COMPARISON BETWEEN R-PHYCOCYANIN-LABELED AND R-PHYCOERYTHRIN-LABELED MONOCLONAL ANTIBODY (MAb) PROBES FOR THE DETECTION OF *ENTAMOEBA HISTOLYTICA* TROPHOZOITES

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Abstract. A comparison between R-phycocyanin (R-PC)-labeled monoclonal antibody (MAb) probe and R-phycoerythrin (R-PE)-labeled MAb probe for the detection of the three standard reference strains of the cultured-derived *Entamoeba histolytica* trophozoites, namely HK-9, HM-1:IMSS, and HTH-56:MUTM were evaluated by using direct immunofluorescence antibody (DIFA) assay five times for each strain. Under the blue irradiation of the fluorescent microscope, both R-PC-labeled and R-PE-labeled MAb probes showed consistently greenish-yellow trophozoites and golden-orange trophozoites, respectively. The R-PE-labeled MAb probe stained the trophozoites more brightly and clearly than those stained by the R-PC-labeled MAb probe of the same Eh208C2-2MAb. When observed under the green irradiation, both probes showed the same intensity of brightly red color at the trophozoites of all three strains of *E. histolytica*. The sensitivity of both tests was 100%. Since this Eh208C2-2MAb could recognize specifically *E. histolytica* pyruvate: ferredoxin oxidoreductase (PFOR) enzyme, therefore, our two antibody probes would be valuable for use as a rapid, easy and sensitive test for diagnosis of invasive amebiasis. Further applications of these two probes directly onto the fecal sample spots and to more culture-derived strains *of E. histolytica/E. dispar* of known zymodemes in collaboration with the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDRB), Dhaka, Bangladesh, are under investigation.

INTRODUCTION

Amebic dysentery and extraintestinal amebiasis such as amebic liver abscess is a public health problem around the world (Garcia and Bruckner, 1993). It was estimated to annaully result in 50 million cases of colitis and liver abscess and 40,000-100,000 deaths (WHO, 1997; Walsh, 1986). Human amebiasis was ranked as the second after malaria due to leading parasitic cause of death worldwide (WHO, 1997). Amebiasis has been evidenced long time ago. In 1855, Lambl found and described in some detail amebae in the stool of a child in Praque who died of infantile diarrhea, in 1875 Fedor Alexsandrovich LÖsch (Lesh) found amebae in a stool of a 24-years-old farmer with chronic dysentery who admitted to his clinic in November 1873 in St Petersberg, and in 1883 Koch observed five cases of dysentery in Egypt, two of them complicated with abscess of the liver displaying numerous amebae (Kean, 1988) and there was evidence of this disease in Thailand since 1886 during the Reign of King Rama III (Thammapalerd *et al*, 1993). The disease was found both in human and animals (Bowman, 1995; Mahannop and Mahannop, 1998). Although it is not difficult to treat and cure the disease, the diagnosis is still problematic such as time consuming, high cost, and low sensitivity (Singh *et al*, 1999). There is an urgent need for specific, rapid and simple diagnostic test method which is appropriate for developing countries where amebiasis is a main problem.

Phycobiliproteins (PBPs) are the water soluble fluorescent proteins derived from cyanobacteria, rhodophyta, and cryptophyta. The R-phycocyanin (R-PC) is a blue-colored PBP which carries phycocyanobilin (PCB) chromophoric group. In contrast, the phycoerthrin (R-PE) subunits are deep rose chromoproteins and carry phycoerythrobilin (PEB). The R-PC contains a PEB in addition to the phycocyanobilin (PCB) chromophore and has $(\alpha\beta)3$ molecular structure. The PEB makes R-PC appear slightly more purple to the eye than the purer blue but

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it produces a similar red fluorescence. The R-PE has three types of subunits: α, β and γ and a subunit structure of (αβ)6γ. The α subunit of R-PE contains only the PEB chromophore, while the β and γ subunits contain both PEB and phycourobilin (PUB). However, phycoerythrin 566 (PE 566) is obtained from cryptomonads. Unlike the other PBPs, these pigments are not organized into phycobilisomes (PBsomes) within the organism. They appear as (αβ)2 dimers and the chromophores of PE 566 are phycoerythrobilin (PEB) and cryptoviolin (CV) (Wedemayer *et al*, 1991). PBsomes of *Gracilaria chilensis* contains the three proteins R-PE, R-PC and R-allophycocyanin (R-APC). These PBP conjugates can be applied to fluorescence-activated cell sorting and analysis, fluorescence microscopy, and fluorescence immunoassay (Oi *et al*, 1982). In the present study, R-PC and R-PE from the red algae *Gracilaria fisheri* that are commonly found along both sides of the Gulf of Thailand and in brackish water lakes opening to Andaman Sea were extracted and purified. Both of them are recognized as value-added product derived from agar and agarose production. Previously, we have shown that R-PE-labeled MAb probe specifically stained either culture-derived or stool-derived trophozoites of *E. histolytica* (Thammapalerd *et al*, 1996c). We, therefore, conduct this further study to apply the R-PC, another fluorescent dye to identify the *E. histolytica* trophozoites by MAb-based direct immunofluorescent antibody (DIFA) technique. Its sensitivity and effectiveness will be compared with the well-established R-phycoerythrin (R-PE)-labeled MAb probe.

MATERIALS AND METHODS

Amebae and culture conditions

Three standard strains of *E. histolytica*, namely HK-9, HM-1: IMSS and HTH 56: MUTM were cultured according to the technique of Diamond (1982). They were grown axenically in screw-capped tubes in TYI-S-33 medium in the presence of 10% heat-inactivated bovine serum. The HK-9 strain was kindly provided by Dr Louis S Diamond, NIH, Maryland, USA, and the HM-1: IMSS by Professor Gordon B Bailey, Morehouse School of Medicine, Atlanta, Georgia, USA, while the HTH-56: MUTM strain was locally axenized at the Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand (Thammapalerd *et al*, 1993).

Anti-*E. histolytica* MAbs

Murine MAbs were raised against the three pathogenic strains of *E. histolytica*, namely HK-9, HM-1: IMSS and HTH-56: MUTM using SP2/0 myeloma cells according to the method of Galfre and Milstein as described in Thammapalerd and Tharavanij (1991). Several antibody-secreting hybridoma clones were obtained, among which Eh208C2-2 MAb was used throughout for the detection of *E. histolytica* trophozoites in this study. Ascites fluid from pristane-primed BALB/c mice was collected and centrifuged at 900*g* for 5 minutes and the supernatant was kept at -20˚C. The IgG fraction of the ascites fluid (MIgG) was purified by using Protein A Sepharose CL-4B^{R} affinity chromatography as described earlier (Wonsit *et al*, 1992).

Purification of R-PE and R-PC

Both R-PE and R-PC were short term extracted from red algae, *Gracilaria fisheri* from Pattani Province, Thailand. The method was modified from previous report (Thammapalerd *et al*, 1996b). They were extracted with 50 mM sodium phosphate buffer, pH 7.0, followed by precipitation with 0-30%, 30-45% and 45-60% final concentration of saturated ammonium sulphate solutions at 4˚C for 2 hours each, respectively. The precipitates were then further purified by passing through DEAE-cellulose (DE-52) and Sephacyl S-200 column chromatography.

Preparation of R-PC- and R-PE-labeled MIgG probes

SPDP conjugation of R-PC and R-PE: To 1.0 ml (10 mg) of either R-PC or R-PE of *Gracilaria fisheri* in a coupling buffer (0.1 M sodium phosphate, 0.1 M NaCl, pH 7.4) was added a fresh solution of 10 µl (0.13 mg) N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) in anhydrous methanol. After 60 minutes at room temperature, the reaction mixture was applied to a column of Sephadex G-25 (2.0 x 9.0 cm), equilibrated, and eluted with coupling buffer. The frontally eluted R-PC- and R-PE-S-S-pyridyl derivatives were kept at room temperature or stored lyophilized at 4˚C if not being used immediately.

SPDP conjugation of IgG fraction of Eh 208C2-2 MAb: To 1 ml (1 mg) of MIgG in the above coupling buffer, was added 10 µl (0.13 mg) of SPDP in anhydrous methanol. After incubation of the reacted mixture for 60 minutes at room temperature, the mixture was applied to a column of Sephadex G-25 (2.0 x 9.0 cm), equilibrated and eluted with coupling buffer. The frontal eluates which contained MIgG-PDP were reduced by the addition of 1 M dithiothreitol (DDT) solution to a final concentration of 0.05 M of DDT in sodium phosphate buffer, pH 7.4. After a further 20 minutes incubation, the reaction mixture was applied to a column of Sephadex G-25 (2.0 x 9.0cm)

and eluted with coupling buffer, fractions collected and then lyophilized.

SPDP disulfide crosslinking procedures: The SPDP disulfide crosslinking procedure used was modified from those of Kronick and Grossman (1982) and Oi *et al* (1982). Lyophilized thiolated MIgG was mixed together with the lyophilized R-PC- or R-PE-S-S-pyridyl derivatives and dissolved in the total volume of 5 ml of coupling buffer. After 18 hours incubation at room temperature, the iodoacetamide was added into the reaction mixture to a final concentration of 0.03 M for 15 minutes. The reaction mixture was passing through a Sephadex G-25 column, fractions collected with the addition of bovine serum albumin (BSA, Cohn Fraction V, Sigma Chemical Company, St Louis, USA) to a final concentration of 1% lyophilized and stored in the deep freezer (-78˚C). When used, they were reconstituted with PBS buffer containing 0.05% Tween 20 (PBST).

Purification of R-PE- and R-PC-labeled MAb probes

Each conjugate was dissolved in PBS, pH 7.4 and 0.05 ml applied to an FPLC Superose® HR 10/30 column (Pharmacia) connected to a Pharmacia Biopump. The samples were eluted with 0.1 M NaCl, PBS buffer, pH 7.4 at a flow rate of 0.2 ml/minute (12 ml hr^{-1}). The absorption and fluorescent emission spectra were recorded using UV/visible spectrophotometer (LKB Bromma 2141, variable at 200-700 nm). They were preserved in 10% sucrose solution and kept frozen at -78˚ C in small aliquots.

For a rapid purification of R-PC- and R-PE-labeled MAb probes, the fractions after passing through Sephadex G-25 column were collected, concentrated by using solid sucrose (Mitrphol, Thailand), and passed through Sephacryl S-200 column. Thereafter, fraction collected concentrated with sucrose again (Mitrphol, Thailand) until the viscosity of sucrose inside and outside of the dialysing tube was equal to approximately 10%.

Preparation of antigen coated slides

Antigen coated slides were prepared according to the method described by Garcia *et al* (1982) with slight modification. Trophozoites of the KH-9, HM-1: IMSS and HTH 56:MUTM strains from a 48-72 hour culture were chilled in an ice bath for 10 minutes to dislodge the parasites, followed by washing 3 times with 0.15 M NaCl solution (NSS) by centrifugation at 120*g* at room temperature for 5 minutes each. Thereafter, the cell sediment was adjusted in NSS to give approximately $2x10^6$ cells/ml. Two µl aliquots of the cell suspension were dropped on a clean and dry microscopic slide (25 mm x 75 mm) to form 16 spots. Smear slides were dried on a warm plate at 37˚C for 30 minutes and then fixed in cold absolute ethanol for 10 minutes. Prepared slides were wrapped with tissue paper and was then put in polyethylene bags containing a dehydrating agent (Silica-gel) and sealed. They were stored at -20˚C until used.

The DIFA technique

Antigen fixed slides were drawn from the freezer and dried in an incubator at 37˚C for 10 minutes. Ten microliters of R-PC-labeled-MAb probe, 10 µl of R-PE-labeled-MAb probe, 10 µl each of unconjugated R-PE and R-PC, and 10 µl of phosphate buffer solution (PBS), were dropped onto the spots of *E. histolytica* coated slide, serving as one positive and three negative controls for each strain of *E. histolytica* on one slide. The slides were incubated in a humid box at 37˚C incubator for 2 hours. They were washed with cold PBST solution in a coplin jar and shaked for 5 minutes for 3 times. They were air-dried, mounted with 70µl of freshly prepared mounting solution comprising 5% n-propyl gallate in glycerol plus 20% 2M Tris base (Giloh and Sedat, 1982) and covered with the coverslip. They were examined with a Zeiss fluorescent microscope (Zeiss, West Germany) equipped with a phase contrast and equi-illumination system at 100 and 400 times magnification. The fluorescent intensity was recorded as negative (-) or positive (1+-4+). Photographs were taken with KODAK color ASA 400 film with automatically adjusted exposure time.

RESULTS

Purification of R-PE and R-PC

Using this short term extraction with 50 mM sodium phosphate buffer, pH 7.0 for 2 hours at 4˚C in place of overnight extraction at 0˚C, we could be able obtain high yields of both R-PE and R-PC very easily. Chlorophyll, R-PC and R-PE were precipitated out at 0-30%, 30-45% and 45-60% saturated solution of ammonium persulphate, respectively. After further purification by passing through Superose[12] or DE-52 and Sephacryl S-200 column chromatography, the R-PC- and R-PE-MAb probe showed absorbance maxima of 617 nm and 565 nm, and fluorescence emission at 637 nm and 573 nm, respectively (data were not shown).

R-PE- and R-PC-labeled MAb conjugates

Effective conjugates between purified R-PC/R-PE and Eh208C2-2 MIgG could be produced using the heterobifunctional cross-linking reagent, SPDP. They were preserved in 10% sucrose solution in which excellent stability could be achieved.

The DIFA test for the detection of *E. histolytica* trophozoites

Detection of *E. histolytica* trophozoites by using DIFA method with local antibody probes, R-PE- and R-PC-labeled MAb showed successful staining with 3 standard strains of *E. histolytica*, namely HK-9, HM-1: IMSS and HTH-56: MUTM. Under the blue irradiation of the fluorescent microscope, the R-PC-labeled MAb probe stained every trophozoites with greenish-yellow color whereas the R-PE-labeled MAb probe showed golden-orange trophozoites. However, under the green irradiation, both probes gave the similar intensity of brightly red color at the trophozoites of all three strains. They stained efficiently every fixed trophozoites.

DISCUSSION

Having experiences with the production of the antibody probe between R-PE and Eh208C2-2 MIgG, very effective conjugates between purified R-PC and Eh208C2-2 MIgG could be produced more easier using the heterobifunctional cross-linking reagent, SPDP. When used in the DIFA test, both probes can be diluted up to 100 folds. The R-PC-labeled MAb probe stained trophozoites with greenish-yellow color whereas R-PE-labeled MAb probe showed golden-orange trophozoites under the blue irradiation of the fluorescent microscope. The staining intensity of R-PE-lebeled probe appeared brighter and more noticable than those of R-PC staining. However, under the green irradiation, both probes gave the same intensity of brightly red color at the trophozoites of all three strains. Every fixed trophozoites were stained efficiently.

Both probes are highly stable and not so sensitive to light and temperature which are due to using high concentration of sucrose as effective preservative. The UV light of fluorescent microscope could make the fluorescence color fade during observation because of photobleaching effect. The addition of N-propyl gallate

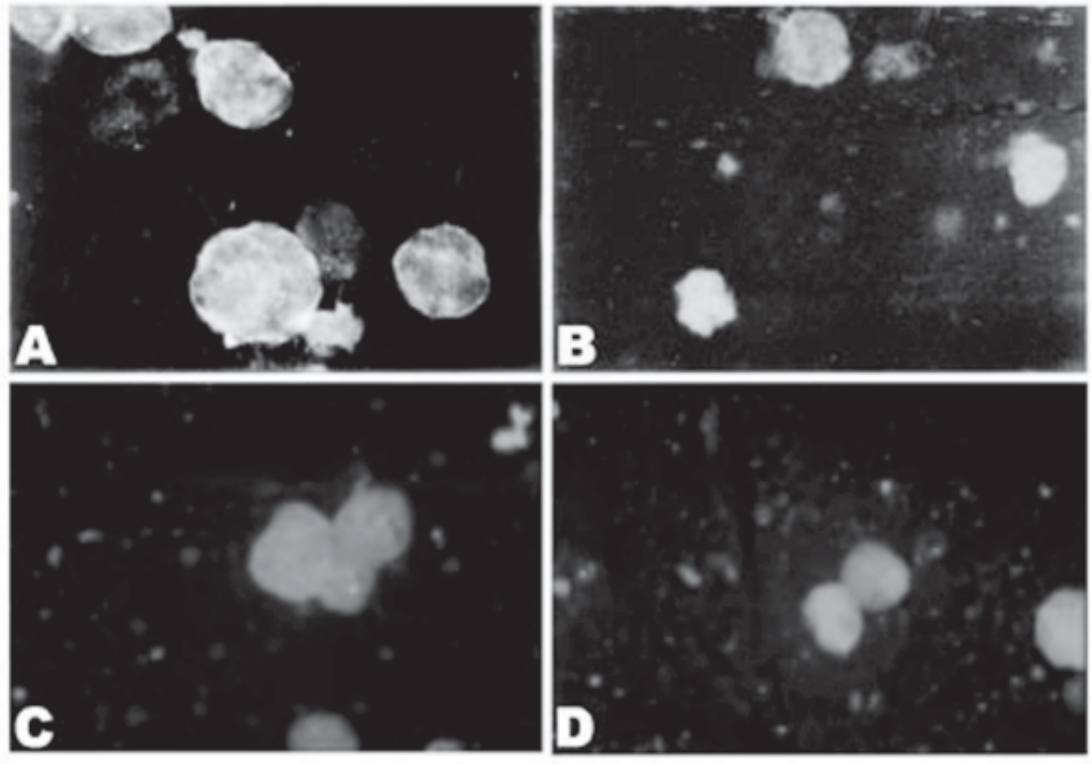

Fig.1- *Entamoeba histolytica* trophozoites, strain HTH-56:MUTM derived from culture displayed brightly greenish-yellow (A) and golden-orange fluorescence (B) after staining with the R-PC- and R-PE-labeled Eh208C2-2 MIgG conjugates, respectively by DIFA under ultraviolet light irradiation of the fluorescent microscope. When observed under the green irradiation, both probes showed the same intensity of brightly red color at the trophozoites (C and D). The photographs were taken by Ziess fluorescence microscope (Ziess, West Germany) with Kodak color ASA 400 film after automatically exposure.

in the mounting solution could sustain the stability of the fluorescence color for upto one week or more if the cover slips were sealed with the nail enamel, wrapped with aluminium foil and kept the slides in the refrigerator. These slides can be used later on as teaching materials. The high sensitivity was another advantage of these assays. Moreover, the assays were less time-consuming to perform. Therefore, it should be accommodated in a diagnostic laboratory service to detect *E. histolytica* in autopsy/biopsy specimens in stool and even in liver pus samples. Fresh stool samples from patients with amebic dysentery or amebic liver abscess were just spotted or smeared directly onto microscopic slide, dried and fixed the same way as those cultured amebae (Thammapalerd *et al*, 1996c). Further applications of these two probes directly onto the fecal sample spots and to more culture-derived strains of *E. histolytica/E. dispar* of known zymodemes in collaboration with the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDRB), Dhaka, Bangladesh, are under investigation.

Diagnostic and therapeutic applications of Eh208C2-2 MAb have been clearly shown (Thammapalerd and Tharavanij, 1991; Wonsit *et al*, 1992; Scherchand *et al*, 1994; Thammapalerd *et al*, 1996b; 1996c; Sosa *et al*, 1995; 2000). This Eh208C2-2 MAb specifically recognized the amitochondriate *E. histolytica* pyruvate:ferredoxin oxidoreductase (PFOR) (Thammapalerd *et al,* 1996a), the key enzyme responsible for the decarboxylation of pyruvate and energy production (Kerscher and Oesterhelt, 1982). PFORs were found distributed within the trophozoites as well as membrane associated (Sosa *et al*, 1995; Samarawickrema *et al*, 1997). An immunotoxin (IT) comprising Eh208C2-2 MAb and the deglycosylated toxic moiety of ricin A (RA) chain, was prepared and test both *in vitro* and *in vivo* (Sosa *et al*, 1995; 2000). The purified IT could specifically inhibit the proliferation of the trophozoites of *E. histolytica* (Sosa *et al*, 1995) as well as protection against invasive *E. histolytica* in hamster models (Sosa *et al*, 2000).

Recently, the R-PE from red algae *Griffithsia monilis, Gracilaria chilensis* and *Polysiphonia urceolata*, the R-PC from *Polysiphonia urceolata*, the C-PC from *Spirulina platensis* were successfully crystallized (Ritter *et al*, 1999; Contreras-Martel *et al*, 2001; Chang *et al*, 1996; Jiang *et al*, 2001; Wang *et al*, 2001) and recombinant C-PC produced (Cai *et al*, 2001). To our best knowledge there are three PE structures deposited in the Protein Data Bank: R-PE from *Polysiphonia urceolata* at 2.8 Å resolution (PDB code 1 lia) solved by Chang *et al* (1996), R-PE from *G. monilis* at 1.9 Å resolution (PDB code 1b8d) solved by Ritter *et al* (1999), R-PE from *G. chilensis* at 2.2 Å resolution solved by Contreras-Martel *et al* (2001). These will allow one to evaluate its fluorescence properties for applications in biochemical and immunological studies in many infectious diseases.

Moreover, the first crystal structure of PFOR from bacterium *Desulfovibrio africanus* was reported providing significant new information on its structural organization and redox chemistry (Chabriere *et al*, 1999a, b; Pieulle *et al*, 1997; 1999a, b; Charon *et al*, 1999). PFORs belong to a large group of enzymes that depend on thiamin pyrophosphate (TPP) for the cleavage of carbon-carbon bonds adjacent to a carbonyl group, are potential targets for specific drug design because they are restricted to microorganisms that include several anaerobic human pathogens like *E. histolytica*, *Giardia lamblia* and *Trichomonas foetus* (Thammapalerd *et al*, 1996a; Samarawickrema *et al*, 1997; Upcroft and Upcroft, 1993; Johnson, 1993). It is, therefore, very interesting for us to further study the structure and catalytic mechanism of these enzymes when interact with these chromophores and its thiamine-based mechanism of catalysis in pyruvate decarboxylation in *E. histolytica* or in IT against invasive amebiasis.

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