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Study of Antiamoebic Antibody in Amoebiasis Using ELISA and RID Techniques

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Abstract

To study the humoral immune response to *Entamoeba histolytica* infection, sera prepared from different cases of amoebiasis from gastroenterology ward of B.H.U hospital, including15 cases with amoebic liver abscess (ALA), 10 cases with acute amoebic dysentery (AD) and 10 controls were examined by Enzyme Linked Immuno Sorbent Assay (ELISA), using crude amoebic antigen prepared from *Entamoeba histolytica* NIH: 200 grown axenically and its three chromatographed fractions, i.e.fraction I (FI), fraction II (FII) and fraction III (FIII). Efficacies of different antigens in detecting anti amoebic antibodies were compared. Estimation of immunoglobulin G (IgG), immunoglobulin M (IgM) and immunoglobulin A (IgA) levels were also carried out employing Single Radial Immuno Diffusion (RID) technique. The results indicated that crude amoebic antigen and fraction I (FI) were more efficient in antibody detection while, potencies of fraction II (FII) and fraction III (FIII) were lower as antigens for serodiagnoses. On estimating different immunoglobulin levels, it was found out that there was significant increase in IgG level in amoebiasis group while, no significant increase was observed in IgA level. IgM level was increased only in amoebic liver abscess cases compared to negative controls.

Keywords: *Serodiagnosis, Antibody titre, Immunodiagnosis, Amoebiasis*

Introduction

Amoebiasis due to *Entamoeba histolytica* is world wide in its distribution and has a spectrum of clinical manifestations (1).

E. histolytica is a protozoan parasite that causes amoebic colitis and liver abscess in some developing countries such as Mexico and India (2). Most often, infection is symptomless but in approximately 10% of human hosts, invasion to gut mucosa and extra intestinal sites lead to dysentery, amoebic liver abscess, pulmonary abscess and involvement of other organs (3). Amoebiasis is responsible for 50,000-100,000 deaths annually (4). In spite of effective treatment against amoebiasis, morbidity and mortality due to amoebic infection is being reported which suggests ways to be find out for limiting or eradicating the disease. By inoculation of live or fixed trophozoites, the evolution of the hepatic lesion and the participation of some humoral factors in the development of hepatic amoebic lesion was studied (5).Humoral and amoebicidal cell mediated immune responses have been documented in patients recovering from invasive *E. histolytica* infection (6). Serological tests are valuable adjuncts to the diagnosis and epidemiology of amoebiasis (7) and these assays are performed with *E. histolytica* whole cell lysate as antigen. The whole cell extract contains a number of antigens of various specificities (8). To define the specificity and their usefulness for the serological tests, the present work has been undertaken and also to characterize the serological specificities of the various isolated fractions. Apart from these, serum immunoglobulin levels were also evaluated for the patients' sera.

Materials and Methods

Serological tests for the detection of anti amoebic antibodies were carried out in different

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groups of clinically confirmed amoebiasis and well matched controls, using different amoebic antigens by employing ELISA.

Antigen preparation Crude amoebic extract was prepared from *E. histolytica* NIH: 200 grown axenically in TPS-1 medium (9). Ultrasonicated extract of *E. histolytica* was prepared and labeled as crude amoebic antigen. By column chromatography three fractions were obtained.These fractions were labelled as FI, FII and FIII. 200 µl of each antigen, i.e. crude, FI, FII and FIII, were used for coating ELISA plates. The protein content of each antigen was determined by Lowry method (10).

Sera i) Amoebic liver abscess: blood was collected from 15 patients with liver abscess. They were either acutely ill or had symptoms lasting for months. These patients were admitted to gastroenterology ward of B.H.U (Banaras Hindu University, Varanasi, India) hospital. Their cases were diagnosed on the bases of clinical and ultrasonography findings and confirmed by aspiration of anchovy sauce pus from the liver abscess. Parasitological investigations were carried out for stool specimens and aspirated pus.

ii) Acute amoebic dysentery: 10 cases having the history of dysentery accompanied by varying amount of blood stained mucous in the faeces. Clinical diagnosis was confirmed by clinical signs like 3-10 times bowel movement per day, discomfort in lower abdomen for 1 to 7 days or more. Microscopic examination of stool demonstrating the haematophagous trophozoites of *E. histolytica* was carried out.

iii) Control group: Ten individuals of this group looked apparently healthy with no illness related to amoebiasis. Three consecutive microscopic examination of their stools or formol-ether concentration method (11) was found to be negative for *Entamoeba histolytica* cyst.

Sera prepared from above mentioned cases were diluted serially from 1:100 to 1:12800.

O-phenylene Diamine Dihydrochloride (OPD) was used as substrate (pH=3.7) and the conjugate was Horse Radish Peroxidase antihuman Immunoglobulin (Sigma,USA).

Based on clinical analysis of the O.D values of the healthy controls, a cut-off point equivalent to the mean +2 SD O.D values were calculated (2). Any test sample with O.D value of more than cut-off point, i.e. 0.474 was regarded as positive.

Quantitation of the immunoglobulins in cases and controls were used as the marker for study of humoral immune responses. Serum immunoglobulin levels were evaluated by Single Radial ImmunoDiffusion technique using tripartigen plates provided. Procedure followed as per instruction provided by the manufacturer (Hoechst pharmaceuticals, India Ltd).

IgA and IgM were estimated by using undiluted sera while for estimation of IgG the sera were diluted to 1:30 dilutions.

Loading of the plates A total of 15 immunodiffusion plates containing 12 wells, 5 plates for each immunoglobulin, were used. In each plate wells were numbered clockwise, and the first well was filled with 5µ l of the control sera supplied by the manufacturer. Wells numbered 2 to 12 were the test wells. These were filled with 5 µl of the sera prepared from the study cases. After filling the wells, plates were left at room temperature for 2-3 days. A diffusion time of 72 hours was needed for IgM. After incubation period was over, measurements were taken. Results were considered reliable only when the values for the control serum poured in well No. 1 was within confidence range. In case of IgG the values found were multiplied by the dilution factor 30.

Results

Using the cut-off value of O.D as 0.474, when crude amoebic extract was employed for detecting antibody in different cases, it was observed that in amoebic liver abscess group, 14 cases were positive (93.33%). In the case of

acute amoebic dysentery, antibody was detected in 7 out of 10 cases (70% positivity).

As is shown in Table 1, using FI for detection of antibody in the sera of different groups, antibody was detected in 93.33% cases of the first group . Using the same antigen for antibody detection in amoebic dysentery cases, there were 7 positive (70%) cases. Using fraction II in 9 cases (60%) of amoebic liver abscess, antibody could be detected. In amoebic dysentery cases antibody was detected in 5 cases (50%) only. Using FIII antigen, 7 cases of first group were positive (46.6%). In second group, antibody was detected in 5 cases (50%). Using different antigens, no antibody could be demonstrated in any of healthy controls. Serum IgG, IgM and IgA were estimated quantitatively from the different categories of amoebiasis cases and controls, but IgE was not estimated because their role, as reported in protozoal infection, is minimal. The mean IgG levels were elevated in all the study groups with a maximum rise in amoebic liver abscess followed by amoebic dysentery cases. Patients with amoebic liver abscess had mean IgG level of 1725 + 237.12 mg/dl while, it was 1602 + 158.31 mg/dl in case of dysentery patients. There was highly significant difference (P<0.001) between IgG level in amoebic liver abscess patients when compared to control group with mean IgG value of 1304 + 238.52 mg/dl. Significant difference was observed when IgG value of amoebic dysentery cases was compared with control group (P<0.001). When the IgA value of liver abscess patients as well as dysentery patients was compared with control group, no significant difference was observed. Significant difference (P<0.001) was found out on comparing IgM values of first group with control group. Difference was significant when IgM values of dysentery patients were compared with control group which was 133 + 26.4 (Table 2). However, the overall picture of the immunoglobulins in amoebiasis cases and controls, as is shown in the bardiagram, revealed that IgG was increased in the intestinal and extra-intestinal amoebiasis groups, and the IgM was elevated in amoebic liver abscess group only. Though, IgA also showed rise in both amoebic liver abscess and dysentery groups but, it was not significantly different from that of control group.

Table 1: Antibody detection in different cases using different antigens

		Crude antigen		**FI**		**FII**		**FIII**	**-**
Clinical Categories	**Total No. tested**	**Positive cases**	**Percent Positivity**	**Positive cases**	**Percent Positivity**	**Positive cases**	**Percent Positivity**	**Positive cases**	**Percent Positivity**
Amoebic Liver Abscess	15	14	93.33	14	93.33	9	60.00	7	46.60
Acute Amoebic Dysentery	10	7	70.00	7	70.00	5	50.00	5	50.00
Control	10	0	-	0	-	0	-	0	-

Taking the cut-off value of 0.474

Table 2: Estimated serum immunoglobulin levels in different groups

Clinical Category	No. in each group	MeanIgG+S.D mg/dl	MeanIgA+S.D mg/dl	MeanIgM+S.D mg/dl
Amoebic Liver Abscess	15	1725+237.12	261+72.35	168+28.5
Acute Amoebic Dysentery	10	1602+158.31	231+59.35	162+28.1
Control group	10	1304+238.52	219+58.1	133+26.4

P (1:2) t=1.43 NS P (1:2) t=0.4 NS P (1:2) t=0.51 NS
P (1:3) t=4.92<0.001 P (1:3) t=1.40 NS P (1:3) t=3.05<0.01
P (2:3) t=3.90<0.001 P (2:3) t=1.71 NS P (2:3) t=2.34<0.05

Discussion

Depending upon the calculated O.D values, the positive and negatives were differentiated.

i) Antibody detection in amoebic liver abscess patients: Out of 15 amoebic liver abscess patients in 14 anti amoebic antibody were detected (93.33%) using crude amoebic antigen and FI. Other workers also recorded 80-100 percent positivity (5).

The O.D value taken in this study is higher than the O.D taken by other workers (12, 7). This could be the reason for 1 negative case in which antibody could not be detected. FII could detect antibody in 9 out of 15 cases. While using FIII antigen, only 7 cases were positive. This finding revealed poor antigenicity of these antigens in detecting antibody.

ii) Antibody detection in acute amoebic dysentery cases: By using crude amoebic antigen and FI for antibody detection, antibody was detected in 7 out of 10 cases (70%). Ganguly et al (1) found 87.5% positivity and Agarwal et al (7) obtained 62.96% positivity. In negative cases probably, early onset of infection might not have caused sufficient tissue invasion to stimulate enough antibody response to be detected. The other factor attributable is that they might have been less reactive individuals or low responders. While by employing FII and FIII antigens for antibody detection, in 5 out of 10 cases antibody was detected. This shows poor antigenicity of these antigens in detecting antibody and higher O.D values taken in this study.

There was no positive case in individuals belonging to the control group because they were never exposed to amoebiasis so the question of antibody does not rise. No false positive result was obtained with any of the antigens. Therefore, ELISA, by testing with all the amoebic antigens, showed 100 percent specificity. The potency of FII and FIII is lower as an antigen for serodiagnosis since the sensitivity of the test reduces hence unsuitable for serodiagnosis. Quantitation of the serum immunoglobulin G revealed highly significant difference in both amoebiasis groups as compared to control. This finding is in accordance with the findings of Schulz et al (13), Sepulveda et al (14) who reported increase in IgG level in amoebiasis as a whole. The findings of invasive amoebiasis groups are well corroborated with above mentioned workers. In this study, mean serum immunoglobulin A level was also estimated in both amoebiasis groups. Comparison of mean IgA value of amoebic liver abscess group with control group reveals no significant increase in IgA level of either of the amoebiasis groups and the values were comparable to the control group. Serum immunoglobulin M level was also quantitated in all the study cases. Mean serum immunoglobulin M level showed elevation in

amoebic liver abscess group. The difference between IgM values of amoebic liver abscess group when compared to that of control showed significant difference. In acute amoebic dysentery group also, mean IgM value showed elevation although, the difference between IgM value of these patients compared to control group was found to be non-significant ($P<0.05$). Increase in serum Ig M level in case of amoebic liver abscess has been reported by previous workers (15). The rise in IgM may be due to the fact that amoebic liver abscess is an extra intestinal manifestation of invasive amoebiasis where the antigens come in contact with the immune system of the body. Since the patients were studied as soon as they were admitted in the hospital, the IgM, being the earliest antibody, probably was detected at a significantly higher level than dysentery and control groups. However, the overall picture of the immunoglobulins in amoebiasis cases and controls showed that IgG was increased in the intestinal and extra intestinal (ALA) amoebiasis groups and the IgM was elevated in amoebic liver abscess group only. While, though IgA also showed rise in both amoebic liver abscess and dysentery groups but, it was not significantly different from that of control group.

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