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Identification of surface antigen of *Trichomonas vaginalis*

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Abstract: Plasma membrane proteins of a Korean isolate of *Trichomonas vaginalis* HY-1 were fractionated for antigen analysis. Homogenates of *T. vaginalis* were fractionated by the differential centrifugation using sucrose step-gradient method. The interface layer from the 25%/45% sucrose was collected as a plasma membrane fraction and its purity was examined by transmission electron microscopy. The antigenicity of plasma membrane fraction was analysed by enzyme-linked immunoelectrotransfer blot technique with immune rabbit serum and compared with surface antigen labelled with N-hydroxysuccinimide-biotin. The fluffy fraction of 25%/45% sucrose interface was homogeneous and membrane particles were present as extended sheet and concentric vesicles showing typical trilamellar appearance under transmission electron microscope. Seven fractions at 40, 50, 60, 110, 130, 140 and 150 kDa were identified as the antigenic membrane proteins in EITB with anti HY-1 rabbit serum. The common band at 60 kDa was detected both in antigenic fractions of plasma membrane and surface protein labelled with NHS-biotin. This result indicates that this protein is considered as a major surface antigen of *T. vaginalis*. The role of this surface antigen at 60 kDa should be studied further.

Key words: *Trichomonas vaginalis*, differential centrifugation, sucrose step-gradient, plasma membrane fraction

INTRODUCTION

Trichomonas vaginalis is a flagellated parasitic protozoa in human, which is responsible for trichomoniasis, a sexually transmitted disease causing significant worldwide morbidity.

Surface antigens of parasitic protozoa play the important roles in disease pathogenesis by serving as attachment factors, direct virulence factors, or mediating evasion of the host immune response through direct suppression or antigenic variation (Scott and Snary, 1979; Peterson and Alderete, 1982; Ryu *et al.*, 1992). Adhesion of the organisms to mucosal cells was considered a first and pre-requisite step for *T. vaginalis* infections (Alderete and Pearman, 1984). Attachment of non-proliferating *T. vaginalis* to HeLa cells and to cultured vaginal epithelial cells in serum free culture system was mediated by proteinaceous adhesin (s) on the parasite surface (Alderete *et al.*, 1988).

Variation in intrinsic virulence among isolates of *T. vaginalis* has been demonstrated in both animal models (Honigberg, 1961) and tissue culture systems (Kreiger *et al.*, 1983) and geographic variation among isolates of *T. vaginalis* was demonstrated by using monoclonal antibodies and the indirect immunofluorescence technique (Kreiger *et al.*, 1985). It is important to analyze surface

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antigens in detail to study antigenic variation, as it is assumed that this phenomenon is due to the surface antigen of the organisms and may play pivotal roles in evasion of host immune response or susceptabilty to reinfection (Scott and Snary, 1979; Ryu *et al.*, 1992). However few experimental data on antigenicity of surface anitgen and antigenic heterogeneity have been available. Recently, Woo *et al.* (1993) tried to tag the surface protein of *T. vaginalis* by biotinylation of live organisms with N-hydroxysuccinimido-biotin (NHS-biotin) which is not permeable to live cells and incorporate to surface protein via lysin residue. In their study they identified antigenic fractions of surface proteins by an immunoprecipitation study with immune rabbit sera and proposed that the six peptides of surface protein at 46, 60, 68 (70), 90 (96), 125 and 220 kDa showed an antigenicity.

This study was designed to isolate plasma membrane of *T. vaginalis* HY-1, by centrifugation on a sucrose step-gradient and to analyze their antigenic fractions by an immunoelectrotransfer blot. The antigenic bands of membrane fractions were compared with biotinylated surface antigens of *T. vaginalis*.

MATERIALS AND METHODS

1. Culture

T. vaginalis isolate HY-1 was obtained from a vaginal swabs of female outpatients attending the Gynecology and Obstetrics Clinics at Hanyang University Hospital. Parasite was cultured at 37°C in Diamond's TPS-1 medium supplemented with 10% heat-inactivated bovine serum and subcultured at 2-day intervals (Diamond, 1957).

2. Antiserum

Antisera against *T. vaginalis* HY-1 were generated in 3.5-*kg* New Zealand white rabbits as described by Min *et al.* (1993). Briefly, rabbits were injected intradermally with a mixture of 0.5 *ml* of trichomonal suspension containing 2×10^7 organisms and 0.5 *ml* of Freund's complete adjuvant (Difco, Mich). Booster injections were performed 3 weeks later with equal volumn of organisms in Freund's incomplete adjuvant. Rabbits were bled 2 weeks after booster immunization. Control rabbit sera were obtained before immunization. The sera were stored at -70°C until use.

3. Membrane isolation from *T. vaginalis*

Four liters of cultured parasites were harvested, washed in PBS and centrifuged at 4,800 × *g* for 15 min at 4°C. The cell pellet was resuspended in 20 times its volume of hypotonic buffer (10 mM Tris/HCl, pH 7.4, 30 mM $MgCl_2$, 1mM PMSF) for 10 min at 0°C. The swollen cells were lysed with serrated pestle (AA, Thomas, NJ) in tight dounce homogenizer. The homogenate was then centrifuged at 1,000 × g for 1 min at 4°C in a swing-out rotor to remove nuclei and any whole cells resisting homogenization. One milimole PMSF was added in each step as an antiprotease. The partially cleared supernatant was removed and recentrifuged. The supernatant contained crude plasma membrane was then made to 25% sucrose by adding 50% sucrose. Twenty mililiter aliquots of this sample were layered onto 10 ml of buffered 45% sucrose and centrifuged in a swing-out rotor (TST28.38, Kontron Instruments) at 100,000 × g for 60 min at 4°C. After centrifugation, the fluffy band which had collected at the 25%/45% sucrose interface was removed, diluted and recentrifuged at 100,000 × g for a further 20 min at 4°C (Clark and Holberton, 1986).

4. Transmission electron microscopy

The pellets of membrane fraction were fixed in 3% glutaraldehyde, post-fixed in 1% OsO_4, dehydrated in ethanol and embeded in Epon 812. The preparations were sectioned with Sorval MT-2B Ultramicrotome and observed with transmission electron microscope (Hitachi H-500).

5. Biotinylation and immunoprecipitation

Live *T. vaginalis* HY-1 was labelled with NHS-biotin to tag the surface proteins as described by Woo *et al.* (1993). One mililter of trichomonal suspension containing 1×10^7 organisms was biotinylated by adding 1 mM NHS-biotin at 4°C for 30 min. Cells were then

washed three times with cold PBS to terminate labelling and solubilized with 1 ml of lysis buffer (10 mM CHAPS and antiprotease mixture:1 mM PMSF, 1 mM TLCK, 50 μg/ml TPCK) on ice. Solubilized parasites were centrifuged at 10,000 × *g* for 30 min. The supernatant was concentrated with centricon (cut off value: 10 kDa; Amicon, MA) and used immediately whenever possible, or frozen at -70°C until required.

The labelled antigen extract (125 μl/675 μg) was immunoprecipitated with 50 μl of anti-*T. vaginalis* rabbit sera at 4°C overnight. Two hundred microliters of a 50% protein A-Sepharose solution in lysis buffer were added and incubated for 1 h. Immune complexes bound to protein A-sepharose beads were washed four times in lysis buffer before analysis by SDS-PAGE and electroblotting.

6. Enzyme-linked immunoelectrotransfer blot (EITB)

Trichomonal fractionated membrane proteins and biotinylated antigen preparations were solubilized in electrophoresis dissolving buffer containing 60.5 mM Tris-HCl (pH 6.8), 2% β-mercaptoethanol, 10% glycerol and 2% SDS. As a control, total trichloroacetic acid (TCA; 10% final concentration)-precipitated proteins of extensively washed organisms were prepared (Min *et al.*, 1992). Each antigen samples were electrophoresed through 7.5% polyacrylamide minigel kit (Hoeffer Sci., Calif) in discontinuous buffer system as described by Laemmli (1970).

Transfer was carried out as described elsewhere (Towbin *et al.*,1979) in 25 mM Tris, 192 mM glycine and 10% (v/v) methanol buffer (pH 8.3) for 3 h at 70 V at 4°C. Sheets were saturated for 2 h in PBS (pH 7.2) containing 5% (w/v) skimmed milk, washed and then incubated for 1 h in anti-*T. vaginalis* rabbit sera diluted 1:1,000 (v/v) in PBS. The sheets were subsequently incubated for 1 h with peroxidase conjugated antirabbit Ig G (Cappel, PA) diluted 1:1,000 (v/v) and then were visualized using 0.05% diaminobenzidine.

In case of biotinylated proteins transferred sheets were incubated for 1 h in peroxidase conjugated antibiotin (Vector, Calif) diluted 1:1,000 after 2 h incubation with blocking solution. Then the sheets were incubated in the peroxidase substrate, diaminobenzidine to visualize the protein bands.

RESULTS

1. Electron microscopic observation of plasma membrane fractions

Plasma membranes obtained from trichomonad by sucrose gradient method were examined by transmission electron microscope. The fraction from the 25%/45% sucrose interface appeared fairly homogeneous with membranes present both as extended sheets and as concentric vesicles. Many membrane structures showed typical trilaminal appearance (Figs. 1 & 2).

2. Antigen analysis of plasma membrane fraction in EITB

Antigens in the isolated plasma membrane fraction were detected by EITB. Seven bands of plasma membrane antigens were reacted with antirabbit sera at 40, 50, 60, 110, 130, 140 and 150 kDa (Fig. 3, lane 3), whereas about twenty antigens were reacted in whole cell proteins (Fig. 3, lane 2).

Surface antigenic peptide tagged with NHS-biotin were observed at 46, 60, 68, 90, 125 and 220 kDa (Fig. 3, lane 6).

In these assays of an isolated plasma membrane fraction and biotinylated surface

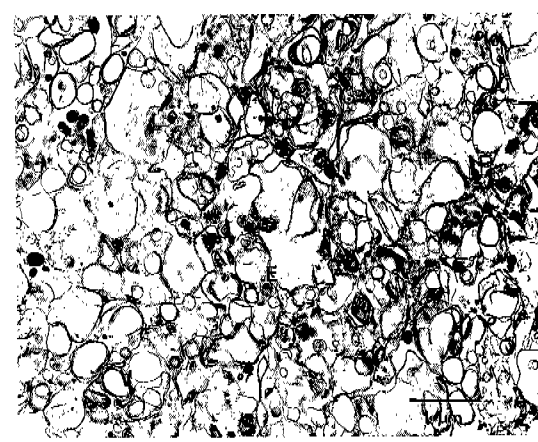

Fig. 1. Plasma membrane fraction of *T. vaginalis* HY-1 isolated by homogenization and sucrose step-gradient method. Membrane particles are present as extended sheet (E) and concentric vesicle (V) (× 10,000).

protein, the band at 60 kDa revealed as a common reactive peptide which originated from surface membrane constituents (Fig. 3).

DISCUSSION

T. vaginalis is a sexually transmitted parasite with strain-to-strain heterogeneity (Alderete *et al*, 1985, Garber *et al*. 1986, Min *et al*, 1992) and has geographic variations (Kreiger *et al*, 1985). Recently, particular interest is being paid to identifying membrane antigens that may be important in the development of immunity in trichomoniasis and that play a role in antigen heterogeneity. Information on trichomonal membrane antigens has been available by the indirect means, such as extrinsic radiolabelling (Peterson and Alderete, 1982) or surface biotinylation (Woo *et al*, 1993). However no attempt has been made to isolate trichomonal plasma membrane until the present time. Therefore we tried direct isolation of plasma membrane first from *T. vaginalis* in this study.

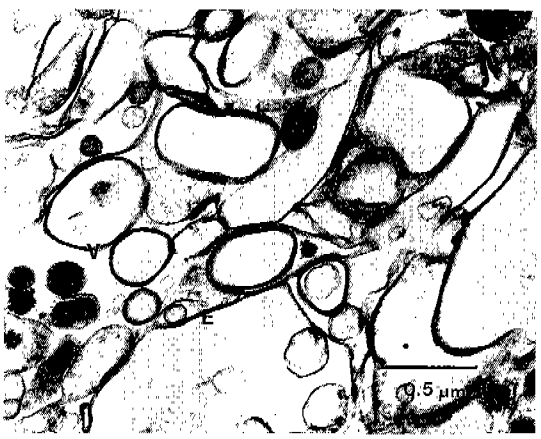

Fig. 2. Magnification of Fig. 1. (× 30,000).

Although it is not possible to assess critically the purity of plasma membrane preparations by means of electron microscopy, in general trichomonad possessed only limited amounts of glycogen in rosette appearance (Honigberg, 1990) and membrane fractions obtained from the 25%/45% sucrose interface appeared

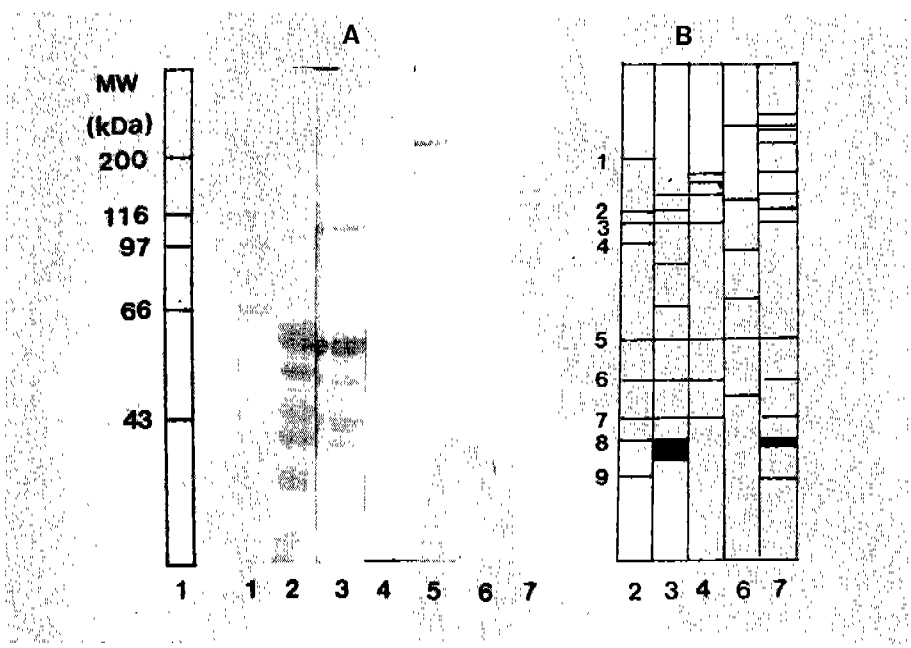

Fig. 3. Western blot of *T. vaginalis* HY-1 plasma membrane antigens purified by sucrose step-gradient centrifugation (lane 4) reacted with immune rabbit serum in EITB and immunoprecipitated surface antigen biotinylated prior to lysis (lane 6). Lane 1: High M.W. marker. Lane 2: Amido black stain of TCA precipitated proteins of *T. vaginalis* HY-1. Lane 3: Immunoblot of whole lysates probed with immune rabbit serum. Lane 5: Biotinylated whole lysate. Lane 7: Immunoprecipitated whole lysate (lysed prior to biotinylation). A: Western blot patterns. B: Schematic diagram of A.

nearly homogeneous with membranes. Our result was similar to that of Clark and Holberton (1986) who isolated plasma membrane of *Giardia lamblia* in their experiment. Thus it was thought that the membrane fractions of this study were highly purified and the subsequent study on purity of fractionated proteins will be proceeded.

In EITB assays interestingly the similarities in the antiserum reactivity of whole cell protein and isolated membrane fraction were observed. Among seven bands at 40, 50, 60, 110, 130, 140 and 150 kDa of membrane fraction four peptides at 40, 50, 60 and 110 kDa shared common bands with whole cell antigens when probed with rabbit antisera (Min *et al.*, 1993). This suggests that the four common antigens at 40, 50, 60 and 110 kDa of whole cell antigens may be membrane origin and be important in expression of antigenicity of trichomonads.

Totally seven membrane antigens were detected in isolated membrane fractions by EITB at 40, 50, 60, 110, 130, 140, and 150 kDa and six surface antigens were reacted with immune rabbit sera by immunoprecipitation at 46, 60, 68, 90, 125 and 220 kDa. These different results can be explained that EITB technique cannot recognize some structural epitopes not reformed after blotting, and in contrast immunoprecipitation allows the recognition of these structural epitopes and NHS-biotin recognizes only peptides containing lysin residue (Andrews and Bjorvatn, 1991).

In this study reactive membrane peptide with a molecular weight of 60 was very specific and common in EITB and biotinylation assays. This result is quite similar to those of Alderete (1983) with radioimmunoprecipitation study and Woo *et al.* (1993) with biotinylation and immunoprecipitation technique. Thus it is considered that the protein with a molecular weight of 60 is one of a major surface antigen. Furthermore Neale and Alderete (1990) demonstrated that a 43 kDa protease, which was a subunit of an extracellular protease at 60 kDa, was associated with cell surface of *T. vaginalis* and in recent studies this protease was deeply related with the pathogenesis of this protozoan infection (Garber and Lemchuk-Favel, 1989 & 1994). It is very interesting that the 60 kDa of the surface protein in this study is same in molecular weight from the results of other study groups eventhough the protease at 60 kDa was extracellular, so further studies on the biological and biochemical characterizations of 60 kDa protein in this study should be done.

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=국문초록=

질편모충의 표면항원 분석

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질편모충 항원 분석의 일환으로 막항원의 분석을 시도하였다. 수확 세척된 질편모충 HY-1의 homogenate를 sucrose step-gradient를 이용하여 differential centrifugation하였으며 25%/45%의 sucrose 경계면으로부터 막분획을 얻었다. 분리된 막분획은 transmission electron microscopy를 통하여 순수 분리되었는지 확인하였고 효소면역 전기영동 이적법(EITB)을 이용하여 항원성을 관찰하였으며 그 결과는 다음과 같았다. 분리된 막분획은 투과전자 현미경상에서 extended sheet나 concentric vesicle의 형태가 거의 균질하게 분포하고 있었으며 막 분획에서 특징적으로 나타나는 trilaminal appearance를 보여 질편모충의 막분획이 순수 분리된 것으로 간주할 수 있었다. 분리된 막분획은 EITB상에 토끼의 항혈청과 반응하였을 때 46, 60, 110, 120, 130 및 150 kDa에서 항원성이 있는 반응대가 관찰되었으며 N-hydroxysuccinimido-biotin으로 표지하여 분리된 표면항원의 분획과 비교하였을 때 60 kDa의 항원 분획이 서로 일치하는 것으로 나타났다. 따라서 60 kDa의 항원 분획은 표면 항원임을 확신할 수 있었다.

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