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Immune complexes in Indian kala-azar

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Patients with Indian kala-azar were investigated for the presence of circulating immune complexes by the platelet aggregation test, complement deviation test, and polyethylene glycol precipitation test. Circulating antibodies were tested by the conventional indirect immunofluorescence test using leptomonad forms of Leishmania donovani *and* Crithidia luciliae. *The serum complement level (C3) was measured using the Mancini technique. The results indicate that a large number of patients with Indian kala-azar carry circulating immune complexes with a significant lowering of complement levels in their sera. These complexes may be intimately linked with the depressed cell-mediated immune responses that are commonly observed in these patients. The study warrants a detailed immunohistopathological examination of the kidneys for the presence of tissue-bound complexes in chronic patients. Further, it is revealed that* Crithidia luciliae *and* Leishmania donovani *share common antigens and the former can be used as a substitute for determining anti-leishmania antibody by the indirect fluorescence assay.*

Since 1968, when the very significant observations of Dixon and coworkers (*1*) on the role of immune complexes in causing renal disease were published, advances have been made in knowledge about the various antigens that trigger this reaction. Recent studies indicate that, in addition to many bacterial antigens, parasites like *Plasmodium malariae* and *P. falciparum* are important causative agents of renal disease in man (*2*, *3*). Similar lesions have been reported to occur in schistosomiasis (*4*) and other parasitic infections as well (*5*, *6*), although there is a paucity of data concerning kala-azar (visceral leishmaniasis). The latter is caused by an intracellular haemoflagellate and gives rise to a bewildering variety of clinical syndromes in different parts of the world. In India, *Leishmania donovani* causes widespread visceral lesions and the disease has a high morbidity and mortality. While the clinical syndrome of kala-azar has been well recognized (*7*), some of its characteristics were recently evaluated by Aikat et al. (*8*, *9*) in studies of an epidemic in India. However, information about the presence of immune complexes and their role in the pathogenesis of this disease has been conspicuously scanty. The present study was therefore planned with the object of investigating the serum of patients with kala-azar for direct or indirect evidence of the presence of circulating immune complexes in these patients.

PATIENTS AND METHODS

A total of 313 cases of suspected kala-azar were investigated between October 1977 and February 1978 during an epidemic in the state of Bihar, India. Detailed clinical and haematological (and a part of the immunological) studies on 149 proven cases have already been published (*8*, *9*, *10*). Sera from these patients were stored at −70 °C and the following investigations were performed on selected groups:

(i) β_1C globulin levels were estimated by the conventional single radial immunodiffusion technique using antiserum.[a] Sera from 20 healthy laboratory workers served as controls and the results were expressed as a percentage of the mean normal pool.

(ii) The platelet aggregation test (PAT) was performed according to the method advocated by Pentinnen (*11*). Briefly, 20 ml of heparinized blood was drawn in a plastic syringe, transferred to a disposable centrifuge tube, and the platelet-rich plasma was collected immediately after centrifugation. The platelets were washed twice with pyrogen-free saline and once with buffered salt solution (Ca- and Mg-free, with 1.0 mmol/l glucose at pH 7.8), and then suspended in phosphate-buffered saline (PBS) to give a concentration of 200 000/mm^3. The test was performed in 'U' microplates[b] by adding 0.25 ml of the platelet suspension to an equal volume of doubling dilutions of the patient's serum. The sedimentation pattern was recorded after incubating the plates for 18 h at 4 °C; any button formation was read as negative.

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(iii) Anticomplementary activity was tested as described before (*12*). Washed sheep red cells were sensitized with an optimum dilution of amboceptor and 120 million of these cells were labelled with 100 μCi (or 3.7 Bq) of ^{51}Cr (sodium chromate) according to the method of Sanderson (*13*). The washed, sensitized, labelled erythrocytes were adjusted to a final concentration of 120 million/ml. In small round-bottom tubes, the reagents were then added in the following order:

(1) 50 μl heat inactivated serum + 50 μl CFT buffer[c]	— incubated for 30 min at 4 °C
(2) + 100 μl complement[d]	— incubated for 15 min at 4 °C
(3) + EA[e] 2.5 million + 250 μl CFT buffer[c]	—incubated for 15 min at 37 °C, then centrifuged, and radioactivity in the supernatants determined in a gamma counter.

EA cells,[e] incubated in distilled water for maximal release and in CFT buffer for spontaneous release, were the controls. The percentage inhibition of chromium release was calculated after appropriate corrections. All samples were coded and duplicated, and the results of the various investigations subjected to statistical analysis.

(iv) The polyethylene glycol (PEG) precipitation test was carried out to assess the presence of circulating immune complexes, as advocated by Digeon et al. (*14*). Briefly, a 1 in 25 dilution of each patient's serum in borate buffer (0.1 mol/litre, pH 8.4) was allowed to interact with an equal volume of 7% PEG (relative molecular mass 6000) for a period of 18 h at 4 °C. The tubes were centrifuged while cold at 20 000 *g* for 20 min and the precipitate was washed in 3.5% PEG solution and then suspended in 5 ml of 0.1 mol/litre sodium hydroxide. The optical density (OD) of the precipitated proteins was assessed in a Pye Unichem SP spectrophotometer at 280 nm. As a standard, human gammaglobulin[f] was aggregated at 63 °C for 20 min and dilutions ranging from 1.25 mg to 10 mg/ml were prepared. These were similarly precipitated with 3.5% PEG solution and their OD measured. A curve was drawn with OD (on the ordinate) against protein concentrations (on the abscissa). Similar concentrations of unaggregated globulins were also precipitated with PEG and their optical densities determined.

(v) Anti-*Leishmania* antibodies were estimated by conventional indirect immunofluorescence assays (*15*), using leptomonad forms of *Leishmania donovani* (cultured in the laboratory) as well as *Crithidia luciliae*. The latter was a gift from Professor L. A. Aarden in Amsterdam and was cultured in the laboratory according to the method of Boné & Steinert (*16*). No attempt was made to find the end-titre of each serum.

[c] Complement fixation test buffer (Oxoid Ltd, code N°. BR16).

[d] 1:30 dilution of fresh guinea pig serum was the source of complement.

[e] EA = amboceptor treated erythrocytes.

[f] From Curewell (India) Ltd.

RESULTS

Data from studies carried out within 6–8 weeks of storage at −70 °C of serum (from patients with Indian kala-azar) indicated that the complement (C3) levels were reduced. The serum controls from subjects in the endemic area revealed a value of 95.6% (S. E. ± 3.4%) of the mean normal C3 level, as opposed to 54.3% (S. E. ± 3.9%) in the kala-azar patients. The difference was statistically significant ($P < 0.001$).

The platelet aggregation test (PAT) was also abnormal in these patients. The test, though requiring meticulous handling of platelets, was found to be adequately reproducible. The arithmetic mean titre in the controls was less than 2, while in the kala-azar patients it was 193.1 (± 51.9), even higher than those observed in patients with systemic lupus erythematosus (108 ± 36.1). Similarly, the geometric mean titre in the kala-azar patients (25.1 ± 1.04) was much higher than that in the controls (0.5 ± 0.02).

The complement deviation test likewise corroborated the PAT findings because the sera from a large number of kala-azar patients inhibited the release of chromium from the EA cells on exposure to guinea pig complement. The test is based on anti-complementary activity but is much more sensitive than the conventional test so that precise values can be calculated with accuracy. The mean percentage inhibition of chromium release (immune lysis) in healthy controls was 13.6 (± 3.07), as opposed to 29.6 (± 7.3) in the controls from the endemic area and 82.5 (± 4.3) in the patients with kala-azar. The difference between the test group and the controls was highly significant ($P < 0.001$).

The PEG precipitation test revealed a mean optical density of 0.77 (»0.07) in the group of kala-azar patients, compared with 0.31 (»0.02) in the controls. The standard curve obtained with different concentrations of heat-aggregated gammaglobulin (AHG) showed a linear correlation between the amount of AHG and the optical density. Normal gammaglobulin, without heat aggregation, failed to give significant precipitation with polyethylene glycol. The difference in PEG values between the controls and the patients was also statistically significant ($P < 0.01$) although the range was much wider among the patients.

The sera of all patients with kala-azar revealed a varying degree of positive immunofluorescence with the leptomonad forms (Fig. 1 and 2). Further, as expected, a significant percentage of the control samples from the endemic area also revealed a positive fluorescence. The patients' sera also revealed a diffuse positive reaction with *Crithidia luciliae*. The staining was more marked on the membrane and basal body; nuclear or kinetoplast staining could not be delineated. Sera from local healthy individuals did not reveal a positive reaction.

DISCUSSION

Immune complexes have been incriminated in the pathogenesis of a large number of diseases. The resurgence of kala-azar in North Bihar offered a unique opportunity to study certain aspects of this disease. The annual rainfall in the affected area was between 100 and 200 cm which is ideal for the propagation of *Phlebotomus argentipes*, the vector of this disease in India.

Our earlier studies indicated that in Indian kala-azar, the immunological spectrum is reminiscent of that seen in lepromatous leprosy where abundant intra-macrophage bacilli, coupled with intense B-cell activation and depressed T-cell function, form an integral part of the process.

The sera of these patients regularly reveal a high titre of antibodies to *Leishmania* antigens. It is not difficult to imagine that these sera would contain significant amounts of immune complexes as well. Surprisingly, no detailed immunopathological studies of renal lesions are available in the literature. One of the possible reasons could be that the patient's non-renal problems were so serious that detailed clinical studies of the kidney were given a low priority. Infection with *Leishmania donovani* is a well recognized method for producing experimental amyloid disease (*17*). In humans, renal amyloidosis in kala-azar has been noted as a passing reference. The kidney may be infiltrated by amastigotes and, rarely, the urine of these patients may contain parasites; yet no authentic reports of an immune-complex type of nephritis are available.

Our study demonstrates that a large proportion of kala-azar patients do have circulating immune complexes as judged by the platelet aggregation, PEG precipitation, and complement deviation tests. The platelet aggregation test has given consistently high values; in fact, they are even higher than those observed in patients with systemic lupus erythematosus or lepromatous leprosy. Similarly, the polyethylene glycol precipitation test has also offered corroborative evidence in favour of such a phenomenon. Though ideally the present studies should have been supplemented by RAJI cell assay or a Clq-binding assay, the evidence is fairly convincing in favour of the presence of abundant immune complexes. Concentrating the soluble complexes (using PEG precipitation) revealed the presence of anti-*Leishmania* antibodies by indirect immunofluorescence, but further characterization was not undertaken. In fact, Casali & Lambert (*18*) also successfully identified purified complexes (formed *in vivo*) in the serum of a patient with kala-azar. Such isolated complexes were found to contain complement components, possible antigen (IgG) and anti-*Leishmania* antibodies. The levels of anti-*Leishmania* antibodies were very high in our patients too, because these antibodies could easily be picked up in 80% of the patients (using counterimmunoelectrophoresis) and the incidence of the latex reaction was also high (*10*). Despite this, the lack of significant renal damage, as reported in the literature, points to some mechanism protecting the kidneys from such an insult, e.g., the type of immune complexes generated may be different. It has earlier been documented that large complexes with excess Fc binding sites are taken up by the reticuloendothelial system more avidly than the smaller complexes with Ig excess. It is likely that such complexes are of a relatively larger size, with more free Fc receptors available to be mopped up by the aggressive reticuloendothelial system of these patients. In fact, Parish et al. (*19*) have stressed the direct relationship of heavy complement binding and Fc groups exposed on immune complexes to the degree of macrophage binding. The presence of immune complexes has recently been reported by Desjeux et al. (*20*) in mucocutaneous leishmaniasis also.

Further, a marked leucopenia, absolute lymphopenia, and relative lymphocytosis are fairly conspicuous and constant features in patients of kala-azar, yet there is invariably an intense hyper-gammaglobulinaemia, mainly involving the IgG and IgM classes of immunoglobulins. The cell-mediated responses, as judged my MIF (migration inhibition factor) assays and recall antigens, are depressed in these patients during the acute phase of the disease (*9*). The main effect thus appears to be both on the suppressor cells and on the cells involved in delayed-type hypersensitivity responses rather than on cells with a helper function. Small (*21*) and Perry et al. (*22*) have suggested that free antigens or immune complexes can induce suppressor cells. These can also generate anti-receptor antibody, as postulated by Huber & Lucas (*23*). But so far there is no evidence in the literature to indicate the generation of suppressor cells in kala-azar. One is tempted to speculate that intercurrent infections, so commonly observed in these patients, are perhaps due to the marked leuco-

penia and a T-cell defect. Such a defect may be further augmented by immune complexes blindfolding the T lymphocytes.

The findings of immune complexes in this study warrant another look at the renal tissues of these patients, especially those in whom the disease has become chronic. The study also illustrates a close cross-reactivity of *Leishmania donovani* and *Crithidia luciliae*, as judged by the intense immunofluorescence observed with the sera of patients suffering from kala-azar when *Crithidia* is used as a substrate.

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RÉSUMÉ

LES IMMUN-COMPLEXES DANS LE KALA-AZAR INDIEN

Chez des malades atteints de Kala-azar indien, on a cherché à mettre en évidence la présence d'immun-complexes circulants par agrégation plaquettaire, fixation du complément et précipitation au polyéthylène-glycol. Les anticorps circulants ont été étudiés par l'épreuve classique d'immunofluorescence indirecte au moyen des formes leptomonas de *Leishmania donovani* et *Crithidia luciliae*. Les taux sériques de complément (fraction C3) ont été déterminés par la technique de Mancini. Les résultats montrent qu'un grand nombre de malades atteints de Kala-azar indien sont porteurs d'immun-complexes circulants et présentent un abaissement sensible des taux sériques du complément. Il se pourrait que la présence de ces complexes soit en rapport étroit avec la dépression des réponses immunitaires à médiation cellulaire que l'on observe couramment chez ces malades. Les résultats obtenus justifient un examen immuno-histopathologique minutieux du tissu rénal à la recherche de complexes liés aux tissus chez les malades porteurs d'une infection chronique. Par ailleurs, l'étude a montré que *Crithidia luciliae* et *Leishmania donovani* possèdent des antigènes communs et que la première peut être également utilisée pour la détermination des anticorps anti-leishmania par immunofluorescence indirecte.

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Fig. 1. A typical indirect immunofluorescence reaction, observed with the serum of a kala-azar patient, using *Leishmania donovani* as the substrate.

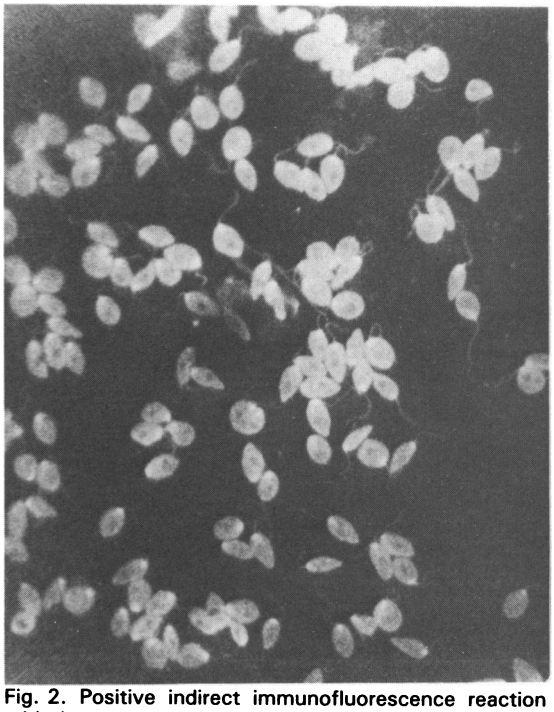

Fig. 2. Positive indirect immunofluorescence reaction with the same sample as in Fig. 1, using *Crithidia luciliae* as the substrate.