Comparison of immunoperoxidase staining with indirect immunofluorescence, ELISA, and Western blotting assays for detecting anti-HTLV-I antibodies in systemic lupus erythematosus

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ABSTRACT Serum antibodies against human T cell leukaemia virus type I (HTLV-I) were investigated in 12 patients by four methods: indirect immunoperoxidase staining, indirect immunofluorescence, enzyme linked immunosorbent assay (ELISA), and strip radioimmunoassay based on the Western blotting assay. Seven patients had systemic lupus erythematosus (SLE) and five various autoimmune diseases with one or more circulating autoantibodies. Serum samples from three patients were found to be HTLV-I-positive by the ELISA assay and sera from five patients showed a non-specific reaction by indirect immunofluorescence. These sera were negative when tested by indirect immunoperoxidase staining and Western blotting assay. All four methods gave positive results when tested with samples from 19 HTLV-I carriers and 16 patients with adult T cell leukaemia. Indirect immunoperoxidase staining and Western blotting assay are probably useful and more specific assays for the detection of anti-HTLV-I antibodies in samples from patients with autoimmune diseases.

Several methods to detect antibodies against HTLV-I—namely, indirect immunofluorescence using fixed MT-I cells,¹ membrane immunofluorescence, solid phase immunocompetition assay,^{2,3} enzyme linked immunosorbent assay (ELISA),⁴ gelatin particle agglutination,⁵ and strip radioimmunoassay, based on the Western blotting assay,⁶ have been reported. There are still difficulties, however, in detecting specific anti-HTLV-I antibodies in patients with autoimmune disease with circulating autoantibodies. Indeed, with indirect immunofluorescence and ELISA assays it is not always easy to exclude a non-specific reaction. In this study we investigated the presence of antibodies to HTLV-I in the sera from patients with systemic lupus erythematosus and other autoimmune diseases by several of the above methods and a new immunoperoxidase technique.

Material and methods

PATIENTS WITH AUTOIMMUNE DISEASE

Sera from seven patients with systemic lupus erythematosus and five patients with progressive systemic sclerosis (n = 1), idiopathic thrombocytopenic purpura (n = 1), Sjögren's syndrome (n = 1), polymyositis, (n = 1), and rheumatoid arthritis (n = 1), treated in Kumamoto (an area where the incidence of HTLV-I infection is high) were studied. All were women aged between 28 and 67 years who had one or more circulating autoantibodies (anti-DNA, anti-mitochondria, thyroid autoantibodies etc). As positive controls, sera from 16 patients with adult T cell leukaemia (ATL) and 19 HTLV-I carriers were tested by indirect immunofluorescence, and sera from 26 healthy subjects were used as negative controls.

SCREENING OF SERA FOR ANTI-HTLV-I ANTIBODIES

Serum samples were surveyed for anti-HTLV-I antibodies by indirect immunofluorescence, ELISA,

Western blotting assay and indirect immunoperoxidase staining. The procedure for indirect immunofluorescence was as follows.¹ MT-1 cells fixed in acetone⁷ were reacted with diluted sera and then fluorescein isothiocyanate conjugated goat antihuman IgG was added and incubated at 37°C for 30 minutes. The MT-1 cell line was derived from peripheral blood leukaemic cells from a patient with ATL. As described previously,¹ cells bearing HTLV-I associated antigens (ATLA) in MT-1 cells are few (1–2%), and almost all MT-1 cells do not express ATLA. Anti-HTLV-I antibodies, positive, and negative reference sera were included in each test as parallel controls. Sera showing bright fluorescence at a 1/10 dilution were regarded as positive. The fluorescence was somewhat granular, often appearing as large aggregates in the cytoplasm but not in the nucleus of the cells. On the other hand, reactions showing diffuse stain in both cytoplasm and nucleus were regarded as non-specific.

The procedure for ELISA (Eisai Kit, Tokyo, Japan) was as follows.⁴ Coated with antigen solution, extracted MT-2 cells⁸ were reacted with diluted sera in plastic cups and then with alkaline phosphate labelled mouse antihuman IgG monoclonal antibody at 37°C for 60 minutes. The MT-2 cell line was established from cord blood lymphocytes cocultivated with leukaemic cells from a patient with ATL. Most MT-2 cells release a large number of virus particles. After adding a *p*-nitrophenyl phosphate solution at 37°C for 60 minutes the reaction was stopped by the addition of 4 N NaOH. The absorbance at 405 nm was measured using a spectrophotometer. The cut off value of ELISA was the mean value for serum samples obtained from 104 healthy subjects determined to be antibody negative by the indirect immunofluorescence

assay plus three times the standard deviation. Absorbance readings of more than 0.400 were considered to be positive.

Western blotting assay was performed as follows. Cell lysates were obtained by incubating 10 million MT-2 cells in 1 ml of lysing buffer (10 mM Tris-hydrochloric acid, pH 7.2, containing 0.9% sodium chloride, 0.5% NP-40 and 2 mM phenylmethylsulfonyl fluoride) for 20 minutes on ice. After removing cell debris at 150 000 rpm for 10 minutes recovered proteins were diluted 1/1 with Laemmli sample buffer and electrophoresed on 12% polyacrylamide in the presence of sodium dodecyl sulphate (SDS). The separated protein was electroblotted on nitrocellulose sheets by the Western blotting technique. The virus strip radioimmunoassay was done by the methods described previously,⁹ using non-fat dried milk instead of bovine serum albumin to prevent non-specific binding of proteins to the nitrocellulose.

Indirect immunoperoxidase was performed as follows.¹⁰ C91/PL cells fixed in acetone were reacted with diluted sera for 30 minutes at 37°C in a moist chamber and then with peroxidase conjugated rabbit antihuman immunoglobulin (Dako, Denmark) at 37°C for 30 minutes. The C91/PL cell line was established from cord blood lymphocytes cocultivated with HTLV-I positive neoplastic T cells.¹¹ The C91/PL cell line is also a high producer of HTLV-I viral particles. The cytochemical reaction was developed for five minutes with 30 mg of diaminobenzidine tetrahydrochloride (DAB, Sigma) in 50 ml of PBS and 20 µl of hydrogen peroxide (30 vol), and then washed in PBS and distilled water and counterstained with Harris's haematoxylin. Sera showing peroxidase activity in a multiple granular pattern were considered to be positive.

Table HTLV-I antibodies in autoantibody positive sera

Case No	Age/Sex	Diagnosis	ELISA	Indirect immunofluorescence	Western blotting assay	Indirect immunoperoxidase staining
1	35/F	Systemic lupus erythematosus	—	—	—	—
2	28/F	Systemic lupus erythematosus	—	—	—	—
3	41/F	Systemic lupus erythematosus	—	NS	—	—
4	36/F	Systemic lupus erythematosus	—	—	—	—
5	39/F	Systemic lupus erythematosus	—	—	—	—
6	45/F	Systemic lupus erythematosus	—	NS	—	—
7	38/F	Systemic lupus erythematosus	0.676	NS	—	—
8	52/F	Progressive systemic sclerosis	—	NS	—	—
9	37/F	Idiopathic thrombocytopenic purpura	—	—	—	—
10	62/F	Sjögren's syndrome	0.566	—	—	—
11	46/F	Polymyositis	0.623	NS	—	—
12	67/F	Rheumatoid arthritis	—	—	—	—
ATL		(n = 16)	0.870-overscale*	+	+	+
HTLV-I carriers		(n = 19)	1.043-overscale	+	+	+
Healthy subjects		(n = 26)	< 0.400	—	—	—

*range; NS, non-specific staining.

Fig 1 Photomicrographs of detection of anti-HTLV-I antibodies by indirect immunoperoxidase staining: (a) positive (patient with ATL); (b) negative (healthy subject); (c) negative (patient with systemic lupus erythematosus).

Fig 2 Detection of anti-HTLV-I antibodies by Western blotting method: lane a) sera of healthy subject; lane b) sera of patient with ATL; lane c) sera of patient with systemic lupus erythematosus.

Fig 3 Scheme of anti-HTLV-I antibody positive patterns by Western blotting method: (a) 90% of positive sera show both anti-gag and env gene products of HTLV-I; (b) 5% of positive sera show only anti-gag gene products; (c) <5% of positive sera show only anti-env gene products.

Results

The table shows the incidence of anti-HTLV-I antibodies in the sera from the 12 patients with autoimmune disease by ELISA, indirect immunofluorescence, Western blotting assay, and indirect immunoperoxidase staining. Three of the 12 sera were positive in the ELISA assay and five showed a non-specific reaction by indirect immunofluorescence, but none was found to be positive by Western blotting assay or indirect immunoperoxidase staining. All sera from patients with ATL and HTLV-I carriers shown to be positive by ELISA and indirect immunofluorescence assays were also positive by Western blotting assay and indirect immunoperoxidase staining. Sera from healthy subjects, however, were negative by all assays. Fig 1a illustrates the reactivity of a patient with ATL (HTLV-I antibody positive serum); fig 1b, a patient with systemic lupus erythematosus (HTLV-I antibody non-specific reaction by ELISA and indirect immunofluorescence assays); and fig 1c, HTLV-I antibody negative serum from a healthy subject by indirect immunoperoxidase-staining. Fig 2 shows the profiles of recognised virus related proteins obtained by Western blotting assay. Usually, positive sera reacted with all or some of the HTLV-I glycoprotein gp46, p28, p24, and p19¹²; sera from patients with autoantibodies did not show antibodies to HTLV-I specific proteins—instead many non-specific bands were observed. Fig 3 shows the characteristic profiles obtained by Western blotting assay in HTLV-I positive patients, which are easily distinguishable from the non-specific bands shown in patients with autoantibodies.

Discussion

It has been shown previously that sera from patients with systemic lupus erythematosus or other autoimmune diseases and malaria may react with HTLV-I-positive cell lines when studied by indirect immunofluorescence and ELISA methods.^{13,14} This is likely to be caused by a false positive reaction due to the recognition of unknown cellular proteins on HTLV-I-positive cells rather than the detection of virus specific antigens, as the findings have not been confirmed when applying more specific assays such as Western blotting assay (fig 2). Therefore, although indirect immunofluorescence, ELISA, and particle agglutination assays are relatively simple first line techniques for the screening of blood donors, there is always the need to confirm the positive results by Western blotting assay.

In this study we have shown that a simple indirect immunoperoxidase staining method, which is commonly used to detect cytoplasmic antigens and the nuclear enzyme terminal deoxynucleotidyl transferase

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(TdT), may be useful and more specific than indirect immunofluorescence and ELISA to detect antibodies to HTLV-I. Thus sera from all six patients which showed a non-specific reaction for HTLV-I antibodies when assessed by indirect immunofluorescence or ELISA, or both, were shown to be unreactive with the indirect immunoperoxidase staining method. The indirect immunoperoxidase staining method always showed clearly granular positive patterns in sera from patients with ATL and in HTLV-I carriers. These results were further confirmed by the Western blotting assay.

A similar indirect immunoperoxidase staining method has previously been reported to be useful in detecting the presence of antibody to HTLV-III,¹⁵ and it has been shown that results obtained by indirect immunoperoxidase staining are comparable with those of the indirect immunofluorescence method. The indirect immunoperoxidase staining method, however, has not been applied systematically to investigate sera from patients with autoantibodies, which may show a non-specific pattern of reactivity by indirect immunofluorescence and the ELISA methods. In this study we have shown that the indirect immunoperoxidase staining method may be more specific than the indirect immunofluorescence and ELISA methods as, unlike indirect immunofluorescence and ELISA, a non-specific reaction has not been detected by indirect immunoperoxidase staining. In relation to the ELISA assay, we performed the standard ELISA test by an antiglobulin technique; it is possible, however, that a competitive ELISA technique might avoid some of the difficulties (non-specific staining) derived from the standard ELISA test and therefore might be as specific as the indirect immunoperoxidase staining method. We have also shown that indirect immunoperoxidase staining is equally sensitive for testing samples of HTLV-I-positive subjects (healthy carriers and ATL patients). In fact, the indirect immunoperoxidase staining method described here seems to be reliable, relatively simple, and reproducible, and can be used as a first line screening method for HTLV-I antibody.

Although a role for viruses in the aetiology of systemic lupus erythematosus has been regarded as feasible,¹⁷ the role of HTLV-I has not been confirmed.¹⁸ Our data further suggest that HTLV-I does not play a part in the pathogenesis of systemic lupus erythematosus.

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