Molecular Heterogeneity of a Lymphocyte Glycoprotein in Immunodeficient Patients

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Abstract

Previous evaluation of lymphocytes taken from patients with Wiskott–Aldrich syndrome (WAS) and other X-linked immunodeficiencies has revealed deficiencies of a lymphocyte sialoglycoprotein with a relative molecular mass of 115 kD (designated gpL-115) found in normal lymphocytes. The development of monoclonal antibodies to gpL-115 has permitted the detection of molecular heterogeneity in gpL-115 from the lymphocytes of immunodeficient patients. When lymphocytes from normal individuals were analyzed by immunoblotting, gpL-115 with only a single molecular species (115 kD) was detected. Lymphocytes from 17 immunodeficient patients were analyzed after overnight incubation. Two patients had no gpL-115 with an M_r of 115 kD, but gpL-115 with an M_r of either 95 or 135 kD was detected. Nine patients had gpL-115 with M_r equally of 95 and 115 Kd. Other patients exhibited gpL-115 with combinations of 95, 115, and 135 kD. The heterogeneity of the degraded gpL-115 suggests that WAS and other X-linked immunodeficiencies are due to a series of abnormalities, all of which involve gpL-115, and may explain the clinical heterogeneity of the diseases.

Introduction

We have previously reported the deficiency of a normal sialoglycoprotein with a relative molecular mass of 115 kD (designated gpL-115) in the lymphocytes of patients with Wiskott–Aldrich syndrome (WAS)[1] and other X-linked T lymphocyte immunodeficiencies (1–3). The production of a monoclonal antibody (designated L-10) to gpL-115 has permitted us to evaluate the molecular heterogeneity of the lymphocyte gpL-115 abnormalities (2). Normal lymphocyte gpL-115 is stable in vitro whereas gpL-115 from lymphocytes of immunodeficient patients undergoes partial degradation. The heterogeneity of the gpL-115 degradation suggests that patients with gpL-115 deficiency represent a spectrum of disorders, all of which are characterized by the abnormal degradation of lymphocyte gpL-115.

Methods

Patients. Patients with WAS were males, whose diagnosis was based upon thrombocytopenia with platelets of reduced size and function and the variable presence of eczema and/or immunodeficiency. The patients with T lymphocyte immunodeficiency resulting from the deficiency of gpL-115 have been previously described (3).

Purification and separation of lymphocytes. Peripheral blood was collected in either preservative-free heparin (100 U/ml) or acid-citrate-dextrose (National Institutes of Health, Formula A, 0.17 ml/ml). Blood specimens were processed either immediately (fresh) or after overnight air shipment or storage at room temperature (overnight). Mononuclear cells were isolated on Ficoll-Hypaque gradients. After collecting the mononuclear cells, they were washed in Hanks' balanced salt solution, resuspended in Hanks' with 5% inactivated fetal bovine serum (Irvine Scientific, Irvine, CA), and incubated at 5×10^6 cells/ml in 100-ml tissue culture dishes (Falcon 1029, Falcon Labware, Cockeysville, MD) for 1 h at 37°C in a 5% CO_2 incubator. The nonadherent cells (lymphocytes) were collected. In some experiments whole blood samples were centrifuged, and the plasma was removed. The patients' plasma was added to the erythrocytes-leukocytes of normal individuals, and the plasma from normal individuals was added to erythrocytes-leukocytes from the patients. After resuspension, the samples were maintained at room temperature overnight before leukocyte separation. T and non-T lymphocytes were prepared by bulk erythrocyte rosette formation. Lymphocytes were incubated at 4°C for 2 h with neuraminidase-treated sheep red blood cells. The lymphocytes were centrifuged at 150 *g* for 10 min, gently resuspended, and separated on a Ficoll-Hypaque gradient. Interphase cells (non-T lymphocytes) contained < 10% erythrocyte rosette-forming cells, whereas the pelleted lymphocytes after lysis with 0.87% Tris-ammonium chloride contained > 90% erythrocyte rosette-forming cells (T lymphocytes). Thymocytes were obtained from thymus removed as a routine component of open heart surgery. Granulocytes were obtained by the dextran sedimentation of peripheral blood leukocytes. The leukocyte-rich supernatant was separated on Ficoll-Hypaque gradients; the pelleted cells contained > 80% granulocytes. Cell lines (CEM and U937) were routinely grown in RPMI 1640 with 10% heat inactivated fetal bovine serum with 1×10^{-2} M glutamine and penicillin/streptomicin added.

Sialidase treatment. Lymphocytes at 1×10^7/ml were treated with *Vibrio cholerae* sialidase as previously described (2).

Cell lysis. Cells were routinely lysed at a concentration of 24×10^6 cells/ml except for thymocytes, which were lysed at 48×10^6/ml. The lysis buffer contained of 0.5% NP-40 in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM phenylmethylsulfunyl fluoride, and 3 mM iodoacetamide. The lysates were filtered through 0.2-μm Millipore filters (Millipore Corp., Bedford, MA) and stored at −70°C until use.

Monoclonal antibodies. L-10 is a murine IgG2a monoclonal antibody which reacts with both the sialo and asialo forms of gpL-115. L-2 is an IgE monoclonal antibody which reacts with only the sialo form of gpL-115. The production of the monoclonal antibodies has been previously described (2). The antibodies were generously provided by E. Remold-O'Donnell (Center for Blood Research, Boston, MA).

Polyacrylamide gel electrophoresis and immunoblot analysis. NP-40 extracts were solubilized by heating with an equal volume of 2% sodium dodecyl sulfate (SDS) in 16 mM Tris-glycine buffer, pH 6.8, with 2% 2-mercaptoethanol for 2 min at 100°C. Samples (75λ) were electrophoresed in a 7% SDS polyacrylamide gel with a 4% stacking gel at 25 mA for 6 h (4). After the completion of the electrophoretic run, the proteins were transferred from the polyacrylamide gel to a nitrocellulose membrane by overnight electroblotting. The transfer buffer was 25 mM Tris-192 mM glycine, pH 8.3, diluted 4:1 with methanol. After transfer, the ni-

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1. *Abbreviations used in this paper:* WAS, Wiskott–Aldrich syndrome.

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trocellulose membranes were blocked for 1 h with gelatin buffer (3% gelatin in 20 mM Tris, 500 mM NaCl, pH 7.5) and then incubated for 3 h with a 1:10 dilution of L-10 or L-2 tissue culture supernatant or control mouse ascites. After washing, the L-10–reacted nitrocellulose membranes were incubated with a 1:2,000 dilution of peroxidase-labeled goat anti-mouse IgG (Bio-Rad Laboratories, Richmond, CA). With L-2, the nitrocellulose membranes were incubated with a 1:100 dilution of rabbit anti-mouse IgE for 1 h, followed by washing and incubation with a 1:1,000 dilution of peroxidase-labeled goat anti-rabbit IgG (Bio-Rad Laboratories). After washing, the nitrocellulose membranes were developed with 4-chloro-napthol (Bio-Rad Laboratories), and the immunoblots were photographed. M_r were calculated from high molecular weight marker standards (Bethesda Research Laboratories, Gaithersburg, MD).

Results

Antigenic reactivity of L-10 and L-2. L-10 and L-2 monoclonal antibodies were developed by the immunization of BALB/c mice with a T lymphoblast cell line, CEM. L-10 reacts with both the sialo and asialo forms of gpL-115, whereas L-2 only reacts with the fully sialated form by immunofluorescence and immunoprecipitation (2). After the gel electrophoresis of cell lysates and their transfer to nitrocellulose membranes, L-10 reacts predominantly with a single molecular species from all lymphoid cells (peripheral blood lymphocytes, T lymphoblast cell lines, thymocytes, and B lymphoblast cell lines) and with multiple molecular species from cells of myeloid origin (granulocytes, monocytes, and U937) (Fig. 1). L-10 does not react with nonnucleated cells of hematopoietic origin (erythrocytes) or with nucleated cells of nonlymphohematopoietic origin (fibroblasts and hepatocytes) (data not shown).

The M_r of the L-10–reactive glycoprotein on mature T lymphocytes is 115 kD in its sialated form and 150 kD in its desialated form; the desialated form does not transfer to nitrocellulose as quantitatively as the sialated form. Analysis of the L-10–reactive glycoproteins from thymocytes and CEM revealed two bands; the primary band has an M_r of 115 kD and the secondary band an M_r of 127 kD, presumably resulting from varying degrees of sialation (5). L-10 reacts with glycoproteins from cells of myeloid origin with M_r of 125 and 135 kD; no reactivity with glycoproteins with an M_r of 115 kD is found in lysates of myeloid cells.

To determine the stability of lymphocyte gpL-115 in vitro, peripheral blood samples were either processed immediately (fresh) or maintained at room temperature overnight. L-10 analysis of both fresh and overnight lymphocytes from five normal males showed no significant degradation of gpL-115 (Fig. 2). Peripheral blood samples of normal individuals shipped overnight by air freight also showed no significant degradation of gpL-115 (data not shown).

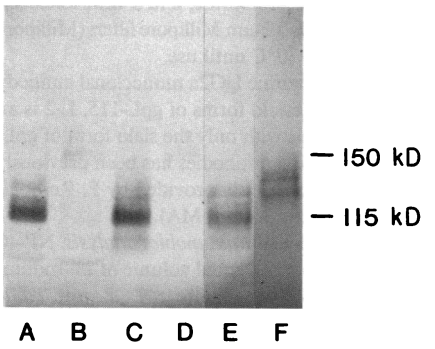

Figure 1. Immunoblotting of various cell types with L-10. Lane: *A*, CEM, untreated; lane *B*, CEM, sialidase-treated; lane *C*, peripheral blood lymphocytes, untreated; lane *D*, peripheral blood lymphocytes, sialidase-treated; lane *E*, thymocytes; lane *F*, granulocytes.

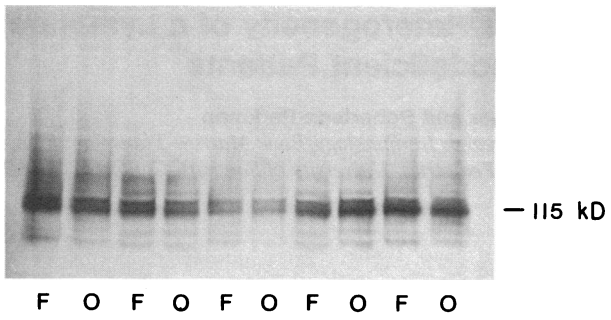

Figure 2. Immunoblotting with L-10 of paired samples of fresh (*F*) and overnight (*O*) lymphocytes from five normal males.

Antigenic analysis of gpL-115 in immunodeficient patients. When lymphocytes of patients with WAS were collected and isolated on the day of collection, no abnormalities in gpL-115 were noted after immunoblot analysis with L-10 (Fig. 3). When, however, WAS lymphocytes were isolated on the day after collection, heterogeneity in gpL-115 by immunoblot analysis with L-10 was detected. Immunoblot analysis of lymphocytes from representative WAS patients are shown in Fig. 4. A total absence of gpL-115 was detected in some patients with the appearance of L-10–reactive bands with higher or lower M_r (Fig. 4, lanes *A*, *E*, and *K*). Lymphocytes from other patients had detectable gpL-115 with the presence of additional L-10–reactive bands with either higher or lower M_r. In total, 17 immunodeficient patients were analyzed (Table I). Nine patients had a pattern characterized by L-10–reactive glycoprotein with M_r of both 95 and 115 kD (Fig. 4, lanes *F–J*). Three patients (in Fig. 4, lane *D* is representative) had three L-10–reaction bands, 95, 115, 135 kD; two patients had L-10–reactive bands of both 115 and 135 kD; and individual patients reacted uniquely with single bands of 135 kD (lane *A*) and 95 kD (lane *K*). Thus, gpL-115 from immunodeficient lymphocytes can degrade to glycoproteins with M_r of 95 kD, 135 kD, or both. The degradation was not due to desialization in that immunoblotting with L-2 gave identical results (data not shown). Samples from affected siblings had similar L-10–reactive patterns.

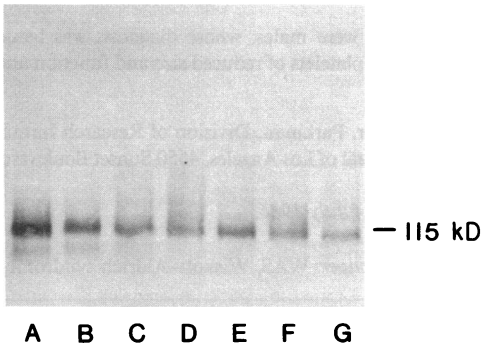

Figure 3. Immunoblotting with L-10 of fresh lymphocytes from WAS patients.

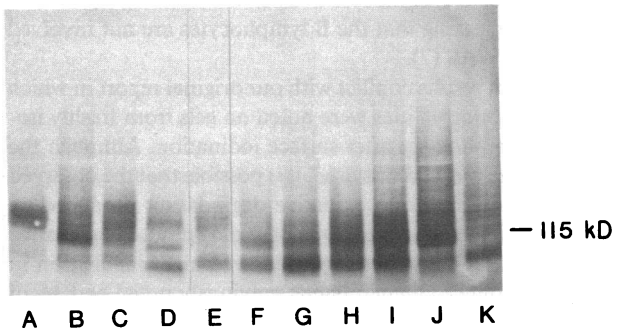

Figure 4. Immunoblotting with L-10 of overnight lymphocytes from WAS patients.

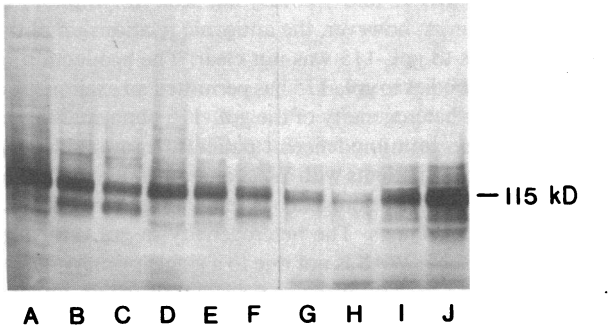

Figure 5. Immunoblotting with L-10 of lymphocytes from patients and normal individuals. Lane *A*, patient 1, fresh; lane *B*, patient 1, overnight in autologous plasma; lane *C*, patient 1, overnight in normal plasma; lane *D*, patient 2, fresh; lane *E*, patient 2, overnight in autologous plasma; lane *F*, patient 2, overnight in normal plasma; lane *G*, normal, fresh; lane *H*, normal, overnight in normal plasma; lane *I*, normal, overnight in patient 1 plasma; lane *J*, normal, overnight in patient 2 plasma.

To determine the basis for the molecular heterogeneity in gpL-115 of patients, lymphocytes taken from patients were isolated on both the day of collection and after overnight incubation. When the samples were analyzed simultaneously, no abnormality in gpL-115 of patients was detected in the fresh lymphocytes whereas multiple L-10–reactive glycoproteins were detected after overnight incubation. To determine whether the degradation of gpL-115 in plasma of patients was due to the presence of enzymes that might degrade lymphocyte gpL-115, the samples from patients were centrifuged, the plasma was removed, and plasma from normal individuals was added. Conversely, the plasma from the patients was added to erythrocytes-leukocytes from normal individuals. After overnight incubation at room temperature, the leukocytes from both patients and normal individuals were isolated as usual. Degradation of the gpL-115 from patients occurred regardless of whether the lymphocytes of patients were incubated in plasma of patients or normal individuals (Fig. 5, lanes *B, C* and *E, F*). No degradation of gpL-115 occurred when lymphocytes from normal donors were incubated with plasma from patients (Fig. 5, lanes *I* and *J*). These results indicate that the degradation seen in gpL-115 from patients was not due to the presence of abnormal plasma enzymatic activity. Equivalent degradation of gpL-115 from patients occurred when freshly isolated lymphocytes from patients were incubated overnight in heat-inactivated fetal bovine serum (data not shown).

gpL-115 analysis of lymphocyte subsets. To determine whether the observed molecular heterogeneity in gpL-115 was present in both T and non-T lymphocytes, WAS (Fig. 4, lane *C*) and normal peripheral blood lymphocytes were separated into T and non-T lymphocytes by bulk erythrocyte rosette formation after overnight incubation. The isolated populations were lysed and analyzed (Fig. 6). Whereas the non-T lymphocytes from both patients and normal individuals exhibited no significant degradation, the T lymphocytes of the patient gave two L-10–reactive bands (M_r 95 and 115 kD) and the T lymphocytes from normal donors gave a single band with M_r of 115 kD. The results indicate that the primary source of the gpL-115 heterogeneity seen in WAS patients is from the T lymphocytes and that WAS B lymphocyte gpL-115 is relatively stable.

Discussion

We have previously reported deficiencies of a lymphocyte sialoglycoprotein (gpL-115) in patients with WAS and other X-linked T lymphocyte immunodeficiencies (1–3). The original assays consisted of surface iodination of peripheral blood lymphocytes followed by gel electrophoresis and indicated an absence of gpL-115 in the lymphocytes from some patients and a decrease in gpL-115 in others (1). Some patients exhibited additional

Table I. Heterogeneity of Lymphocyte gpL-115

Molecular species (M_r)	Normal (n = 12)	Immunodeficient (n = 17)
kD		
115	12	0
95	0	1
135	0	1
95 = 115	0	9
95, 115, 135	0	3
95, 135	0	1
115, 135	0	2

Lymphocytes from immunodeficient patients and normal individuals were collected after overnight incubation and analyzed by immunoblotting with L-10. Immunoblots were photographed, and M_r was calculated.

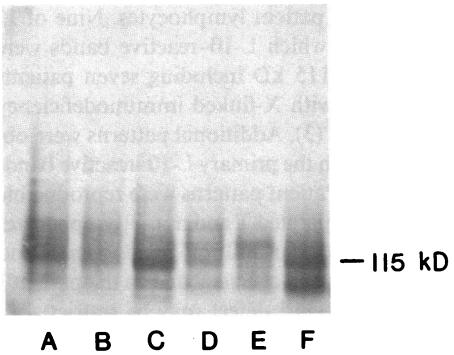

Figure 6. Immunoblotting with L-10 of separated lymphocytes after overnight incubation. Lane *A*, normal, whole; lane *B*, normal, non-T; lane *C*, normal, T; lane *D*, patient, whole; lane *E*, patient, non-T, lane *F*, patient, T.

iodinated bands (M_r 95 and 135 kD) not seen in lymphocytes from normal donors; however, the antigenic relationship of the additional bands to gpL-115 was not clear. The production of monoclonal antibodies to gpL-115 has permitted an examination of the molecular heterogeneity of the gpL-115 abnormalities in lymphocytes from immunodeficient patients. Immunoblotting has revealed that the patients with WAS and other T lymphocyte immunodeficiencies are heterogeneous, exhibiting a series of gpL-115 degradation products. The heterogeneity in gpL-115 degradation suggests that WAS is not due to a single primary defect but may represent a class of deficiencies, all of which involve abnormalities of lymphocyte gpL-115. The heterogeneity of gpL-115 deficiency may explain the clinical heterogeneity seen in WAS patients and other patients with X-linked T lymphocyte immunodeficiencies, who lack platelet abnormalities (3).

L-10 and L-2 antibodies react with all nucleated cells of lymphohematopoietic origin. Mature T and B lymphocytes have a single glycoprotein with an M_r of 115–120 kD. Analysis of thymocytes and CEM, which has a thymocyte-like phenotype, with L-10 reveals an additional band with a higher M_r, which may be due to the undersialation of some gpL-115 on immature T lymphocytes (5). After the treatment of peripheral blood lymphocytes or lymphoid cell lines with sialidase, L-10 detects a single moiety with an M_r of 150 kD, whereas L-2 displays no reactivity in that L-2 reacts with only the sialated form of gpL-115. The transfer of the desialated form of gpL-115 to nitrocellulose membranes is not so quantitative as the sialated form even though both are equally immunoprecipitated by L-10 (2). Analysis of myeloid cells reveals L-10– and L-2–reactive bands with higher M_r than that of lymphoid cells. The multiple bands are not due to the proteolysis of gpL-115 because identical patterns were obtained when U937 and granulocyte membranes were prepared by sonication and isolated on a sucrose cushion instead of by NP-40 lysis.

Normal lymphocyte gpL-115 is stable in vitro with little degradation and a low rate of turnover, < 10% per day (Parkman, R., unpublished data). Normal peripheral blood lymphocytes cultured for 4 d with or without phytohemagglutinin stimulation continued to express a single L-10–reactive band. Thus, normal lymphocyte gpL-115 displays little degradation in vitro.

Inasmuch as deficiencies in lymphocyte gpL-115 were noted in our initial surface iodination studies with WAS patients, it was surprising that fresh WAS lymphocytes were antigenically normal when analyzed by immunoblotting with L-10. Whereas gpL-115 from normal lymphocytes showed no significant degradation after overnight incubation, marked alterations were found in the immunoblots of patient lymphocytes. Nine of 17 patients showed a pattern in which L-10–reactive bands were found equally at M_r 95 and 115 kD including seven patients with WAS and two brothers with X-linked immunodeficiency without platelet abnormalities (3). Additional patterns were observed in other patients in which the primary L-10–reactive bands had M_r of 95 and/or 127 kD. Patient patterns were reproducible on repeat testing. Analysis of separated T and non-T lymphocytes from one patient demonstrated that multiple bands were found in the patient's T lymphocytes and a single band in the patient's B lymphocytes. Thus, the gpL-115 defect may be restricted to the T lymphocytes of WAS patients, a finding consistent with clinical results that have demonstrated that the immunodeficiency and eczema of WAS patients can be corrected by the selective engraftment of normal donor T lymphocytes but not donor B lymphocytes (6). Further allelic exclusion studies have shown a lack of B lymphocyte exclusion in some obligate heterozygotes, suggesting that the B lymphocytes are not involved in all cases of WAS (7).

The present results conflict with our original report in which glycoprotein abnormalities were noted on gels from freshly isolated WAS lymphocytes after surface iodination. Although the lactoperoxidase method was used, it is possible that the observed abnormalities were due to oxidative degradation produced by the lactoperoxidase. The lactoperoxidase may induce degradative changes similar to those observed after overnight incubation in that the additional iodinated bands previously noted had M_r of 95 and 135 kD.

Axelsson et al. (8, 9) have described a chicken antibody to a lymphocyte glycoprotein (LSGP) that has many similarities to gpL-115 (M_r, cellular specificity, etc.). The antibody was raised to the asialo form of the LSGP, and its reactivity is reciprocal to the reactivity of L-2 in that the LSGP antibody reacts with only the asialoglycoprotein, whereas L-2 reacts only with the sialated form of gpL-115. The asialo form of LSGP has an M_r of 150 kD whereas the sialated form of LSGP has an M_r of 115 kD in T lymphocytes and 120 kD in B lymphocytes, values similar to that of gpL-115. When purified LSGP degrades in vitro, it gives rise to one glycoprotein with an M_r of 95 kD and a second with M_r of 18 kD. At present it is not certain whether the 95 kD glycoprotein seen in WAS lymphocytes after overnight incubation is similar to the degradated form of LSGP.

The mixing experiments, in which WAS patient leukocytes were incubated overnight in normal plasma, etc., demonstrated that the degradation of WAS gpL-115 is not due to the presence of serum enzymes that might react with lymphocyte gpL-115 and indicates that the degradation of WAS patient gpL-115 is due to an intrinsic abnormality of the glycoprotein or other components of the lymphocyte. The heterogeneity of the degraded gpL-115 suggests that the primary site of degradation or the rate of degradation may vary from patient to patient. At present, whether the heterogeneity is due to differences in the primary defects (amino acid substitutions in gpL-115) or to differences in the sites of gpL-115 attachment is not known.

We have noted morphologic abnormalities in the peripheral blood lymphocytes of WAS patients by scanning electron microscopy (10). The abnormalities consisted of an increased percentage of lymphocytes without microvilli and the shortening of the microvillus processes on lymphocytes displaying microvilli. These morphologic abnormalities suggest that lymphocyte surface membrane is being removed in vivo, presumably by the spleen. The absence of degraded gpL-115 in freshly donated WAS lymphocytes may be due to the in vivo removal of the abnormal gpL-115 by the spleen. After overnight incubation in vitro, the degraded gpL-115 accumulates because the mechanisms that usually remove the degraded glycoprotein in vivo are not present. The analysis of WAS lymphocytes after overnight culture, therefore, permits the detection of the degraded gpL-115 which is not possible when freshly isolated lymphocytes from WAS patients are analyzed.

We have previously suggested that gpL-115 may have a role in lymphocyte survival (1). Axelsson and his colleagues (8, 9) have also suggested that LSGP may have a role in lymphocyte survival. Because they could not produce antibodies to LSGP in rodents or rabbits but only in chickens, they believed that LSGP is a phytogenetically maintained protein. In vitro experiments with L-10 has revealed no effect of L-10 antibody incubation on in vitro lymphocyte function (mitogen or antigen

blastogenesis, cytotoxicity, natural killer cell function [R. Parkman, unpublished data]). Thus, the defects in gpL-115/LSGP may have no direct effect on lymphocyte function. If gpL-115/LSGP of the lymphocytes of immunodeficient patients is intrinsically unstable and degrades to biochemical forms that are removed in vivo, the resultant lymphocytes may have a shortened in vivo survival leading to an absence of long-lived T lymphocytes and a lack of immunologic memory.

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