Clin. exp. Immunol. (1989) **76**, 391–397

Serum lymphocytotoxic activity in leprosy

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(Accepted for publication 9 January 1989)

SUMMARY

Sera from 167 patients across the spectrum of leprosy and 46 endemic controls were screened for lymphocytotoxic activity (LCA). The Terasaki microdroplet lymphocytotoxicity assay was performed at 37°C and 15°C to test sera for LCA against a panel of lymphocytes from 50 donors which represented most known HLA-ABC antigens. Raised complement-dependent LCA at 15°C was seen in leprosy patients with histories of erythema nodosum leprosum (ENL) or reversal/Type I (I) reactions. Eighty-six per cent of lepromatous (LL) patients with a history of ENL ($n=21$, $P<0{\cdot}001$), 83% of borderline lepromatous (BL) and 88% of borderline tuberculoid patients (BT) with a history of Type I reactions ($n=12$, $P<0{\cdot}01$ and $n=24$, $P<0{\cdot}001$ respectively) had LCA compared to 39% of endemic controls ($n=46$). LCA was attributed to IgM on the basis of reduced activity when serum was treated with both dithiothreitol or absorbed with antiserum for IgM. Removal of immune complexes and rheumatoid factor did not influence LCA. LCA-positive sera reacted similarly with allogeneic lymphocytes from either healthy donors or leprosy patients. Moreover LCA-positive sera reacted with autologous lymphocytes. Specificities for HLA-ABC antigens were not identified. The potential role of these autoantibodies, manifested in leprosy patients with hypersensitivity reactions remains speculative.

Keywords leprosy lymphocytotoxic autoantibodies IgM

INTRODUCTION

Serum lymphocytotoxic activity (LCA) is found in healthy individuals (De Horatius & Messner, 1975; Mendius *et al.*, 1976) with a higher prevalence in patients with systemic lupus erythematosus (SLE) (Butler *et al.*, 1972), rheumatoid arthritis (Levo, Waisman & Ehrenfeld, 1980), inflammatory bowel disease, ulcerative colitis (Strickland *et al.*, 1977), multiple sclerosis (Rumbach *et al.*, 1982), Hodgkins disease (Mendius *et al.*, 1976), HIV infection (Kloster, Tomar & Spira, 1984; Stricker *et al.*, 1987), Crohn's disease (Van Leeuwenhoek, 1981), Graves' Disease, Hashimoto's thyroiditis (Pruzanski *et al.*, 1984), pernicious anaemia (Carmel *et al.*, 1981), primary biliary cirrhosis (Pares *et al.*, 1985), renal disease (Deierhoi, Ting & Morris, 1984), and with acute *Plasmodium falciparum* and *P. vivax* (Wells *et al.*, 1980) infections.

In patients with SLE, LCA correlates with the clinical course of the disorder (Michlmayar *et al.*, 1976; Butler *et al.*, 1972; Winfield *et al.*, 1975) and has been implicated in tissue damage (Ooi *et al.*, 1974; Butler *et al.*, 1972). In this disease, LCA has also been associated with abnormalities in lymphocyte numbers (Utsinger, 1976; Morimoto *et al.*, 1984). A relative depletion of circulating $CD4^+$ T cells has been found in association with anti-CD4 antibodies (Morimoto *et al.*, 1983, 1984). It has been suggested that these antibodies may contribute to the pathology of SLE by disrupting T cell regulatory circuits, thereby precipitating hyperglobulinaemia with autoantibody formation and suppressing cell-mediated immunity (Burnet & Holmes, 1964; Barthold, Kysela & Steinberg, 1974; Abdou *et al.*, 1976; Krakauer, Waldmann & Strober, 1976; Sakane, Steinberg & Green, 1978; Huber, Pfister & Stingl, 1982).

Raised levels of LCA have also been described in the sera of leprosy patients (Kriesler *et al.*, 1975; Serjeantson & Dry, 1980; Naik *et al.*, 1987). Discrepancies exist in these reports as to the type of patient within the disease spectrum with greatest serum lymphocytotoxic activity.

Lepromatous leprosy patients are characterized by hyperglobulinaemia with high titres of anti-*Mycobacterium leprae* antibodies and autoantibodies, a lowered $CD4^+/CD8^+$ ratio in their lesions and impaired cell-mediated responses to *M. leprae* antigens. Changes in $CD4^+/CD8^+$ cells in lesions have also been reported in leprosy patients undergoing hypersensitivity reactions of either the erythema nodosum leprosum (ENL) (Modlin *et al.*, 1983; Rea *et al.*, 1984) or reversal/type I reactions (I) (Modlin *et al.*, 1983; Narayanan *et al.*, 1984). We hypothesized that the LCA found in leprosy patients would be more likely to exist in patients towards the lepromatous end of the spectrum or

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those in reaction and perhaps contribute to the dysregulation of appropriate cell-mediated responses and B cell stimulation seen in these patients. We evaluated LCA in serum from a range of leprosy patients across the spectrum of disease with and without histories of reactional episodes. The patients tested for this study were living in malaria endemic areas. Since one of the highest prevalences of LCA is found in patients with clinical malaria (Wells *et al.*, 1980), serum anti-Plasmodial species antibodies were determined. Similarly, as a negative association between the presence of anti-HBsAg in leprosy patients with LCA has been described, these antibodies were also measured. Initial steps have been taken to characterize the mediator of this activity.

MATERIALS AND METHODS

Patients and controls

Sera were collected from 169 patients diagnosed with leprosy and 46 endemic controls at the Public Health Clinic, Georgetown, Guyana, Lileta Hospital, Lusaka, Zambia and the Marie Adelaide Centre/The Aga Khan University, Karachi, Pakistan. All leprosy patients were classified using clinical and bacteriological criteria specified by the Ridley and Jopling scale (Ridley & Jopling, 1962, 1966; Ridley & Waters, 1969). Patients in hypersensitivity reactions either at the time of bleeding or up to 1 year prior to bleeding were classified separately as shown. Patient and control subjects of both sexes ranged from 10–65 years of age. Lymphocytes were isolated from patients at the Marie Adelaide Centre. These were cryopreserved (Williams *et al.*, 1983) for testing in London.

Lymphocytotoxicity screening

Lymphocytotoxic activity in sera was measured using a modification of the Terasaki microdroplet cytotoxicity assay (Terasaki & McClelland, 1964; Mittal *et al.*, 1968). Target lymphocytes were obtained from 50 normal donors representing most known HLA-ABC antigens and from 40 leprosy patients. Lymphocytes were separated from 20 ml of defibrinated venous blood by Ficoll-Hypaque gradient centrifugation. Washed lymphocytes were suspended in Complement Fixation Test Media (CFT), (Oxoid Ltd., UK) at $1{\cdot}25 \times 10^6$ cells/ml. One microlitre of the cell suspension was added to the same volume of sera diluted 1:2 in CFT in wells of the Terasaki microtitre plates. After a 30 min incubation period, 5 μl of rabbit complement diluted 1:2 in CFT were added to each well and viability was subsequently assessed using inverted phase contrast microscopy. Tests were conducted in parallel at 15°C and 37°C. At 37°C the standard incubation period of 90 min was employed. At 15°C this was extended to 3 h. As 15°C was found to be the optimum reaction temperature during the initial screening experiments, it was used for all subsequent assays. A pool of AB sera was used as a negative control in each Terasaki plate. Cytotoxicity was scored using a 6 point scale from 0 to 8. A score of 1 corresponds to a killing of 10–20% of lymphocytes; a score of 2 = 20–40%; 4 = 40–60%; 6 = 60–80%; and 8 = 80–100%. LCA-positive sera were defined on the basis of reactivity to > 25% of the panel of cells tested.

Anti-Plasmodial immunofluorescence assay

Serum antibodies to Plasmodial antigens were determined using an indirect fluorescent antibody test (IFAT). Briefly, sera diluted 1/16 in PBS were layered onto parasitized human erythrocytes (donated by Dr C.C. Draper, Ross Institute, London School of Hygiene and Tropical Medicine) which were preabsorbed onto glass slides. After 1 h non-specific antibodies were rinsed off, and bound antibody was complexed with a fluorescent labelled polyvalent anti-human immunoglobulin serum conjugate (Wellcome MF01) diluted 1/120 in PBS and 1% Evans blue. Positive and negative control sera were employed on each slide.

Anti-Hepatitis B surface antigen enzyme linked immunoassay

Serum antibodies to HBsAg were determined using an indirect enzyme linked immunoassay. A yeast-derived recombinant HBsAg (Merck Sharpe and Dohne) was used to coat Immunolon I plates at a concentration of 0·55 μg/well in PBS. Post-coated plates were blocked with 1% bovine serum albumin in PBS. Sera diluted 1/10 in PBS were added in duplicate to wells which were washed after an incubation step. Peroxidase-conjugated anti-human IgM (Tago Inc) at 1/5000 in PBS was added to each well. Subsequently ortho-phenylenediamine was introduced as a substrate. The colour change was arrested with 30% hydrogen peroxide and read spectrophotometrically. All incubation steps were conducted for 1 h at 37°C with the exception of the antigen coating step which was conducted overnight at 4°C. Four changes of PBS-tween 20 were used for washing.

Inactivation of serum IgM

To remove IgM activity, the Terasaki assay was performed in the presence of IgM-depleted sera using two pretreatment techniques. One microlitre of 0·01 M dithiothreitol (DTT) (Sigma chemical company) in CFT was added to 1 μl of each test serum and incubated for 30 min at 37°C. The second method employed sera absorbed twice with insoluble anti-human IgM conjugated to sepharose at 4°C for 1 h. Treated sera were then used in the Terasaki assay.

Removal of immune complexes

To remove immune complexes, serum samples were incubated with an equal volume of 6% polyethylene glycol (PEG; 6000 m.w.; BDH Chemicals) in PBS for 1 h at 4°C and centrifuged at 10 000 rpm for 10 min. The supernatant serum was then used in the Terasaki assay.

Analysis and removal of IgM rheumatoid factor

Rheumatoid factor was detected by the slide latex test (Ortho Diagnostic Systems, Belgium). To deplete sera of rheumatoid factor, two absorptions with insoluble human IgG conjugated to latex particles were conducted at a ratio of two volumes of serum to one of packed latex at 4°C for 1 h.

Experimental design

All serum samples processed from endemic controls and leprosy patients were tested against lymphocytes from 50 healthy donors. In addition, sera were screened for anti-plasmodial and anti-HBsAg antibodies. Subsequently, sera positive for LCA (defined as positive on the basis of lymphocytotoxicity towards > 25% of the panel of donors tested) were used to characterize this activity. Five sera from patient groups LL + ENL, BL + I and BT + I, which responded with the highest LCA scores, were chosen for study and compared with negative sera from endemic control subjects. Sera were treated as indicated and screened

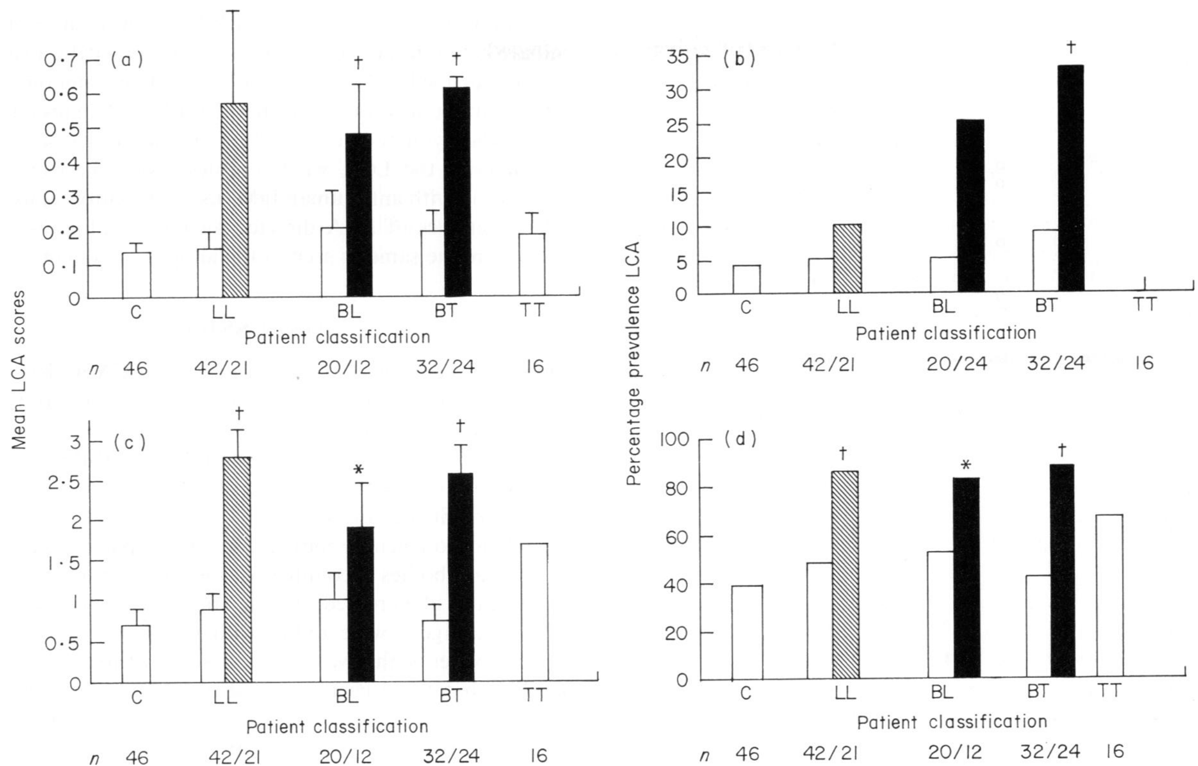

Fig. 1. The mean LCA scores and prevalence of LCA at 37°C (a & b) and 15°C (c & d) across the leprosy spectrum. The results in (a) and (c) are expressed as the mean LCA score for each patient group ± s.e.m. These were determined by averaging individual scores for each serum when tested against panel cells from 50 individuals. The mean score for each patient group was then calculated. The results in (b) and (d) are expressed as the percentage prevalence (reactivity to lymphocytes from more than 25% of the donors tested) of LCA-positive sera in each group. The number of sera tested in each classification (n) is shown. Unpaired t-tests were used to compare leprosy patients with endemic controls; * = $P < 0{\cdot}01$, † = $P < 0{\cdot}001$. LL lepromatous leprosy, BL borderline lepromatous, BT borderline tuberculoid, TT tuberculoid leprosy, ENL erythema nodosum leprosum, I type I reactions, C controls. Patient classification: without reaction (□); with ENL reaction (■); with Type I reaction (■).

against a randomly selected panel of allogeneic cells from 5 or 10 donors as stated.

In the autologous studies, sufficient numbers of patients were not available to permit analysis of each classification with and without evidence of clinical reaction. These experiments are therefore limited to LL, BL and BT patient groups only. Sera were tested in five replicates against autologous lymphocytes.

Statistical analysis
Paired and unpaired t-tests were employed for data analysis.

RESULTS

Serum LCA across the leprosy spectrum
Serum LCA in leprosy patients (Fig. 1) was greater at 15°C than at 37°C. Activity was complement-dependent in all cases and neither sex nor treatment status affected the prevalence of LCA in our study. Weak correlations (Fig. 2a & b) were found between increased LCA scores and IFAT scores of anti-plasmodial antibodies as well as increased optical density readings for anti-HBsAg antibodies ($P = 0{\cdot}0145$ and $P = 0{\cdot}0116$ respectively). Such correlations were not found when LCA was compared with the same antibodies in control subjects (LCA/anti-plasmodial antibodies: $P = 0{\cdot}3644$, $n = 40$; LCA/anti-HBsAg antibodies $P = 0{\cdot}8960$, $n = 25$).

The results of screening sera from leprosy patients against the panel of 50 allogeneic lymphocytes for LCA are shown in Fig. 1 in which sera are grouped according to leprosy status. The mean LCA scores were significantly raised in those patients classified in reaction LL+ENL, BL+I, and BT+I at 15°C when compared to the endemic controls ($P < 0{\cdot}0001$, $P < 0{\cdot}01$ and $P < 0{\cdot}0001$ respectively).

When data were recalculated on the basis of the prevalence of LCA-positive sera, defined as reactivity to lymphocytes derived from more than 25% of the donors tested, the same patient groups LL+ENL, BL+I and BT+I were significantly raised ($P < 0{\cdot}001$, $P < 0{\cdot}01$ and $P < 0{\cdot}001$ respectively).

Characterization of LCA
To identify the class of antibody responsible for LCA, selected serum samples were treated with DTT and absorbed with anti-IgM and selected for activity. Five sera representing positive lymphocytotoxic activity from patient groups LL+ENL, BL+I and BT+I and endemic controls were assayed against a panel of 10 allogeneic lymphocytes before and after treatment. Reactivity was removed entirely by treating serum with DTT (Fig. 3a). When serum samples were absorbed with anti-IgM-coated sepharose (Fig. 3b), LCA was significantly reduced indicating LCA was IgM in nature.

To exclude the possibility that rheumatoid factor was interfering with the Terasaki assay, the same sera were absorbed

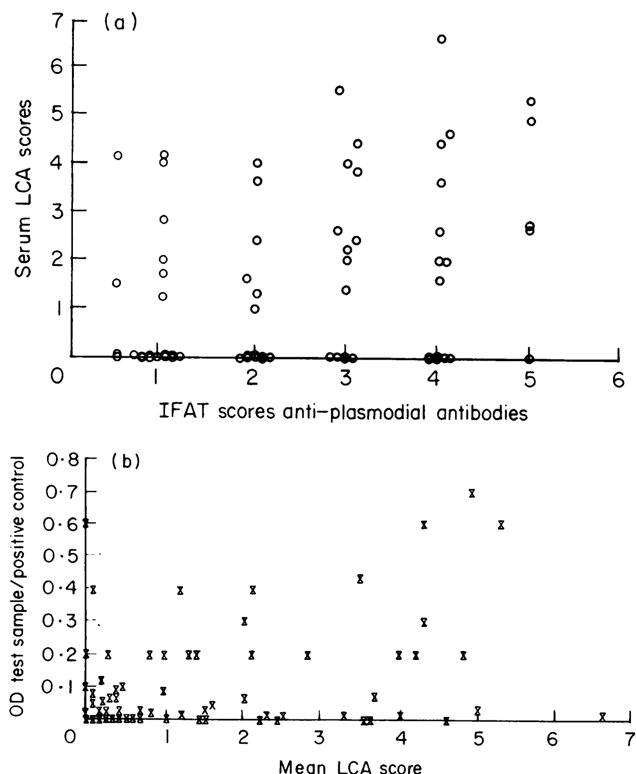

Fig. 2. Correlation of LCA scores at 15°C with levels of antibody for plasmodial (a) and HBsAg (b) antigens in leprosy patients.

Table 1. Comparison between serum LCA against target allogeneic lymphocytes derived from healthy donors and leprosy patients

Source of sera	Source of lymphocytes			
	Control ($n=50$)	LL/LL+ENL ($n=14$)	BL/BL+I ($n=11$)	BT/BT+I ($n=12$)
LL+ENL ($n=5$)	5·4±0·3	4·6±0·5	4·7±0·5	5·5+0·4
BL+I ($n=5$)	5·5±0·3	4·4±0·5	4·2±0·4	5·0±0·3
BT+I ($n=5$)	5·0±0·3	2·9±0·5	3·6±0·5	5·0±0·5

Results are expressed as mean LCA scores±s.e.m.

LL Lepromatous leprosy, BL borderline lepromatous, BT borderline tuberculoid, ENL erythema nodosum leprosum, I type I reactions.

with insoluble human IgG. No significant difference in LCA was seen (Fig. 3c). In addition, another aliquot of the same sera was treated with PEG to remove immune complexes. No significant change in LCA was detectable (Fig. 3d).

LCA against allogeneic/autologous lymphocytes

Sera from the three patient groups exhibiting reactions (LL/ENL, BL/I and BT/I) were tested against lymphocytes from control donors and leprosy patients to determine if patient cells were more susceptible to lysis. LCA however, did not discriminate between the control or patient lymphocytes (Table 1).

Sera defined as LCA-positive or negative using the allogeneic system retained the same characteristics when tested against autologous lymphocytes (Fig. 4). The assays described above to characterize the LCA were repeated (with the exception of absorption with anti-human IgM) using the autologous system. Characteristics of LCA directed against autologous lymphocytes were the same as seen in the allogeneic systems.

DISCUSSION

Our studies confirm a high prevalence of LCA in the serum of patients with leprosy. The data was highly variable both in terms of the reactivity of a single serum to lymphocytes derived from each of the 50 donors, and amongst sera within any given classification of leprosy. Sex and treatment status were not found to influence LCA.

In addition, neither the presence of anti-plasmodial nor anti-HBsAg antibodies accounted for the variation seen. Although correlations of increased levels of antibodies for plasmodial and HBsAg antigens were found with increasing LCA scores in leprosy patients, this was not the case for control subjects. This implies that these correlations are reflections of some other immunological process which is peculiar to leprosy patients and not to control subjects. To confirm that in our hands we would also find high levels of LCA in patients with clinical malaria as did Wells *et al.*, (1980), sera from 12 such patients from the Hospital of Tropical Diseases were screened. Using our criteria for positivity a prevalence of 67% was calculated. It would appear that infection with malaria, and not immunological memory of the same, influences serum lymphocytotoxicity. Where Serjeantson & Dry (1980) had found an inverse relation of HBsAg antibodies with lepromatous patients with LCA, we found the reverse. An examination of all leprosy patients and lepromatous patients reveals that the few outlying samples which express high levels of LCA in addition to high titres of anti-HBsAg antibodies greatly distort the results. As such the trends described here may only be of mathematical significance.

When patients were classified in relation to their history of reaction in addition to leprosy status, it was found that the interaction of leprosy, lymphocytotoxicity and reactions was highly significant and accounted for the individual variation of cytotoxicity seen.

This LCA was attributed to the IgM fraction of sera as activity was depleted through treatment with dithiothreitol and absorption with antisera specific for IgM. LCA also acted in a fashion characteristic of low affinity IgM, in that activity was best observed not at 37°C, but at the lower temperature of 15°C.

In addition, LCA was found to be complement-dependent in all cases. These findings agree with those of other investigators who have also found LCA to be mediated predominantly by complement-dependent IgM with a temperature optimum of 15°C (Terasaki *et al.*, 1970; Winfield *et al.*, 1975; Kriesler *et al.*, 1970; Korsmeyer *et al.*, 1975; Goldberg *et al.*, 1972; Browning *et al.*, 1977).

Polyethylene glycol was used to precipitate immune complexes, to ensure that the IgM involved was not non-specifically binding lymphocytes in complex form. In parallel, rheumatoid factor was tested for and absorbed from sera. Neither factor was found to contribute significantly to LCA.

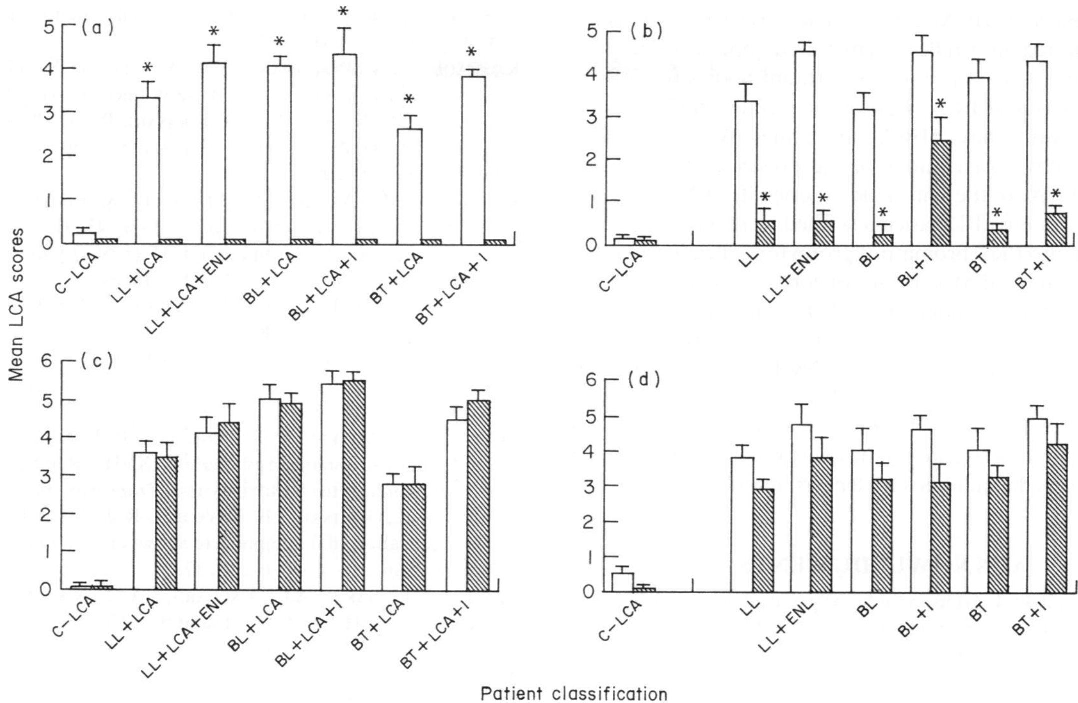

Fig. 3. Characterizing the nature of LCA. Five sera from each patient group were either treated with DTT (a), absorbed with anti-human IgM-sepharose (b), treated with 3% PEG (c) or absorbed with insoluble human IgG (d), before testing against allogeneic lymphocytes from five donors. Results are expressed as the mean LCA scores for five sera from each patient group tested against five allogeneic target lymphocytes. Untreated sera were compared with treated sera using a paired *t*-test; * = $P < 0{\cdot}0001$. Untreated sera (□); absorbed/treated sera (■). Abbreviations as for Fig. 1.

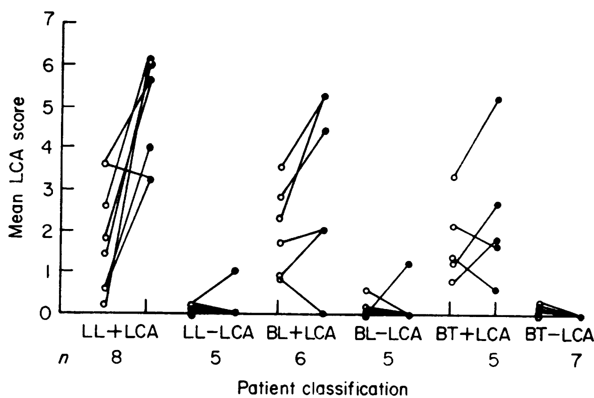

Fig. 4. Comparing the reactivity of sera against allogeneic panel and autologous target lymphocytes. Sera tested for lymphocytotoxicity against allogeneic lymphocytes (○) from 50 normal donors and against autologous lymphocytes (●) (5 replicates). Abbreviations as for Fig. 1.

No discrimination was made by these antibodies in terms of lymphocyte recognition, whether allogeneic target cells were derived from leprosy patients or normal non-endemic healthy controls (Table 1). Reactivity was retained when LCA-positive sera were presented to autologous lymphocytes. This is the first evidence that LCA represents a group of autoantibodies in leprosy patients. These two findings justify the use of an allogeneic system to characterize LCA.

The microcytotoxicity assay used to detect LCA represents an amplified marker system for visualizing antibodies with a tropism for lymphocytes. However, detection of these antibodies *in vitro* does not necessarily imply that these antibodies are lytic to lymphocytes *in vivo*.

LCA was not always directed against lymphocytes from all donors. Sera that had lower mean lymphocytotoxic scores (1–4), were more variable in their reactivity to lymphocytes from the 50 individuals they were tested against. Conversely, sera that had high lymphocytotoxic scores (4–8) reacted to most, if not all, cells tested, with less variation in the degree of lysis seen.

Several explanations could be applied to interpret these findings. One is that sera with high cytotoxic scores recognize an accessible antigen common to most individuals and lymphocytes, whereas those manifesting low cytotoxicity recognize a less common or perhaps a less accessible antigen that could vary with alloantigen expression. A second explanation is that the antigen recognized is common to both serum activities, but that the difference in cytotoxic scores represents a difference in affinity or titre. A third possibility is that LCA represents heterogeneous populations of autoantibodies with different specificities.

The studies reported by Kriesler *et al.* (1975) identified raised LCA in groups LL and BL (52% and 60% respectively). Increased activity was not identified in patient groups BT and TT, however numbers tested were small ($n = 8$ and $n = 3$ respectively). It is possible that if the relationship of LCA prevalence to reactional history had been investigated, higher prevalence may have been found.

Serjeantson and Dry (1980) similarly did not classify patients according to reaction status. They did, however, find an association ($P < 0{\cdot}01$) between increased LCA and LL patients

who were persistent HBsAg carriers. The presence of HBsAg the authors suggested, may reflect an impaired immune response in the host. Conversely, LL patients with antibodies for HBsAg had reduced levels of LCA. Few of the serum samples studied here were shown to have HBsAg antibodies. We are therefore unable to confirm correlations of the presence of these antibodies with LCA. In the same study (Serjeantson & Dry, 1980) the results from nine BL patients studied were masked as they were merged into the lepromatous group for collective analysis. However, tuberculoid patients, in concordance with our results, showed no significant differences when compared to controls. In their study, as with ours, choosing controls from endemic regions resulted in high background levels of LCA. This may perhaps reflect a variety of other subclinical infections.

We are currently characterizing the binding specificities of these autoantibodies to address the relevance of LCA to leprosy patients with erythema nodosum leprosum and reversal or type I reactions.

ACKNOWLEDGMENTS

The authors thank Dr I. Addison for his help and advice in screening for, and working with, rheumatoid factor, Dr P. Fine and Prof M.W. Steward for their helpful discussions and Dr H. Dockrell for her careful reading of the manuscript and valued suggestions. Dr F.N. Rasheed was supported by the British Council.

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