Lymphocyte and neuronal antigens in neuropsychiatric lupus: presence of an elutable, immunoprecipitable lymphocyte/neuronal 52 kd reactivity

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Abstract

Objective—To examine specific lymphocyte or neuronal antigens immuno-precipitated by systemic lupus erythematosus (SLE) sera.

Method—SLE sera were screened for the presence of antibodies binding to surface antigens of CD4(+) HUT-78 or SK-N-SH and IMR-6 neuroblastoma cells using Western blotting or radioimmuno-precipitation.

Results—IgG eluates from both lymphocytes and neuroblastoma cells recognised a 52 kd band in HUT 78 cell lysates. Eight sera studied further using radioimmuno-precipitation also demonstrated binding to a 52 kd antigen (4/8 on HUT-78, 8/8 on SK-N-SH cells), partially depleted by absorption with viable HUT-78.

Conclusion—A 52 kd antigen recognised by SLE sera on lymphocytes and neuronal cells may play a role in the pathogenesis of neuropsychiatric-SLE.

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Sera from patients with systemic lupus erythematosus (SLE) are reactive with many cellular antigens.^{1–3} Attempts to show positive correlations between autoantibodies and disease parameters are often met with frustration; some associations have been found,^{4–10} but a reliable relationship between NP-SLE and cell-surface antigens is yet to be delineated. Technical problems in acquiring pure antigen preparations and the heterogeneity of antibody specificities in SLE sera can be compounded by low-avidity binding and small amounts of cell-surface antigens. In NP-SLE, particular interest has been given to cross-reacting antigens on lymphocytes and neuronal cells.^{7 8 10 11} For the well-characterised intracellular protein antigens such as DNA, Ro, La, Sm and RNP,^{1 2 12 13} there is some evidence of their presence on cell surfaces in vitro, but it is unclear whether or not their appearance is transient and/or relevant in vivo.¹⁴ The investigation presented here is an attempt to characterise which, if any, of the above antigens might be useful markers of NP-SLE; the presence of a lymphocyte/neuronal 52 kd reactivity is documented. Immuno-precipitation experiments are performed to

further clarify this lymphocyte and neuronal 52 kd moiety.

Materials and methods

PATIENTS AND SERA

Venous blood was obtained from patients attending the McMaster University Medical Centre Lupus Clinic who met the 1982 American Rheumatism Association revised criteria¹⁵ for classification as SLE. After separation from blood, serum was aliquoted and stored at -70°C .

CELL CULTURE

HUT-78, a CD4+ human lymphoblastoid T lymphocyte cell line¹⁶ was used as a source of lymphocyte antigens. Cells were passaged as a non-adherent culture and maintained in RPMI 1640 with 10% FCS, HEPES and glutamine. The human neuroblastoma cell lines SK-N-SH (provided by J Fogh, Sloan-Kettering Cancer Research Institute, New York, NY) and IMR-6 (from R Kennett, Department of Genetics, University of Pennsylvania, Philadelphia, PA) were passaged as a monolayer⁵ and maintained in RPMI 1640 with 10% FCS and non-essential amino acids. All cultures were grown at 37°C in 5% CO_2 in air, in a humidified atmosphere. Platelets were provided by Dr J Kelton, McMaster University.

PREPARATION OF DETERGENT LYSATES OF HUT-78 AND SK-N-SH CELLS

HUT-78 cells were harvested and washed three times with Hanks balanced salt solution (HBSS) before solubilisation in PO_4 buffer (pH 7.2) containing 1% Triton X-100 (Bio RAD), 2 mM phenylmethyl-sulfonyl fluoride (Sigma), and 1 trypsin inhibitory U/ml aprotinin. Approximately 1×10^7 cells per ml of lysis buffer were incubated for one hour on ice with occasional vortexing. SK-N-SH cells were solubilised after release from culture with 0.02% EDTA and three washes with HBSS. Lysates were cleared of nuclei by centrifugation at $400 \times G$ for 10 minutes at 4°C . Cytoskeletal components and unsolubilised membranes were removed by ultracentrifugation at $100\,000\text{ g}$ for 60 minutes at 4°C . Aliquoted lysate was stored at -70°C until use.

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WESTERN BLOTTING

Prepared lysates of SK-N-SH or HUT-78 (representing $\sim 6 \times 10^6$ cells) were boiled for five minutes in SDS sample buffer [50 mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulphate (SDS; BIORAD) and 10% glycerol] and electrophoresed under non-reducing conditions using 12% SDS-polyacrylamide slab gels according to the technique of Laemmli.¹⁷ Prestained marker proteins (Bethesda Research) were used to calibrate molecular weight. Electrotransfer of proteins to nitrocellulose sheets¹⁸ was performed overnight at a constant power of 30 volts in transfer buffer [20 mM Tris-HCl (pH 8.2), 150 mM glycine, 20% methanol, 0.01% SDS] using a BIO-RAD Trans-Blot cell.

Nitrocellulose strips were incubated for two hours at room temperature in blocking buffer [20 mM Tris-HCl (pH 7.4), 200 mM NaCl, 0.05% Tween 80, and 5% skim milk powder], washed twice in 20 mM Tris-HCl (pH 7.4), 200 mM NaCl, 0.05% Tween 80, and incubated for 60 minutes at room temperature with constant agitation in test sera at a 1:25 dilution. The strips were washed three times before the addition of alkaline-phosphatase conjugated goat anti-human IgG (H & L) (Biocan Scientific) which was incubated for 60 minutes at room temperature. After three more washes, the strips were developed for colour with BCIP/NBT (BIO-RAD) solution.

ELUATES

Eluates¹⁹ were prepared from HUT-78, SK-N-SH, IMR-6 and platelets by mixing 0.5 ml serum diluted 1:2 with RPM1 with $12\text{--}15 \times 10^7$ cells which had been fixed in 3% paraformaldehyde and incubating overnight at 4°C with gentle mixing. The cells were washed three times in RPM1 at 4°C followed by incubation at 37°C in RPM1 for one hour with gentle agitation. Eluates and supernates were stored at -70°C until used.

RADIOLABELLING

Surface proteins on HUT-78 and SK-N-SH cells were labelled by solid-phase lactoperoxidase/glucose oxidase-catalysed radioiodination using Enzymobeads (BIO-RAD) according to the manufacturer's instructions. Approximately 1×10^7 cells in 250 µl of HBSS were combined with 25 µl of 0.5 M phosphate buffer (pH 7.2), 50 µl 1% β-D-glucose, 0.5 mCi sodium ¹²⁵I and 50 µl Enzymobeads. Following incubation at room temperature for 60 minutes, the cells were washed three times in PBS (pH 7.4) and solubilised in lysing buffer as described for preparation of detergent lysates.

IMMUNOPRECIPITATION AND SDS-PAGE

Preclearance of cell lysates with protein A-Sepharose beads (Pharmacia) did not result in any reduction in non-specific binding and was omitted in these experiments. Aliquots of 300–500 µl of cell lysate were incubated with

30 µl of test serum overnight at 5°C with gentle mixing. Immune complexes were removed by the addition of 10% protein A-Sepharose beads and incubating for a further three hours at 5°C. The beads were washed 5 times in lysis buffer and the complexes dissociated by boiling for five minutes in non-reducing SDS sample buffer. The denatured proteins were electrophoresed by using 12% SDS-polyacrylamide slab gels under non reducing conditions¹⁷ along with prestained molecular weight markers. (Bethesda Research). Gels were fixed in 40% methanol: 10% Acetic acid; 5% glycerol, then dried and autoradiographed with XAR-5 film (Eastman Kodak) for 5–7 days at -70°C.

IMMUNOABSORPTION

HUT-78 and SK-N-SH cells were harvested and washed three times in HBSS to obtain final pellets of 5×10^7 cells with >90% viability as determined by nuclear exclusion of trypan blue. Cell pellets were resuspended in 1 ml of SLE serum variously diluted with RPM1 according to mixed haemadsorption assay (MHA)⁵ titre and incubated for one hour at room temperature followed by at least two hours at 5°C. The procedure was repeated sequentially three times. The final supernatant and diluted serum controls were aliquoted and stored at -70°C until further use.

MIXED HAEMADSORPTION ASSAY

The MHA which has been previously described⁵ and is routinely carried out in this laboratory to screen for the presence of IgG antineuronal antibodies in sera is defined as positive reactivity to one or more neuroblastoma cell lines at a titre $\geq 1:20$. HUT-78 cells as well as neuroblastomas were used in this assay system to test the effectiveness of absorption procedures and the possibility of cross-reactivity with lymphocytes and neuronal cells.

LYMPHOCYTOTOXICITY

Cold reactive anti-lymphocyte (LCA) antibodies were measured by a modification of the micro-droplet method as previously described²⁰ and T cell 30 frozen cell trays (Gen Trak). Sera and cells were incubated for one hour at 4°C followed by the addition of rabbit complement (Gen Trak) and incubation for one hour at 15°C. Results are expressed as a percentage of cells killed.

Results

ELUATE REACTIVITIES WITH LYMPHOCYTES AND NEURONAL CELLS

Serum from a patient with active NP-SLE was used in eluate experiments with HUT-78, SK-N-SH, IMR-6 cells and platelets. Absorption with HUT-78 cells removed most of the 52 kd activity which was recoverable in the eluates (fig 1A). Eluates from the neuroblastoma cell lines SK-N-SH and IMR-6 also demonstrated

Figure 1 (A) Immunoblots showing IgM(M) or IgG(G) reactivities in SLE sera, using goat anti-human IgG or IgM, to 50–52 kD and other proteins on HUT-78 cells. An arrow indicates the 50–52 kD protein band. Lanes 1 and 2, unabsorbed sera; lanes 3 and 4, partially absorbed with HUT-78 cells; lanes 5 and 6, eluates; (B) Control sera showing, with one exception (lane 14), no 52 kD reactivity on immunoblotting of IMR-6 lysates. Lanes 1–6, normal human sera; Lanes 7–14, rheumatoid arthritis sera.

a 52 kD reactivity both in serum and eluate; absorption was incomplete (fig 2). All of the eluates except those from platelets demonstrated LCA activity, but neuronal antibodies were absent except in IMR-6 (table 1). Control sera ($n = 43$) were studied from healthy volunteers or patients with rheumatoid arthritis. Some representative examples of these are shown in fig 1B: 13/14 (93%) were devoid of the 52 kD reactivity on either HUT-78 or IMR-6 lysates.

IMMUNOPRECIPITATION OF LABELLED SURFACE PROTEINS ON LYMPHOCYTES AND NEURONAL CELLS.

In immunoprecipitation experiments, the lysates of I^{125} surface labelled HUT-78 and SK-N-SH cells were incubated with a panel of 8 NP-SLE sera previously used in Western blotting of SK-N-SH lysates, and run in SDS-PAGE. With HUT-78 lysate, four of the sera precipitated a 52 kD band, 2 precipitated a 55 kD band, whereas all eight sera precipitated a 52 kD band with SK-N-SH (fig 3). None of the sera precipitated a 32 kD band against either cell line, and 55 kD was not detected on SK-N-SH with any sera, in contrast to Western blotting results.¹¹ This lack of expression of 55 kD is consistent with previous work in our laboratory (unpublished) using I^{125} labelled neuroblastoma lysates and SLE sera. Table 2 summarises Western blotting and immunoprecipitation experiments.

IMMUNOABSORPTION STUDY

Immunoabsorption experiments were carried out with 4 NP-SLE sera showing 52 kD reactivity, using viable HUT-78 and SK-N-SH cells to confirm the specificity of the 52 kD antigen and to further investigate cross-reactivity. The mixed haemadsorption assay

Figure 2 Immunoblots showing IgG reactivities in SLE sera to 50–52 kD proteins on neuroblastoma cells. Lane 1, unabsorbed serum; Lane 2, absorbed with SK-N-SH cells; Lane 3, eluate from SK-N-SH cells; Lane 4, absorbed with IMR-6 cells; Lane 5, eluate from IMR-6 cells.

was used to test binding activity before and after absorption. Absorption of LCA by HUT-78 cells was demonstrated in two sera. Neuronal antibody was absorbed from two of the four sera, only by SK-N-SH (table 3).

Discussion

The causal role for antibodies directed against cross-reactive, brain-lymphocyte antigens in the pathogenesis of nervous system lupus (NP-SLE) has not yet been determined. Various investigators have proposed roles for neuronal,^{4 5 21} lymphocytotoxic,^{7 9 10 22} ribosomal P²³ phospholipid^{24 25} and glycolipid^{26 27} antibodies in the pathogenesis of NP-SLE, based

on correlations between these antibodies and clinical NP-SLE.

In the current studies, we have attempted to further identify common lymphocyte and neuronal antigens recognised by some SLE sera. This derived from observations on the presence of reactivities to a 50–55 kd as well as a 32–34 kd series of antigens in relation to NP-SLE and, possibly, to cognitive dysfunction.¹¹ We found serum reactivity by Western blotting to 31–32 kd, 50–52 kd and 54–55 kd antigens using CD4⁺, HUT-78 lymphocytes, as well as SK-N-SH neuroblastoma cells. In the current report, the eluates prepared from HUT-78 (lymphocyte line), IMR-6 and SK-N-SH cells (neuroblastoma lines), in contrast to platelet eluates, showed consistent precipitation by IgG antibodies of a 52 kd band; this was especially marked using HUT-78 cell eluates. This 52 kd band was also recognised by a panel of eight sera studied in immunoprecipitation experiments with I¹²⁵ labelled lymphocytes or neuronal cells (fig 3). While these studies were

not performed in a large number of SLE sera, they may nonetheless point out a potential shared lymphocyte/neuronal surface antigen of interest. More definitive, prospective evaluation of this reactivity in a patient cohort is in progress. While indications are that a 50–52 kd immunoreactivity by Western blotting is significantly associated with NP-SLE,¹¹ anti-Ro antibodies by ELISA per se do not correlate with NP-SLE but the presence of both anti-Ro and anti-50/52 kd appears to be associated with NP-SLE; for example, of 11 sera with both Ro and 50/52 kd reactivities, eight (73%) had NP-SLE, as opposed to 16/37 (43%) of sera with Ro-reactivity alone.

Since a significant proportion (37/99 or 37%) of these sera contained anti-Ro reactivity by ELISA, and since 5/6 50–52 kd reactive sera tested in eluate studies were also Ro (+)ve, the possibility exists that the activity seen here in the eluates, as well as directly by radiolabelled immunoprecipitation, represents an anti-52 kd Ro specificity. Numerous investigators have recently commented on the large number of molecular forms of Ro and the genetic polymorphisms encoding this cytoplasmic molecule.^{1 2 12 13} While 52 kd Ro is not normally a component found to be reactive in the immunoassays performed in clinical laboratories, Western blotting may reveal it.^{1 2} Thus it is possible that the lymphocyte-neuronal antigen precipitated by our lupus sera is indeed 52 kd Ro, although definitive molecular proof of this is still lacking. The presence of this antigen on the surface of cells, while not commonly recognised, is possible. Ro can be expressed on the surface of cells such as keratinocytes after ultraviolet radiation and on embryonic cardiac muscle,²⁸ forming the basis for the possible relationships of congenital heart block and photosensitive skin rash with Ro.^{13 14} Whether or not 52 kd Ro can be expressed on the surface of neuronal cells and is related to NP-SLE is still unclear. Recently, a ribosomal P₀ antigen has been shown to be expressed on the surface of SK-N-MC cells; however, the molecular size of this antigen was 38 kd.²⁸

There are several observations in recent reports which speculate on the role of Ro in the possible pathogenesis of certain forms of developmental abnormalities of the nervous system, including dyslexia.³⁰ Indeed, an autoimmune hypothesis of learning disabilities, involving the Ro antigen and based upon a

Table 1 Lymphocytotoxic and neuronal reactivities of eluates demonstrating a 52 kd stained band from NP-SLE serum.

	Lymphocyte reactivity*	Neuronal reactivity**	
	LCA-HUT-78	SK-N-SH	IMR-6
NP-SLE serum (1:2 dilution)	97	++	++++
HUT-78 eluate	60	-	-
SK-N-SH eluate	42	-	-
IMR-6 eluate	57	+	-

*Lymphocytotoxicity expressed as % killing of HUT-78 cells.
**Neuronal Antibody scores as: - <10% positive cells; + 10–25% positive cells; ++ 26–50% positive cells; +++ 51–75% positive cells; ++++ 76–100% positive cells.

Figure 3 Immunoprecipitation of antigens on I¹²⁵ surface-labelled HUT-78 and SK-N-SH cells by SLE sera. Lanes 1–8, sera from individual patients; a 52 kd reactivity is present in each case.

Table 2 Pattern of SLE sera reactivities with lymphocytic and neuronal cell lines by Western blotting (WB) and radioimmunoprecipitation

Serum	HUT-78 (WB) kd bands			HUT-78 (I ¹²⁵) kd bands		SK-N-SH (WB) kd bands		SK-N-SH (I ¹²⁵) kd bands
	32	52	55	52	55	32	52	52
1		X	X	X				X
2	X		X					X
3		X			X		X (very weak)	X
4	X	X					X (very weak)	X
5	X(d)	X		X		X(d)		X
6	X	X					X (very weak)	X
7	X	X	X	X			X	X
8	X	X		X	X	not available		X

X—indicates presence of band.
(d)—indicates presence of doublet.

Table 3 Results of absorption experiments with 4 NP-SLE sera and HUT-78 and SK-N-SH cells in mixed haemadsorption assay

Serum	Lymphocyte antibody*			Neuronal antibody*		
	Pre-abs	Abs with HUT-78	Abs with SK-N-SH	Pre-abs	Abs with HUT-78	Abs with SK-N-SH
1	++++	+	++++	+	++	++
2	+	+	+	++++	++++	+++
3	++++	—	++++	++++	+++	—
4	—	—	—	++	+	—

*Scoring of wells: — <10% positive cells; + 10–25%; ++ 26–50%; +++ 51–75%; ++++ 76–100% positive cells.
abs—absorption.

finding of increased left-handedness and dyslexia in autoimmune disease populations, or vice-versa^{30–31} has been proposed. Our study sample was too small and uncontrolled to draw any conclusions concerning the role of Ro in NP-SLE or the specific cognitive abnormalities which can be associated with it.²¹ The possible relationship of NP-SLE or cognitive dysfunction with specific lymphocyte reactivities, and especially with a 50–52 kd reactivity,¹¹ however, suggests that surface expression of Ro or molecularly related antigens may be involved in the development of NP-SLE and cognitive dysfunction in some patients with SLE. One new neuronal antigen recently shown to be a target of SLE sera, especially in patients with nervous system involvement, is a 50 kd protein whose function has yet to be determined.³²

More work certainly needs to be done on clarifying conditions leading to surface expression of Ro on normal human nervous system components, including neuronal cells, and its relationship to the development of behavioural and cognitive abnormalities. The availability recently of an animal model of cognitive dysfunction in SLE³³ may make this hypothesis directly testable.

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