. Unfortunately, for different target antigens, the balance points require intensive studies to be carried out.

The conventional epoxy resin used for embedding is usually not suitable for immmunoelectron microscopy. A substitute, LR White - a kind of hydrophilic acrylic resin, is now commonly used for the purpose[24]. It can tolerate the presence of water but we rinsed the cell blocks in absolute alcohol to facilitate the infiltration of the resin. The polymerisation temperature was also modified to 37°C to improve especially the preservation of the heat sensitive antigens.

Fungal cell wall is involved in maintaining the integrity of fungal cells. It determines cellular morphology, mediates the attachment to the host epithelium, and is often the targets for host immunity. Extensive molecular analysis and genome studies of *Saccharomyces cerevisiae* revealed that there are more than 50 cell wall proteins performing a variety of functions[25]. Examination of their subcellular localization could significantly enhance the studies of the function of these genes. Our experiment should provide a very good example for such studies.

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