H. Vohra, S. Kaur, A. Wangoo, N. K. Ganguly, R. C. Mahajan

Inhibition of Target Cell Adhesion by Monoclonal Antibody to the 66 kD Surface Antigen of *Entamoeba histolytica*

Summary: The adherence of *Entamoeba histolytica* trophozoites to target cells was studied using a monoclonal antibody to a major surface antigen of 66 kD. Preincubation of trophozoites with monoclonal antibody decreased their ability to adhere to and engulf erythrocytes and to destroy Chinese hamster ovary cells. The monoclonal antibody was specific for the 66 kD antigen, which is possibly a major participant in the adhesion that precedes phagocytosis and cytopathic effects.

Zusammenfassung: *Monoklonaler Antikörper gegen das 66 kD Oberflächenantigen von Entamoeba histolytica hemmt die Adhäsion an Zielzellen.* Die Adhäsionsfähigkeit von *Entamoeba histolytica* an Zielzellen unter dem Einfluß eines gegen das 66 kD Haupt-Oberflächenantigen gerichteten monoklonalen Antikörpers wurde untersucht. Nach Präinkubation mit dem monoklonalen Antikörper verminderte sich die Fähigkeit der Trophozoiten, sich an Erythrozyten anzuheften und Chinese hamster ovary (CHO)-Zellen zu zerstören. Der monoklonale Antikörper erwies sich als spezifisch für das 66 kD Antigen, das wahrscheinlich entscheidende Bedeutung für die Adhäsion hat, die anderen Vorgängen wie der Phagozytose und zytopathischen Effekten vorausgeht.

Introduction

Amoebiasis, an important disease that occurs worldwide, is caused by the cytolytic enteric protozoan *Entamoeba histolytica*. Adherence of *E. histolytica* is a crucial event in colonization and invasion [1]. To identify the membrane molecules involved in adhesion, there is a need to develop monoclonal antibodies that would bind specifically to the related components. Earlier attempts in this direction have resulted in identification of lectin activities in *E. histolytica* [2]. However, the membrane molecules involved cannot be specifically defined using carbohydrates, as different molecules on the cell surface may react with the same carbohydrate. The production of monoclonal antibodies (mabs) that inhibit the lectin activity of *E. histolytica* and the adhesion of trophozoites to Chinese hamster ovary (CHO) cells has now been reported [3]. *Arroyo* and *Orozco* [4] have studied the adherence to *E. histolytica* trophozoites to target cells by using an adhesion-deficient mutant of the parasite. They identified a 112 kD surface protein from a wild type strain, the antibodies to which inhibited the adherence to, the erythrophagocytosis of and the cytopathic effects on cell culture monolayers. Antibodies to this 112 kD polypeptide were found in the sera of patients with hepatic abscess. In the present study, we have attempted to develop monoclonal antibodies to a major surface antigen of *E. histolytica* and to identify those proteins on *E. histolytica* involved in specific attachment of trophozoites.

Materials and Methods

Entamoeba histolytica culture and clones: Axenic *E. histolytica* strain NIH:200, maintained in TPS-I medium, was used in the study [5]. BALB/c mice were injected intraperitoneally with 50 µg of purified plasma membrane antigen [6] in 0.2 ml of phosphate-buffered saline (pH 7.2) along with an equal volume of Freund's complete adjuvant. Mice were boosted twice at six-week intervals. Four days after the last injection, the spleen was removed under sterile conditions and the dissociated immune spleen cells were fused with sp 2/0-Ag-14 cells. Fused cells were cultured in RPMI 1640 medium containing 10% PBS and hypoxanthine-aminopterin-thymidine (HAT). Hybridoma culture supernatants were screened for reaction with purified plasma membrane antigen and were titrated by enzyme-linked immunosorbent assay [7]. Supernatants from hybridoma producing monoclonal *E. histolytica* were added to acetone-fixed trophozoites in a twofold dilution. FITC-conjugated rabbit antimouse immunoglobulin (IgG) was used as the seondary antibody; antibody binding to the trophozoite surface was detected by fluorescence microscopy.

Erythrocyte adhesion and phagocytosis: Erythrocyte adhesion test was carried out as described by *Orozco* et al. [8]. Briefly, the trophozoites were incubated with erythrocytes at 0°C for 10 and 30 min and the number of erythrocytes attached per amoeba counted microscopically. Erythrocytes used were of type B from human donors, washed with sterile saline, adjusted in TPS-I medium and used immediately.

Erythrophagocytosis was measured by the amount of hemoglobin incorporated by the trophozoites following incubation with erythrocytes. Briefly, 5×10^5 live trophozoites were incubated with 5×10^7 erythrocytes for 10 min at 37°C. Then, 10 ml of distilled water was added to the cell mixture to lyse non-ingested erythrocytes. Cells were centrifuged at 360 x g for 5 min and washed twice with PBS. Trophozoites were lysed by incubation with 0.5 ml of 2% Triton X-100 for 10 min. The absorbance (460) was read in a spectrophotometer (Konitron Instruments, Switzerland).

Cytopathic effect of trophozoites on cell culture monolayers: The ability of trophozoites to destroy CHO cell monolayers was

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measured as described [9]. Briefly, live trophozoites were added to cell monolayers and incubated for 1 h at 37°C. After washing the nonadherent trophozoites, the cell monolayers were stained with 1% methylene blue in 0.1 M borate buffer. The monolayers were then washed and remaining dye was extracted from the cells with 0.1 N HCl. The amount of dye in ml^{-1} was read in a spectrophotometer at absorbance 660.

Inhibition assay using the monoclonal antibodies: To evaluate the ability of monoclonal antibody to inhibit adhesion, phagocytosis and cytopathic effects, the trophozoites were preincubated at 0°C for 30 min with mab precipitated from the ascitic fluid by ammonium sulphate [10]. Dilutions of mab were adjusted to 1:10, 1:50 and 1:100 for inhibition assays. After preincubation, the assays were carried out in triplicate as described above.

Gel electrophoresis and protein blotting: The purified membrane antigen [6] was resolved in 10% gels by SDS-PAGE. The separated proteins were electrophoretically transferred onto the nitrocellulose paper [11] and immunoblotting was done with monoclonal antibody as the primary antibody and rabbit antimouse IgG labelled with peroxidase (secondary antibody; Dakopatts, Denmark). The bands were visualized after the addition of substrate (3,3' diaminibenzidine 4 HCl; Sigma, USA).

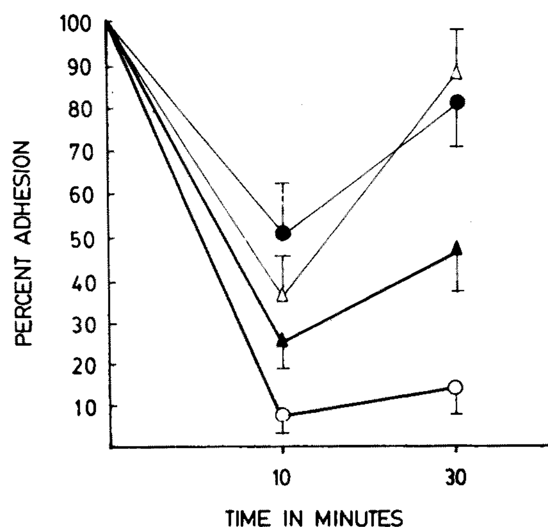

Figure 1: Percent adhesion of erythrocytes to live trophozoites of *E. histolytica* in the presence of monoclonal antibody (O-O) and 1:10 (▲-▲), 1:50 (△-△) and 1:100 (●-●) dilutions.

Results

To identify the monoclonal antibodies directed to the amebic surface antigen, ELISA was carried out using purified membrane antigen (20 µg/ml). Of the 16 clones positive for anti *E. histolytica* monoclonal antibody, only one reacted strongly with the surface antigen in ELISA. This monospecific antibody was further titrated by indirect immunofluorescence using acetone-fixed trophozoites. The monoclonal antibody stained the surface of trophozoites at a titre of 1:256. Furthermore, this monoclonal was assayed as an inhibitor of trophozoite adherence. The erythrocyte adherence assay was carried out in the presence of different concentrations of monoclonal antibody. A mean of 16.6±2.71 erythrocytes per ameba was obtained after trophozoites were incubated with erythrocytes (without antibody) for 10 min at 0°C and the value was taken as 100%. When 1:10, 1:50 and 1:100 dilutions of monoclonal antibody were used in the test system, the values for percent adhesion to erythrocytes were 26.61±18.21, 36.79±13.47 and 50.19±5.46, respectively (Figure 1).

The inhibition of adhesion to erythrocytes by trophozoites was seen after incubation for either 10 or 30 min, though the percent inhibition achieved was greater when incubation with monoclonal antibody was for a longer time.

Figure 2 shows the percent erythrophagocytosis by trophozoites. Preincubation of trophozoites with even a 1:100 dilution of monoclonal antibody inhibited phagocytosis by 50%, followed by 51.2 and 30.8 percent efficiency of inhibition with 1:50 and 1:10 dilutions of antibody, respectively.

The monoclonal antibody inhibited the ability of trophozoites to destroy CHO cell monolayers by 40% at a 1:50 dilution (Figure 2). Typical values of monolayers in

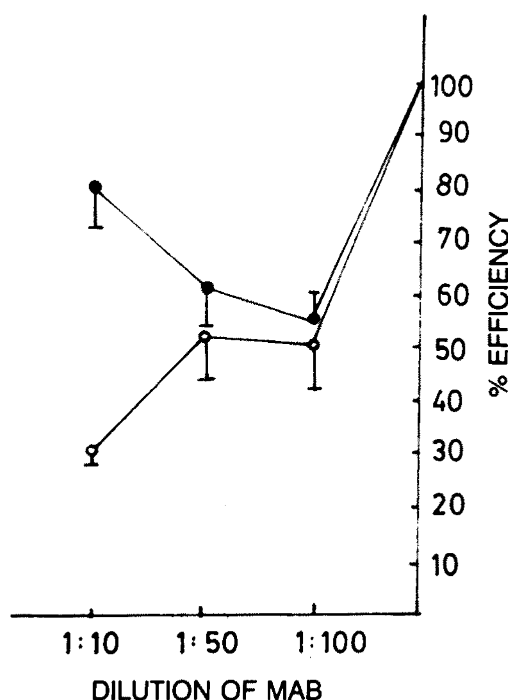

Figure 2: Percent efficiency of live trophozoites to erythrophagocytose (O-O) and destroy CHO cells (●-●) in the presence of monoclonal antibody.

contact with trophozoites that were not incubated with monoclonal antibody were 0.038–0.045 at an absorbance of 660; these were taken as 100% destruction values. Negative controls in which the monolayers were not in contact with trophozoites gave values of 0.46 to 0.52. These values were taken as 0% destruction.

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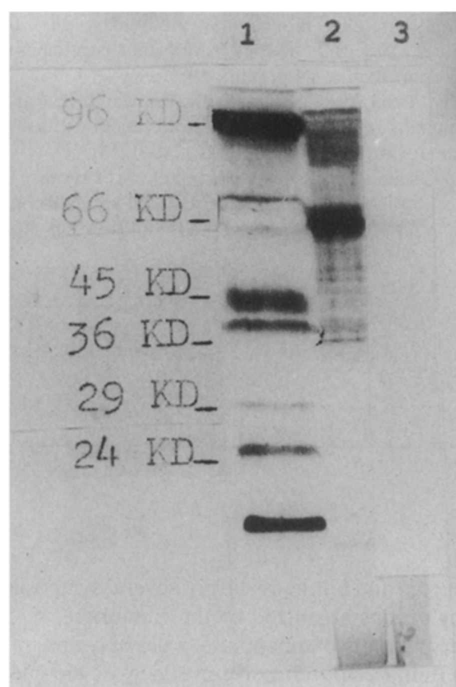

Figure 3: Immunoblotting pattern of total membrane proteins with immune sera (lane 2) and monoclonal antibody (lane 3).

Immunoblotting of total membrane proteins with the monoclonal antibody showed that it reacted with an antigen of relative molecular weight of 66 kD (Figure 3).

Discussion

The plasma membrane of protozoa plays an important role in the interaction of parasite and host. A number of plasma membrane components have been identified that mediate initial parasitic attachment and entry into cells, serve as virulence factors, or interact in an immunomodulatory capacity with the host. *Aust-Kettis* et al. [12], who did not separate the cytoplasmic membrane from the ^{125}I radio-labelled *E. histolytica* strains HK-9 and NIH:200, found 12 polypeptides by autoradiography, the molecular weights ranging from 9 to 150 kD. Monoclonal anti-amoeba antibodies led *Torian* et al. [13] to identify, characterize and purify a 96 kD surface glycoprotein that reacted specifically with sera from patients with amebic liver abscess. Surface proteins that participate in the *in vitro* adhesion of *E. histolytica* trophozoites to human erythrocytes have also been recognised; using different methods, the proteins were found to have molecular weights of 112 kD [14], 170 kD [15] and 220 kD [16].

We report here the production of a monoclonal antibody to a major surface moiety of *E. histolytica*. This membrane constituent is an antigen of 66 kD located on the trophozoite surface as seen in immunofluorescence studies. Both the adhesion of trophozoites to erythrocytes and phagocytosis were specifically reduced when the monoclonal antibody to this 66 kD protein was used in assay systems. The monoclonal antibody reacted strongly with the 66 kD protein in immunoblot studies. The erythrocyte adherences studies were performed to determine whether it was directed to a protein involved in trophozoite adherence to erythrocytes. The inhibition of adherence by the antibody suggests the involvement of this surface antigen in adherence. The protein identified could be one of the many adhesins that participate in the adhesion of trophozoites to the target cells. Trophozoite adherence to the target cells is one of the first events in phagocytosis and cytopathic effects; therefore, it is logical to assume that other adhesion-dependent virulence properties of the trophozoites should also be inhibited. Data obtained on the inhibition of phagocytosis and cytopathic effects correlate with that obtained on inhibition of adhesion, thereby providing more evidence that specific adhesion precedes these events. The results indicate that this 66 kD antigen may be a major participant in adhesion, but the possibility that other surface molecules may be involved in the attachment of trophozoites to target cells cannot be excluded.

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Book Review

S. C. Oaks, V. S. Mitchell, G. W. Pearson, C. C. J. Carpenter (eds.)

Malaria: Obstacles and Opportunities
309 pages, Price: $ 39.95 (hardbound)
National Academic Press, Washington D.C. 1991

This book is a report of the "Committee for the Study on Malaria Prevention and Control," which was formed by the Institute of Medicine (IOM) at the request of the United States Agency for International Development (USAID).

Malaria has made a dramatic resurgence over the past two decades. The eradication programs in the 1950s and 1960s have failed; major problems concerning drug resistance and maintenance of control have evolved. Despite the tremendous efforts in vaccine development, the hopes for a major breakthrough have not yet been fulfilled. The objectives of this study were to present a summary of the current situation and to give recommendations that funding agencies could use to focus their efforts in malaria research, prevention and control. The report comprises the study results and commentary of the 19-member committee and the contributions of another 55 international top experts. Study results and recommendations are based on the detailed analysis of information from a wide range of sources and on the results of several symposia, meetings and working groups organized by the committee.

In 12 chapters with two appendixes, a glossary and an index, the reader will find a comprehensive overview of the current status of malaria research, prevention and control efforts worldwide, including socioeconomical and ethical aspects. Each chapter begins with a statement on expected aims within a foreseeable period of time (Where we want to be in the year 2010). This is followed by a detailed overview on the current status (Where we are today) and ends with a research agenda, which clearly focuses the major problems and denotes the research priorities. Conclusions and recommendations have been summarized in a separate chapter. Readers who are unfamiliar with malaria should start with the two chapters "Background" and "Overview," which will serve as an introduction to the subject. At the moment, this report is one of the most up-to-date overviews of the problems and research needs in malaria. This book provides competent advice to all who are interested in state-of-the-science information and research agendas concerning prevention and control.

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