A COMPARISON OF COUNTERIMMUNOELECTROPHORESIS AND INDIRECT HAEMAGGLUTINATION TESTS FOR THE IMMUNOEPIDEMIOLOGICAL INVESTIGATION OF KALA-AZAR

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INTRODUCTION

The invasive procedures usually required for the parasitological diagnosis of kala-azar generally preclude their application to epidemiological investigations. Serology would, therefore, be of importance for field studies of that disease. Although a variety of serologies such as indirect haemagglutination (IHA) (Bray and Lainson, 1967), indirect fluorescence (IF) (Shaw and Voller, 1964), counterimmunoelectrophoresis (CIE) (Desowitz *et al.*, 1975; Rezai *et al.*, 1977), and enzyme-linked immunosorbent assay (ELISA) (Hommel, 1976) have been applied for the serodiagnosis of kala-azar they appear to differ in specificity and sensitivity and it is still not clear which technique, or combination of techniques, is the most suitable for surveillance purposes. Moreover, a serological test that meets all the theoretical requirements when carried out by well-trained workers in a well-equipped laboratory may not be applicable in the endemic region because of the laboratory conditions existing there.

The reappearance of kala-azar in the Pabna District of Bangladesh has given rise to the concern that the disease which once ravaged East Bengal could again become established in many areas of the country. It was apparent that a surveillance programme should be carried out and that a serological component to that programme would have to be included in order to identify the existing foci of infection and to monitor any changes in the epidemiological pattern. For pragmatic reasons we decided to evaluate and compare two serological tests, CIE and IHA. CIE because of its ease of performance and the availability of reagents and apparatus and IHA because commercially prepared and standardized test kits (Cellognost-Leishmania, Behringwerke) could be obtained and also because, as recently noted by the World Health Organization (WHO, 1984) the commercially available IHAT has not been fully evaluated in the field.

MATERIALS AND METHODS

Population : The study was carried out in a village of approximately 1000 people near Shadjipur, Pabna District, Bangladesh. Examination of the villagers for hepatosplenomegaly revealed 5 cases of possible kala-azar (malaria either does not exist or is of very low endemicity in Pabna). Bone marrow biopsy for slides and culture in N.N.N. medium was made from four cases all of which subsequently proved to be parasitologically positive. Case 5 from which bone marrow could not be obtained was aldehyde test positive. Serum samples were obtained from the above 5 cases and from an additional 34 asymptomatic villagers.

Serologies : CIE was performed in an Austigen apparatus (Hyland, Costa Mesa, California) set at 40 mA for 1 hour. The gel

plates were prepared from 0.75% Type IV agar (Sigma, St. Louis, Missouri) dissolved in barbital-acetate buffer pH 8.2 (0.04M barbital, 0.02M sodium acetate). Following electrophoresis, the plates were soaked overnight in 5% sodium citrate to dissolve any false positive precipitates formed by C-reactive protein (Hillyer and Capron, 1976). The reactions, read by means of incident light illumination and magnifying glass, were scored as +++ (3-4 strong precipitin bands), ++ (2-3 moderately strong precipitin bands), and + (1-2 moderately strong to weak precipitin bands).

Two antigens were used for CIE, one an extract of a Shadjipur strain of *Leishmania donovani* promastigotes maintained in culture, the other an extract of an old, monomorphic strain of *Trypanosoma brucei* trypomastigotes maintained in white rats. The *L. donovani* antigen was made by centrifuging cultures containing numerous organisms and washing the organisms three times with sterile phosphate buffered saline. The pellet was then resuspended in 2-3 ml buffer and the organisms disrupted by repeated free-thawing followed by sonication and centrifugation. The final protein content of the supernatant was estimated to be 2-3 mg/ml.

The *T. brucei* antigen was tested to determine whether it could serve as an alternative reactant in the CIE in areas where kala-azar but not trypanosomiasis is present, such as in Bangladesh. Laboratories in endemic regions often do not have the technical or economic resources to maintain, under sterile conditions, the large scale *in vitro* culture of *Leishmania* needed for diagnostic and immunoepidemiological requirements but conceivably could maintain *T. brucei* in laboratory animals. Desowitz *et al.*, (1975) had reported that antigen from another haemoflagellate, *T. cruzi*, gave a complete cross-reaction with sera from kala-azar patients. *T. brucei*-infected white rats were bled by cardiac puncture into syringes containing heparin when the infections attained a massive parasitaemia. The blood was centrifuged at 1200 rpm (300 xg) for 10 minutes and the buffy layer containing the trypanosomes removed. The trypanosomes were washed 3 times in PBS, resuspended in buffer and disrupted in a Hughes-Colab press. The extract was centrifuged at 10,000 rpm (4,000 xg) for 30 minutes, the supernatant adjusted by Lowry analysis (Lowry *et al.*, 1951) to contain 5 mg protein/ml, lyophilized in 1 ml aliquot and reconstituted to the same volume of sterilized glass-distilled water as needed.

The IHA test was performed using the Cellognost-Leishmania kit (lyophilized sensitized cells, positive and negative control sera) according to the instructions supplied by the manufacturer, Behringwerke Diagnostics, Frankfurt, Germany. The quantitative test (serial serum dilutions) was carried out on the 5 kala-azar cases whereas, for the sake of economy, the presumptive test (one dilution of 1 : 40) was used for the 34 serum samples from the asymptomatic villagers. The quantitative test was then performed on all the presumptive test-positive sera.

RESULTS

All of the 5 sera from the kala-azar cases were CIE-positive. Sera positive to the *L. donovani* antigen were in all instances also positive to the *T. brucei* antigen and probably because of its estimated higher protein content the precipitin lines were usually more distinct and numerous with the latter antigen. None of the sera from the 34 asymptomatic villagers was positive by this test. Table 1 compares the results of the CIE and IHA tests from the 5 kala-azar cases. The notes accompanying the Behringwerke Cellognost-Leishmania kit state, "Serum titres of 1: 64 and above give a clear indication of *L. donovani* infection. Mean serum titres range

Table 1
Comparison of
the counterimmunoelectrophoresis (CIE)
and indirect haemagglutination (IHA)
tests on sera from kala-azar patients.

Case No.	CIE	IHA (reciprocal titre)
1	+++	2048
2	++	2048
3	++	32
4	+	64
5	+++	512

Table 2
IHA titres of presumptive test-positive sera from healthy individuals resident in an area where kala-azar is present.

Titre (reciprocal)	16	32	64	128
No. sera at titre	1	3	7	3

from 1 : 256 to 1 : 2048. Titres lower than 1 : 32 eliminate kala-azar''. According to these criteria it will be seen from Table 1 that 3 of the 5 cases were IHA positive, 1 was borderline, and 1 negative. Nor did the strength of the CIE reactions give good agreement with the IHA titre. Thus, one ++ CIE reaction had an IHA titre of 1 : 2048 (case 2) while another ++ CIE reaction had a 1 : 32 IHA titre (case 3).

The IHA test results from the sera of the 34 asymptomatic individuals are even more difficult to interpret. Fourteen (41%) were positive by the presumptive screening test. When these 14 sera were retested by the quantitative method they gave a titre distribution shown in Table 2. Ten serum samples had titres between 1 : 64 and 1 : 128, levels considered by Behringwerke to be positive for kala-azar.

DISCUSSION

The objectives of immunoepidemiological surveillance of kala-azar are, obviously, to determine whether or not cases are occurring in any particular area and, if so, to determine the prevalence rate. The results of our study suggest that CIE by virtue of sensitivity and simplicity may be the best serological technique to answer these questions. Rezai *et al.*, (1977) in comparing the CIE and IF for the diagnosis of kala-azar came to a similar conclusion.

Further investigations are required to define the role of the more sensitive tests, such as IHA and ELISA, for immunoepidemiological purposes. Although numerous studies have evaluated a variety of serological tests for the diagnosis of kala-azar there has been little work on the application of these tests to immunoepidemiology. At present, the major difficulty in interpreting the results of the more sensitive tests is the inability to account for the positive, albeit relatively low, antibody titres in the sera of individuals residing in an endemic area but who have no signs or symptoms of kala-azar. In our study 10 of the 34 (29%) of the "normal'' sera were IHA positive at titres of 1 : 64 – 1 : 128. Similarly, Srivasta *et al.*, (1979) in applying the ELISA test to a study of kala-azar in Bihar State, India, reported a significantly higher mean optical density value in the "normal'' sera from Bihar (OD 0.11) than in "normal'' sera from Delhi (OD 0.04) where kala-azar is not transmitted. However, similar to our low-positive IHA titres, they also noted that their "normal positives'' had a significantly lower mean OD than that of the sera from confirmed cases (OD 0.60). Srivasta *et al.*, (1979) suggested that the "normal positive'' sera "indicate exposure of the population to infection''. This would imply that a large number of individuals either immunologically terminate the infection or are in a non-clinical infected state. Neither of these hypotheses have been proven but would be of considerable importance. Some IHA borderline positive sera may re-

present early cases that are CIE-negative due to a low level of IgG antibody at that stage. However, the apparently low incidence of kala-azar in Pabna makes this an unlikely cause considering the 29% borderline positivity rate of the "normal sera".

Despite the above uncertainties, for the present, we recommend the use of two serological tests for the immunoepidemiological surveillance of kala-azar; CIE to identify those infected with clinical disease and a more sensitive test such as the ELISA or IHA to detect what seems to be a "background" of exposure. Meanwhile, the questions raised should be resolved by further expanded studies such as longitudinal investigations that would determine the clinical and parasitological status of those with borderline titres and those who sero-convert to those titres.

SUMMARY

Counterimmunoelectrophoresis (CIE) using cell-free extracts of *Leishmania donovani* promastigotes and *Trypanosoma brucei* as antigens and indirect haemagglutination (IHA) using commercially prepared reagents were compared for their diagnostic efficacy and applicability to immunoepidemiological studies in an area of Bangladesh where kala-azar is present. The CIE was positive for all parasitologically confirmed cases whereas the IHA positivity was only 60%. The *T. brucei* antigen was equally as good, if not better, than the *L. donovani* antigen for CIE. The CIE test was negative for all of 34 apparently healthy villagers. For this same group of individuals, 10 (29%) were low titre-IHA positive. The findings suggest that CIE is the more reliable diagnostic test but both methods should be employed for immunoepidemiological investigations.

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