APPLICATION OF MONOCLONAL ANTIBODIES AGAINST
ENTAMOEBA HISTOLYTICA IN TROPICAL MEDICINE RESEARCH
FINAL REPORT

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APPLICATION OF MONOCLONAL ANTIBODIES AGAINST
ENTAMOEBA HISTOLYtica IN TROPICAL MEDICINE RESEARCH.

NITAYA THAMMAPALERD
SAVANAT THARAVANIJ

DEPARTMENT OF MICROBIOLOGY AND IMMUNOLOGY
FACULTY OF TROPICAL MEDICINE
MAHIDOL UNIVERSITY
BANGKOK 10400, THAILAND

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Organization : Department of Microbiology and Immunology
Faculty of Tropical Medicine, Mahidol University
420/6 Rajvithi Road, Bangkok 10400, Thailand
Tel. 2460056, 2460058, 2461272-3, 2461278
Telex 84770 UNIMahi TH, FAX 246-8340

Project Leaders : Nitaya Thammapalerd
Savanat Tharavanij

Co-investigators: Ratri Wonsit, Daroon Kotimanasvanij,
Mullika Charoenpol, Pongsak Techasathirakul,
Srisin Khussmith, Choomani Lamom,
Duangrhudee Chindanond, Danai Bunnag
and Prayong Radomyos

Project Consultant : Nil

Authorized Officers : Natth Bhamarapravati, M.D., Ph.D.
President, Mahidol University
Santasiri Sornmani, M.D., Ph.D.
Dean, Faculty of Tropical Medicine

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EXECUTIVE SUMMARY

Ninety six monoclonal antibodies (MAbs) reacting against three pathogenic strains of *E. histolytica* (strains HM-1:IMSS, HK-9, and HTH-56:MUTM) have been produced. Based on their IFA staining patterns, these MAbs were classified arbitrarily into 5 groups: Group I (43 monoclones) showed generalized staining to all components of trophozoite comprising the cytoplasmic granules (G), the membrane (M), and with or without staining of the released products (RP). Group II (18 monoclones) stained only the granules. Group III (20 monoclones) stained cytosol (C) only. Group IV (13 monoclones) stained membrane and cytosol. Group V (2 monoclones) stained the granules and cytosol.

Zymodeme types of 75 isolates of *E. histolytica* were 23 zymodeme I, 44 zymodeme II, 1 zymodeme XIV, 1 zymodeme XIX, 1 *E. histolytica*-like amebae (Laredo), 3 non-*E. histolytica* amebae and 2 non-typable amebae. Most of them belong to zymodeme II.

Selected murine MAbs raised against crude lysate of the pathogenic HM-1:IMSS strain of *Entamoeba histolytica* were used in the development of an indirect fluorescent antibody (I"A") test for differentiation between pathogenic and non-pathogenic zymodemes. Four MAbs (Eh19C7, Eh19C8, Eh35C1 and Eh35C5) showed positive reactions to more than 75% of ≥23 pathogenic zymodemes and less than 20% of ≥9 non-pathogenic zymodemes and non-reactive to the Laredo strain. The Eh35C1 and Eh35C4 MAbs recognized the same proteins of 183 and 170 Kd whereas the molecules recognized by the 19C7 and 19C8 could not be demonstrated by the Western blot. Another MAb (208C2-2) was used in the MAb-based double sandwich ELISA for the detection of
E. histolytica antigen in the stool; the assay could detect the antigen equivalent to as few as 110 and 280 amebae/ml in PBS of the HM-1:IMSS and HK-9 strains respectively. The assay was applied to single stool samples from 3 groups of individuals comprising 40 group I patients whose stools were positive for E. histolytica cysts and/or trophozoites, 48 group II patients whose stools were negative for E. histolytica but positive for other parasites, and 36 group III parasitologically negative healthy controls. The assay was positive in 77.5%, 2.1% and 2.7% of groups I, II and III samples respectively with specificity, positive predictive value and efficiency of 97.9%, 96.8% and 88.6% respectively.
INTRODUCTION

Amebiasis is still one of the major tropical diseases of public health importance affecting approximately 48 million people (1). Since the majority of amebic infections are asymptomatic, parasite causing symptomatic and asymptomatic infections may be different. Differentiation between pathogenic and nonpathogenic amebae can be accomplished by isoenzyme typing (2), IFA test with MAb to internal granular materials (3), Mr 30 Kd antigen (4), Mr 96 Kd membrane antigen (5), galactose-specific adherence lectin (6), genomic DNA sequence analysis (7) and hybridization of DNA probes (8, 9). We hypothesize that pathogenic amebae have certain antigens not present in nonpathogenic amebae. Demonstration of such antigens should be possible with pathogenic zymodeme-specific MAbs. Such MAbs should also be useful for the development of assays for the detection of E. histolytica antigens. Antigen detection by MAb-based enzyme-linked immunosorbant assay (MAb-based ELISA) (10,11), polyclonal antibody-based ELISA (PAb-based ELISA) (12); and DNA hybridization with DNA probes (13) have been successfully applied to clinical specimens.

RESEARCH OBJECTIVES

Association between pathogenic zymodesmes of E. histolytica and clinical amebiasis has been well documented. We hypothesize that pathogenic zymodesmes should possess certain antigens which are absent in non-pathogenic zymodesmes. If this is the case, it should be possible to raise monoclonal antibodies (MAbs) with
potentials for differentiation between these two types of zymo-
demes. Some of these MAbs could also be used in the development
of the assays to detect the amebic antigens for clinical diagno-
sis of amebiasis. The overall aim and specific objectives of this
study are:

1. To type pathogenic *E. histolytica* by using MAb-based IFA.
2. To develop a MAb-based ELISA for the detection of *E.
   histolytica* antigens in clinical specimens.

Innovative aspects of this study include the development of
a MAb-based IFA for differentiation between pathogenic and non-
pathogenic zymodemes as a substitute for the time-consuming
isoenzyme typing developed by Sargeaunt (2) and for the estab-
ishment of a MAb-based ELISA for the detection of amebic anti-
gens in stool specimens. Our approach to the MAb-based IFA for
characterization of pathogenic zymodeme of *E. histolytica* was in
essence similar to that of Strachan *et al.* (3). Our undertaking
might begin at the same time as Strachan *et al.*, but the progress
made was slower owing to the need to establish the technique for
zymodeme typing. On the other hand, the MAb-based ELISA for the
detection of amebic antigens may not be considered as truly
innovative, since the procedure adopted was similar to that
reported earlier by Ungar *et al.* (10). However, the use of anti-
*E. histolytica* MAbs developed in our laboratory in place of MAbs
from commercial source merits the study. In addition, some of
our anti-*E. histolytica* MAbs may react to 'functional' epitopes
and thus could be useful in an analysis of immune mechanisms
against this protozoa particularly in the future development of a
vaccine against amebiasis.

MATERIALS AND METHODS

Production of MAbs:

Spleen cells from BALB/c mice immunized with axenically-cultivated or monoxenically-cultivated pathogenic strains of *E. histolytica* (HM-1:IMSS, HK-9 or HTH-56:MUTM, respectively) were fused with Sp2/O myeloma cells according to the method of Galfre and Milstein, 1981 (14). The spent medium from growing hybrids was screened for anti-amebic antibodies by IFA and ELISA. Antibody-secreting hybrids were cloned by a limiting dilution technique. IgG fractions of MAbs from ascites or supernatants were purified by Protein A Sepharose CL4B chromatography (15).

Isotyping of mouse MAbs:

Classes and subclasses of MAbs were determined by using mouse hybridoma isotyping kit from Calbiochem Immunochemicals.

SDS-Polyacrylamide gel electrophoresis and Western blot analysis of *E. histolytica* antigens recognized by MAbs:

After fractionation of the trophozoites protein antigens of *E. histolytica* by 7.5-20% gradient sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), the gel was electroblotted onto 0.45 um pure nitrocellulose membrane (Bio-Rad Laboratories) according to the method of Towbin et al., 1979 (16) at a field strength of 300 mA for 18 hours at 26°C. The unreacted sites on the nitrocellulose strips after blotting were blocked
by soaking the strips in PBS containing 2% gelatin 1% BSA, and 0.02% sodium azide (PBS-gel) at room temperature for two hours with gentle rocking. The blotted nitrocellulose strips were treated with immunized mouse serum, normal mouse serum, mouse myeloma Sp2/0 supernatants and monoclonal antibodies with gentle rocking for 1 1/2 hours at room temperature. The strips were then washed with rocking 5x with PBS containing 0.05% Tween 20 and treated with $^{125}$I-labeled rabbit anti-mouse IgG (1 x $10^5$ cpm/ml in PBS/BSA from New England Nuclear) for 1 1/2 hours with agitation at room temperature. The strips were washed as previously described and dried. Autoradiography was done by exposing the strips to X-ray films (Kodak X-Omat RP) with intensifying screen at -70°C.

Preparation of rabbit anti-E. histolytica polyclonal IgG (PIgG):

Two rabbits were immunized with three injections at two week intervals with the physiological saline lysate of the HM-1:IMSS strain of E. histolytica incorporated in Freund's complete adjuvant initially and incomplete adjuvant subsequently. The final IFA titer was 1:640. PIgG was prepared by protein-A Sepharose chromatography (15).

Zymodeme typing of E. histolytica:

This was carried out according to the technique of Sargeaunt et al., 1987 (2).

MAB-based IFA typing of E. histolytica:

Thirty six MAbs were tested against 36 ameba isolates
comprising 25 pathogenic zymodemes II, 7 nonpathogenic zymodemes I, 3 non-\textit{E. histolytica} and one \textit{E. histolytica}-like ameba (Laredo strain) by IFA test using the technique of Garcia \textit{et al.}, 1982 (17) with slight modification.

**MAb-based ELISA for detection of \textit{E. histolytica} antigen:**

Wells of 96-well polystyrene plates were coated with anti-\textit{E. histolytica} MAb IgG prepared from MAb 208C2-2, blocked with non-fat dried milk, reacting sequentially with various concentrations of \textit{E. histolytica} lysate or clinical samples, rabbit polyclonal anti-\textit{E. histolytica} IgG, alkaline phosphatase-labeled anti-mouse IgG and \(p\)-nitrophenyl phosphate substrate with washing at each step. The result was read at OD of 405 nM with an ELISA plate reader (Titertek, Multiskan, MCC/340, Flow Laboratories).

**RESULTS**

**Production of MAbs:** Two hundred and nineteen of 528 hybrids from five fusions were observed to secrete anti-\textit{E. histolytica} antibodies, 96 of which have been cloned. Based on IFA staining patterns, these MAbs could be arbitrarily divided into 5 groups comprising 43 group I MAbs with reactivities against cytosol (C), membrane (M), granules (G) with or without staining of the released products (RP); 18 group II MAbs with reactivities only against granules (G); 20 group III MAbs with reactivities only against cytosol (C); 13 group IV MAbs with reactivities against membrane (M) and cytosol (C); and 2 group V MAbs stained the granules (G) and cytosol (C) (Table 1).
Zymodeme typing: Zymodeme types of seventy five isolates of amebae comprising three axenically-cultivated HK-9, HM1-IMSS and Laredo strains, one monoxenically-cultivated isolate (HTH56:MUTM) and 71 multixenically cultivated amebae were 23 zymodemes I, 44 zymodemes II, 1 zymodeme XIV, 1 zymodeme XIX, 1 E. histolytica-like, 3 non-E. histolytica, and 2 non-typable amebae. Our findings agree with those reported elsewhere showing that most of the pathogenic E. histolytica belong to zymodeme II.

Selection of MAbs for typing of pathogenic zymodemes: Based on IFA reactivities of 36 MAbs against 36 amebic isolates comprising 25 pathogenic zymodemes II, seven nonpathogenic zymodeme I, one E. histolytica-like Laredo strain, and three non-E. histolytica amebae (Table 2), four promising MAbs (Eh19C7, Eh19C8, Eh35C1 and Eh35C5) were identified. These four MAbs reacted to more than 75% of pathogenic zymodemes and less than 20% nonpathogenic amebae and the Laredo strain. Both Eh35C1 and 35C5 MAbs recognized two E. histolytica-specific molecules of 183 and 170 Kd, whereas the molecules recognized by the Eh19C7, Eh19C8 MAb could not be demonstrated by the Western blot. These four MAbs should be more extensively tested against amebic isolates from patients with symptomatic and asymptomatic amebiasis to assess their values in the differentiation between pathogenic and nonpathogenic amebae.

Detection of E. histolytica antigen: Five MAbs comprising Eh208C2-2, Eh116C1, Eh16C1-1, Eh65B8 and Eh35C1-3 were screened by ELISA against antigens deliberately added in PBS,
serum, feces and urine of parasitologically negative feces. High optical density reading was obtained with three MAbs namely Eh208C2-2, Eh65B8 and Eh35C1-3. Eh208C2-2 was chosen for the development of MAb-based ELISA for the detection of *Entamoeba histolytica* antigen. The Eh208C2-2 MAb recognized Mr122, Mr115, Mr111 and Mr65 Kd proteins whereas the molecules recognized by the other two MAbs could not be demonstrated by the Western blot. The limit of sensitivity of Eh208C2-2-based ELISA against the HM-1:IMSS and the HK-9 strains in PBS were 110 and 280 amebae respectively. This assay was applied to single stool samples and was positive in 31 of 40 (77.5%) of fecal samples from patients positive for either *E. histolytica* cysts or trophozoites or both. In contrast, only 1 of 48 (2.1%) of fecal samples positive for other protozoa and/or helminths was positive. Feces from 36 parasitologically negative were positive in one case (2.7%). The specificity, positive predictive value and efficiency of the MAb-PAb-based ELISA are 97.9%, 96.8% and 88.6% respectively (Fig. 1). It should be noted that MAb 208C2-2 could be used to detect both trophozoites and cyst antigens of *E. histolytica*. 
Table 1. Classification of anti-\textit{E. histolytica} MAbs based on IFA staining patterns.

<table>
<thead>
<tr>
<th>Group</th>
<th>Staining patterns</th>
<th>MAb</th>
<th>Isotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Generalized staining of amebae including the granules, cytosol, plasmalemma, internal membrane and with or without released products</td>
<td>208C2-2, 35C1-3, 12/6*, 9/17, 20/1</td>
<td>IgG1, IgG1, IgM, IgM</td>
</tr>
<tr>
<td>II</td>
<td>Cytoplasmic granules</td>
<td>208C2-3, 19C6, 65B8</td>
<td>IgG1, IgG1, IgG1</td>
</tr>
<tr>
<td>III</td>
<td>Cytosol</td>
<td>90, 11/8, 22/8, 26/2*</td>
<td>IgG1, IgG1, IgG1</td>
</tr>
<tr>
<td>IV</td>
<td>Membrane and cytosol</td>
<td>23C17</td>
<td>IgG1</td>
</tr>
<tr>
<td>V</td>
<td>Granules and cytosol</td>
<td>34/2, 116C1</td>
<td>IgG1, IgG1</td>
</tr>
</tbody>
</table>

* Agglutinated with live \textit{E. histolytica} trophozoites
Table 2. Reactivities of monoclonal antibodies to *E. histolytica* isolates from Thailand

<table>
<thead>
<tr>
<th>MAb</th>
<th>Zymodeme</th>
<th>E. histolytica-like amebae and non- <em>E. histolytica</em> amebae</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td>Eh6C1</td>
<td>4/7</td>
<td>18/24#</td>
</tr>
<tr>
<td>Eh6C2</td>
<td>3/7</td>
<td>16/25</td>
</tr>
<tr>
<td>Eh6C2/8</td>
<td>0/6</td>
<td>10/21</td>
</tr>
<tr>
<td>Eh6C12</td>
<td>2/7</td>
<td>18/25</td>
</tr>
<tr>
<td>Eh6C13</td>
<td>1/7</td>
<td>10/24</td>
</tr>
<tr>
<td>Eh6C14</td>
<td>2/7</td>
<td>10/25</td>
</tr>
<tr>
<td>Eh6C18</td>
<td>2/7</td>
<td>16/24</td>
</tr>
<tr>
<td>Eh6C19</td>
<td>2/7</td>
<td>15/23</td>
</tr>
<tr>
<td>Eh6C20</td>
<td>4/7</td>
<td>19/25</td>
</tr>
<tr>
<td>Eh6C22</td>
<td>1/7</td>
<td>14/24</td>
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<td>Eh6C24</td>
<td>1/7</td>
<td>14/24</td>
</tr>
<tr>
<td>Eh6C26</td>
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<td>14/23</td>
</tr>
<tr>
<td>Eh16C1</td>
<td>1/7</td>
<td>12/24</td>
</tr>
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<td>Eh16C2</td>
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<td>Eh19C1</td>
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<td>Eh19C4</td>
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<td>Eh19C5</td>
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<td>Eh19C6</td>
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<td>4/24</td>
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<tr>
<td>Eh19C7*</td>
<td>1/7</td>
<td>19/25</td>
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<tr>
<td>Eh19C8*</td>
<td>1/7</td>
<td>19/24</td>
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<td>Eh19C9</td>
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<td>Eh19C11</td>
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<td>Eh35C1*</td>
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<td>19/23</td>
</tr>
<tr>
<td>Eh35C1/4*</td>
<td>1/6</td>
<td>19/25</td>
</tr>
<tr>
<td>Eh35C3</td>
<td>1/7</td>
<td>12/19</td>
</tr>
<tr>
<td>Eh35C4</td>
<td>0/7</td>
<td>17/25</td>
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<tr>
<td>Eh35C5*</td>
<td>2/7</td>
<td>20/25</td>
</tr>
<tr>
<td>Eh106C1</td>
<td>0/1</td>
<td>9/24</td>
</tr>
<tr>
<td>Eh208C1</td>
<td>4/6</td>
<td>9/14</td>
</tr>
<tr>
<td>Eh208C2</td>
<td>5/6</td>
<td>16/22</td>
</tr>
<tr>
<td>Eh208C3</td>
<td>5/6</td>
<td>21/22</td>
</tr>
</tbody>
</table>

# Number with positive IFA/number tested
* MAb showing >75% IFA positivity against pathogenic zymodemes and less than 20% IFA positivity against the combined non-pathogenic zymodemes and non-*E. histolytica* amebae or *E. histolytica*-like amebae.
Figure 1. Scatter diagram showing the sensitivity of MAb-PAb-based ELISA for the detection of *E. histolytica* in stool.

Group 1: Stool positive for *E. histolytica* and/or other intestinal parasites.

Group 2: Stool negative for *E. histolytica* but containing other parasites: *E. coli*, *G. lamblia*, *T. hominis*, *O. viverrini*, *T. trichiura*, *E. vermicularis*, Ascaris, Hookworm and *E. hartmanni*.

Group 3: Stool negative for all parasites.
Facilities and equipment obtained from USAID grant e.g. ELISA reader, deep freezer, laminar flow and CO₂ incubator have been made available for use by our department staffs and by other faculty members.

Through USAID support, our work has gained better recognition. We have been invited by Professor Dr. Karl Maramorosch, Department of Entomology and Economic Zoology, Cook College, the State University of New Jersey RUTGERS, who is the editor of a volume "Biotechnology for Biological Control of Pests and Vectors" to prepare a chapter on "Biomedical Applications of Monoclonal Antibodies against Entamoeba histolytica". On the other hand, we have been invited to be advisor and co-advisor of the M.Sc. and Ph.D. students of our Faculty and co-advisor of M.Sc. students of other Faculty and other University.

There was one 2nd year M.Sc. student (Tropical Medicine, Mahidol University) involved in this project in the year 1989-1990. The grant was helpful in providing reagents and equipment for her thesis work and enabled her to develop MAb-based ELISA to detect *E. histolytica* antigens in stool specimens.

Technology of MAb-based ELISA to detect *E. histolytica* antigens in stools was transferred in 1990 to a Nepalese M.Sc. student (Tropical Medicine, Mahidol University) as the result of which he could apply this technique to detect successfully *E. histolytica* antigens in sera and hepatic sections of hamsters with experimentally-induced hepatic amebiasis.

Technology for the maintenance of monoclones and produc-
tion of MAbs in tissue culture fluids and as ascites was transferred to another M.Sc. student (Chemistry, Srinakarinwirot University Prasarnmit). The IgG fraction of this MAb will be conjugated with phycoerythrin produced at the Department of Chemistry, Faculty of Science, Srinakarinwirot University Prasarnmit, Bangkok for use in the future development of antibody labeled probe for direct staining of *E. histolytica* from *in vitro* culture initially and in clinical samples subsequently.

Collaborations

We are now collaborating with Dr. Porntip Chaimanee, Department of Chemistry, Faculty of Science, Silpakorn University, Nakorn Pathom 7300, Thailand under the sub-project title, "Susceptibility testing of *E. histolytica* to Bruceine and Related Components in *Brucea javanica* (L) Merr". Dr. Chaimanee is the principal investigator of a USAID-supported project (No. 9.334) entitled "Enzyme-linked Immunoassay for the determination of Bruceine and Related Quassinoid Compounds in *Brucea javanica* (L) Merr".

**PROJECT ACTIVITIES/OUTPUTS**

Lists of meeting, training and publications are shown below

**Meetings**


3. After PSTC Conference on Biotechnology for Health and Agriculture during June 6-9, 1988, Washington, D.C., PI also had an opportunity to meet Dr. Louis S. Diamond, Head, Section of Parasitic Growth and Differentiation NIH/NIAID to discuss cultivation and axenization of E. histolytica and to visit American Type Culture Collection, Rockville, Maryland.


5. Oral Presentation of "Preliminary attempt to axenic cultivation of E. histolytica from liver pus". ABSTRACTS. Current research work of the Faculty of Tropical Medicine, 1989, p. 7

6. Poster Presentation of "Application of monoclonal antibodies against Entamoeba histolytica in tropical medicine research".
Celebration of Mahidol University Anniversary Seminar and Workshop on Goal Oriented Research for Health for All, February 28 - March 2, 1990 at Asean Institute of Health Development, Mahidol University at Salaya.

7. Oral Presentation of "MAb-based ELISA for the detection of E. histolytica antigens in clinical specimens. Current research work of the Faculty of Tropical Medicine, ABSTRACTS. August 3, 1990, p. 18.


Training

The grant has provided an opportunity for PI to be trained in an area which we did not have expertise. PI received training on isoenzyme typing of \textit{E. histolytica} by Mr. Peter G. Sargeaunt, Senior Research Scientist at the Department of Medical Protozoology, London School of Hygiene and Tropical Medicine during February 1987.

Publications


PROJECT PRODUCTIVITY

The project has fulfilled most of the goal. The area that we could not accomplish is the development of techniques to detect cyst antigens of \textit{E. histolytica}. This shortcoming was mainly due to failure to purify large enough number of cysts of \textit{E. histolytica} from the stools of patients to be used for raising and screening of murine MAbs.

FUTURE WORK

Since we have at least 4 MAbs with potentials to differentiate between pathogenic and nonpathogenic \textit{E. histolytica}, we would like to apply these MAbs in the detection and characterization of \textit{E. histolytica} isolates from asymptomatic and symptomatic
patients. The results will be compared with DNA probes for pathogenic and nonpathogenic E. histolytica to be made available to us by Professor David Mirelman, Department of Biophysics, Weizmann Institute of Science, Rehovot, Israel.

LITERATURE CITED


6. Petri WA, Jackson TFGH, Gathiram V, Kress K, Safter LD,


