

# Autoantibodies to T and B cell lines detected in serum samples from patients with systemic lupus erythematosus with lymphopenia and hypocomplementaemia

Masaaki Noguchi, Masao Iwamori, Takao Hirano, Shigeto Kobayashi, Hiroshi Hashimoto, Shun-Ichi Hirose, Yoshitaka Nagai

## Abstract

**Antibodies to lymphocytes in serum samples from 88 patients with systemic lupus erythematosus (SLE) and 15 normal control subjects were examined by a cell enzyme linked immunosorbent assay (ELISA) with four human T and B cell lines as antigens. The antibodies reacted with the Wa B cell line and the T cell lines P12 (CD4<sup>-</sup>, CD8<sup>+</sup>), Jurkat (CD4<sup>-</sup>, CD8<sup>-</sup>), and Hut78 (CD4<sup>+</sup>, CD8<sup>-</sup>). Antibody titres in serum samples from patients with SLE were higher than in those from normal control subjects. Titres of antibodies to P12 were correlated with titres of antibodies to Wa, Jurkat, and Hut78 in serum samples from patients with SLE. IgG antibodies to P12 were associated with lymphopenia and reduced haemolytic complement. By thin layer chromatography immunostaining, the antibodies in serum samples from two of 10 patients with SLE with high titres of IgG antibodies to P12 and lymphopenia were shown to react with three monosialoglycosphingolipids and two neutral glycosphingolipids from P12 cells. Antibodies to lymphocytes in serum samples from patients with SLE react with T and B cell lines, recognise a series of cell membrane glycosphingolipids and are associated with the lymphopenia and hypocomplementaemia typical of active disease.**

Systemic lupus erythematosus (SLE) is an autoimmune disease characterised by the spontaneous production of multiple antibodies to self antigens such as nuclear components<sup>1</sup> and lymphocytes.<sup>2</sup> Among these antibodies, autoantibodies to T cells have been suggested to be closely related with the autoimmune status and course of SLE.<sup>3</sup> Both helper T cells<sup>4</sup> (CD4<sup>+</sup>) and suppressor T cells<sup>5</sup> (CD8<sup>+</sup>) are thought to be important targets of antibodies in the pathogenesis of SLE.

We have already shown that a cell enzyme linked immunosorbent assay<sup>6</sup> (cell ELISA) for the detection of antibodies to lymphocytes can be carried out with four human T and B cell lines as antigens in place of native T and B cells. In this study, antibodies to lymphocytes in serum samples from patients with SLE were detected by a cell ELISA using as antigens four human T and B cell lines that have various lymphocyte surface markers including either helper (CD4) or suppressor (CD8) T cell markers. In this work IgM and IgG antibodies to lymphocytes were correlated with the

number of lymphocytes in peripheral blood and complement levels (CH50) as we expected that IgM or IgG antibodies to lymphocytes might be a useful marker of disease activity.

Antibodies to glycosphingolipids<sup>7</sup> are thought to be important in neurological complications of SLE.<sup>8-9</sup> Autoantibodies to glycosphingolipids on lymphocytes have not yet been reported, though antibodies to other lymphocyte antigens such as  $\beta_2$  microglobulin,<sup>10</sup> HLA,<sup>11</sup> interleukin 2 receptor,<sup>12</sup> DNA,<sup>13</sup> i antigen,<sup>14</sup> and others are known. In this work we attempted to establish whether autoantibodies in serum samples from patients with SLE can react with glycosphingolipids on lymphocytes.

## Patients and methods

### PATIENTS AND CONTROLS

Eighty eight patients (84 women and four men, average age 27.8 years), fulfilling four or more of the American Rheumatism Association criteria for SLE<sup>15</sup> were included in the study. The patients were receiving an average approximate corticosteroid dose of 15 mg/day prednisolone. Fifteen normal control subjects (12 women and three men, average age 26.2 years) were selected from healthy laboratory staff.

### LYMPHOCYTE CELL LINES

Three human T cell lines: P12/Ichikawa<sup>16</sup> (P12) and Jurkat<sup>17</sup> derived from acute lymphoblastic leukaemias, and Hut78 derived from a patient with Sézary syndrome,<sup>17</sup> and one B cell line: Wa<sup>18</sup> from an Epstein Barr virus transformed B cell were maintained in RPMI 1640 medium (Nissui, Tokyo, Japan) containing 10% fetal calf serum. Lymphocyte surface markers on each cell line were determined by flow cytometry<sup>19</sup> (FACStar, Becton Dickinson, CA, USA) after staining each cell line with FITC-labelled monoclonal antibodies to Leu1 (CD5), Leu2a (CD8), Leu3a (CD4), Leu4 (CD3), and HLA-DR (Becton Dickinson).

### CELL ELISA

The cell ELISA was performed as follows. About 10<sup>6</sup> lymphocytes were put in each well (200  $\mu$ l/well) of 96 well polystyrene plates (Falcon, Becton Dickinson) precoated with 0.01 mg/ml poly-L-lysine (Sigma, St Louis, MO, USA) for 30 minutes. The plate was centrifuged at 400 g for six minutes and then incubated with 0.025% glutaraldehyde (Sigma) in phosphate buffered saline for 15 minutes. After washing, the plate was incubated with 2% bovine serum albumin in phosphate buffered saline for one

Department of  
Rheumatology,  
Juntendo University  
School of Medicine,  
2-1-1, Hongo,  
Bunkyo-ku, 113, Japan  
M Noguchi  
T Hirano  
S Kobayashi  
H Hashimoto  
S-I Hirose

Department of  
Biochemistry,  
Faculty of Medicine,  
Tokyo University,  
7-3-1, Hongo,  
Bunkyo-ku, 113, Japan  
M Iwamori  
The Tokyo Metropolitan  
Institute of  
Medical Science,  
3-18-22, Honkomagome,  
Bunkyo-ku, 113, Japan  
Y Nagai

Correspondence to:  
Dr Noguchi.

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hour, followed by washing with 100  $\mu$ l 1:250 diluted test serum samples for one hour. The antibodies bound to the plate were determined by incubation with 1:1000 diluted peroxidase conjugated goat antihuman IgM or IgG antibodies (Cappel, Marvern) for one hour, washing three times and visualising the enzyme activity with 0.04% *o*-phenylenediamine and 0.01% hydrogen peroxide in 0.1 M citric acid-0.2 M disodium hydrogen phosphate buffer (pH 4.6). The reaction was terminated by 4 M sulphuric acid and the absorbance measured at 490 nm.

#### IMMUNOABSORPTION

To remove IgG antibodies to P12, 1:300 diluted serum samples from a patient with SLE with a high titre of the antibody and a normal control subject were absorbed with P12 cells at ratios of  $10^4$ ,  $10^5$ , and  $10^6$ . The antibody to P12 was measured after the immunoabsorption by cell ELISA with P12 cells as the antigen.

#### Lymphocyte surface markers of four human T and B cell lines detected by flow cytometric analysis

Cell lines	Origin	CD designation*				
		CD5	CD8	CD4	CD3	HLA-DR
Wa	B cell	-	-	-	-	++
P12	T cell	+	+	-	-	-
Jurkat	Non-T, B cell	+	-	-	+	+
Hut78	T cell	-	-	+	+	+

\*(-) negative; (+) positive; (++) strongly positive.

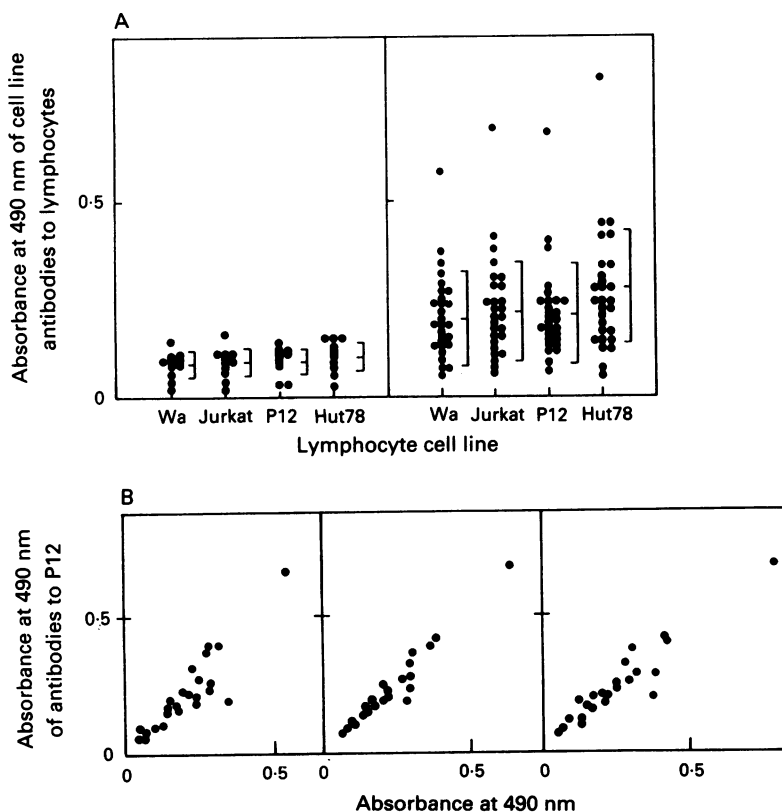


Figure 1 (A) Cell line antibodies to lymphocytes in serum samples from normal control subjects ( $n=11$ ; left hand panel) and patients with SLE ( $n=28$ ; right hand panel) detected by cell ELISA. Results given as absorbance at 490 nm. The central horizontal bar indicates the mean value and the upper and lower horizontal bars the standard deviation. The difference between control subjects and patients with SLE was significant ( $p<0.05$ , Wilcoxon rank sum test). (B) Correlation of titres of antibodies to P12 with antibodies to Wa (left hand panel), Jurkat (centre panel), and Hut78 (right hand panel) in serum samples from patients with SLE ( $n=28$ ) detected by cell ELISA. Correlation with antibodies to Wa,  $r=0.91$ ,  $p<0.01$ ; Jurkat,  $r=0.98$ ,  $p<0.01$ ; Hut78,  $r=0.94$ ,  $p<0.01$ .

#### PREPARATION OF GLYCOSPHINGOLIPIDS

Total lipids were successfully extracted from lyophilised P12 cells with chloroform-methanol-water (20:10:1, 10:20:1, 20:10:1, 10:20:1, and 10:10:1 vol/vol) at 40°C for 20 minutes and were then divided into acidic and neutral glycosphingolipids by DEAE-Sephadex column chromatography.<sup>20</sup> Acidic glycosphingolipids were further fractionated into mono-, di-, and trisialoglycosphingolipids as described previously.<sup>21</sup>

#### IMMUNOSTAINING WITH THIN LAYER CHROMATOGRAPHY

Acidic and neutral glycosphingolipids from 5 mg dry weight of P12 cells were chromatographed on a thin layer chromatography plate (Polygram, Macherey-Nagel, Germany) with chloroform-methanol-water (55:45:10 and 65:35:8 vol/vol) respectively, and were then visualised by orcinol-sulphuric acid reagent and thin layer chromatography immunostaining.<sup>22</sup> For thin layer chromatography immunostaining the plate was incubated with blocking buffer (1% ovalbumin, 1% polyvinylpyrrolidone in phosphate buffered saline) for one hour and then incubated with 1:100 diluted serum samples from 10 patients with SLE and five normal control subjects for one hour. After washing the plate five times with 0.1% Tween in phosphate buffered saline, it was incubated with 1:300 diluted peroxidase conjugated goat antihuman IgM and IgG antibodies (Cappel). The enzyme activity remaining on the plate after five washings was visualised with 4  $\mu$ l hydrogen peroxide, 6 mg 4-chloro-1-naphthol in 2 ml of methanol, and 10 ml of 50 mM TRIS-hydrochloric acid containing 200 mM sodium chloride (pH 7.4). The reaction was stopped by washing the plate with water.

#### STATISTICS

The statistical significance of differences in absorbance between patients with SLE and normal control subjects was determined with the Wilcoxon rank sum test. Correlations between the absorbances of antibodies to P12 and antibodies to other cell lines, the number of lymphocytes in peripheral blood, and complement levels (CH50, number of units determined by 50% haemolysis<sup>23</sup>) were evaluated by Student's *t* test. Statistical significance was defined as a *p* value  $<0.05$ .

#### Results

##### LYMPHOCYTE SURFACE MARKERS

The table gives the cell surface markers detected on the lymphocyte cell lines after staining.

##### ANTIBODIES TO LYMPHOCYTES

Figure 1A shows that the titres of cell line antibodies to lymphocytes reacting with Wa, P12, Jurkat, and Hut78 cells were higher in serum samples from 28 patients with SLE than in those from 11 normal control subjects. In each serum sample the titre of antibodies to P12

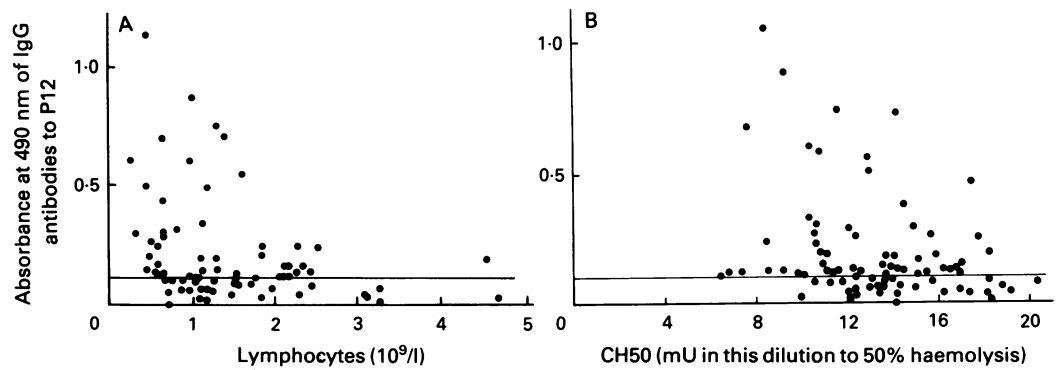


Figure 2 Correlation of titres of IgG antibodies to P12 detected by cell ELISA with (A) peripheral blood lymphocyte count and (B) complement (CH50) level in serum samples from 88 patients with SLE. The horizontal line indicates two standard deviations above the mean absorbance of the normal control serum samples (n=15). (A)  $r = -0.33$ ,  $p < 0.01$ ; (B)  $r = -0.27$ ,  $p < 0.05$ .

was correlated with titres of antibodies to Wa ( $r = 0.91$ ,  $p < 0.01$ ), Jurkat ( $r = 0.99$ ,  $p < 0.01$ ), and Hut78 ( $r = 0.94$ ,  $p < 0.01$ ) (fig 1B). In a larger study of 88 patients with SLE, antibodies of IgM and IgG class reactive with P12 cells were found in 54 and 27 serum samples respectively.

ASSOCIATION OF ANTIBODIES TO LYMPHOCYTES WITH LYMPHOPENIA AND DECREASE OF COMPLEMENT

There was an inverse association between the titre of antibodies to P12 and the number of peripheral blood lymphocytes ( $r = -0.33$ ,  $p < 0.01$ ) (fig 2A) and complement level (CH50) ( $r = -0.27$ ,  $p < 0.05$ ) (fig 2B). All 10 patients with SLE with high titres of IgG antibodies to P12 had lymphopenia (lymphocytes  $< 1.5 \times 10^9/l$ ), but the titres of IgM antibodies to P12 did not correlate with lymphopenia (data not shown).

IMMUNOABSORPTION

Immunoabsorption of IgG antibodies to P12 with  $10^6$  P12 cells reduced the titre to 0 (data not shown), indicating that the antigen is localised on the cell surface.

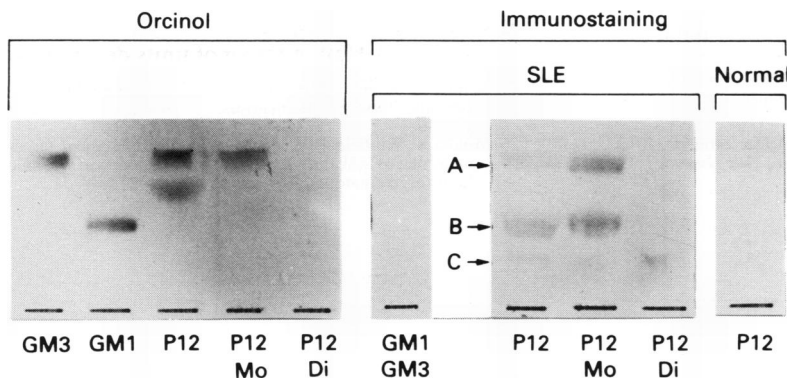


Figure 3 Thin layer chromatography immunostaining of acidic glycosphingolipids from P12 cells with serum samples from a patient with SLE and a normal control subject. The acidic glycosphingolipids from P12 cells are abbreviated to P12. The monosialo-, di-, and trisialo-glycosphingolipids from P12 cells are abbreviated to P12 Mo and P12 Di respectively. The left hand plate was stained with orcinol- $H_2SO_4$  reagent. The right hand plate was immunostained with a serum sample containing the highest detected titre of IgG antibodies to P12 and a normal control. Monosialo-glycosphingolipids A, B, and C were stained by serum samples from the patient but not the normal control subjects.

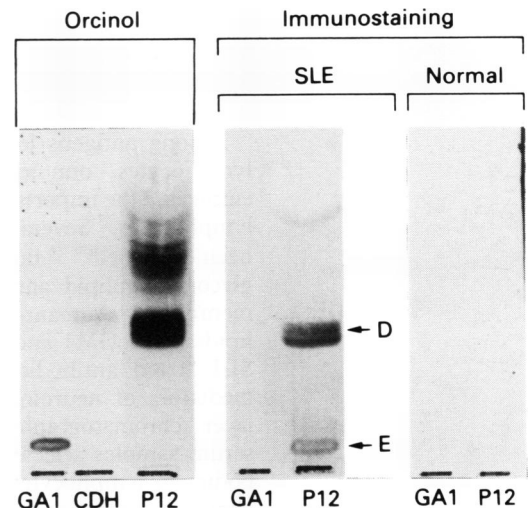


Figure 4 Thin layer chromatography of neutral glycosphingolipids from P12 cells with a serum sample from a patient with SLE and a normal control subject. The neutral glycosphingolipids from P12 cells and asialoGM1 are abbreviated to P12 and GA1 respectively. The left hand plate was stained with orcinol- $H_2SO_4$  reagent. The right hand plate was immunostained with serum samples from the patient with the lowest lymphocyte count and a normal control subject. Neutral glycosphingolipids D and E were stained with a serum sample from the patient, but not the normal control subject. CDH=ceramide dihexosides.

THIN LAYER CHROMATOGRAPHY IMMUNOSTAINING

For thin layer chromatography immunostaining, two high titre serum samples were selected to identify possible surface antigens. Three monosialo-glycosphingolipids migrating close to GM3, GM1, and GD1b (fig 3) and two neutral glycosphingolipids migrating as ceramide dihexosides and close to asialoGM1 (fig 4) were detected by serum samples from patients with SLE, but not by the control serum samples.

Discussion

Antibodies to lymphocytes probably play an important part in the pathogenesis of SLE. Such antibodies can promote complement dependent lymphocyte cytotoxicity<sup>2</sup> and in NZB autoimmune mice there is an autoantibody to thymocytes with complement dependent lymphocyte cytotoxicity activity against suppressor T cells (the so called natural thymocyto-

toxic autoantibody).<sup>24</sup> We have shown previously that human lymphocyte cell lines are suitable targets for the detection of these antibodies. In this work we analysed the relations of titres of antibodies to lymphocytes in serum samples from patients with SLE with the distribution of lymphocyte surface markers on the target cells, peripheral blood lymphocyte count, and serum complement levels. Serum titres of antibodies to lymphocytes in patients exceeded those in normal control subjects and there was a close correlation of titres of antibodies to P12 (CD4<sup>-</sup>, CD8<sup>+</sup>) with those of antibodies to Wa (B cell), Jurkat (CD4<sup>-</sup>, CD8<sup>-</sup>), and Hut78 (CD4<sup>+</sup>, CD8<sup>-</sup>) indicating that autoantibodies to lymphocytes react with T and B cells and are not restricted to reactions with suppressor and helper T cells.

The titre of IgG (but not IgM) antibodies to lymphocytes correlated inversely with lymphocyte count and complement level. High titres could be a clinically useful marker of lupus disease activity.

Among antigens localised on the surface of lymphocytes, our immunoabsorption studies emphasise the importance of surface antigens on lymphocytes. Several protein antigens have been described<sup>25, 26</sup> but this is the first report of glycosphingolipid antigens in the lymphocyte membrane. Autoantibodies to glycosphingolipids (asialoGM1 and GM1) are recognised in SLE,<sup>8</sup> and antibodies to asialoGM1 may be mediators of neurological disorders.<sup>9</sup> By thin layer chromatography immunostaining, two serum samples selected for high titres of antibodies to lymphocytes and associated lymphopenia were reactive with three monosialoglycosphingolipids and two neutral glycosphingolipids from P12 cells. The monosialoglycosphingolipid antigens migrated close to GM3, GM1, and GD1b but are not identical with them. The two neutral glycosphingolipid antigens are dihexosyl ceramides and an unknown glycosphingolipid migrating close to asialoGM1.

IgG antibodies to lymphocytes detected by our sensitive cell ELISA with human cell lines might be a useful marker of disease activity in SLE. Further study is needed to determine the structure of the antigens (including glycosphingolipids) and to analyse the relation of these antibodies to other clinical data.

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