Evaluation of the lysosome-associated membrane protein LAMP-2 as a marker for lysosomal storage disorders

CHI T. HUA,1,2 JOHN J. HOPWOOD,1 SVEN R. CARLSSON,3 RAY J. HARRIS,2 and PETER J. MEIKLE1*

For many lysosomal storage disorders, presymptomatic detection, before the onset of irreversible pathology, will greatly improve the efficacy of current and proposed therapies. In the absence of a family history, presymptomatic detection can be achieved only by a comprehensive newborn screening program. Recently we reported that the lysosome-associated membrane protein LAMP-1 was increased in the plasma from 70% of individuals with lysosomal storage disorders. Here we report on the evaluation of a second lysosome-associated membrane protein, LAMP-2, as a marker for this group of disorders. The median concentration of LAMP-2 in the plasma of healthy individuals was 1.21 mg/L, fourfold higher than the median LAMP-1 concentration (0.31 mg/L). LAMP-2 was increased in 66% of patients with lysosomal storage disorders, and the increases coincided with increased LAMP-1 concentrations. The reference intervals for LAMP-1 and LAMP-2 in blood spots taken from newborns were 0.20–0.54 mg/L (n = 1600) and 0.95–3.06 mg/L (n = 1600), respectively. A high correlation was observed between the concentrations of LAMP-1 and LAMP-2 in both control and affected individuals. The higher concentrations of LAMP-2, relative to LAMP-1, in plasma make LAMP-2 an attractive marker; however, the final selection will be dependent on the availability of new diagnostic markers and their ability to detect disorders currently not identified by LAMP-2.

Lysosomal storage disorders (LSDs) represent a group of at least 40 genetic diseases, each of which results from a deficiency of one or more proteins involved in the degradation of macromolecules in lysosomes. The deficiency causes accumulation of undegraded substrates within lysosomes, which increase in number and size and can severely impair the physiology of the cell.

Most affected individuals are clinically healthy at birth but can present with a wide range of clinical manifestations within the first few years of life. Clinical symptoms depend on the specific disorder and can include central nervous system dysfunction, skeletal abnormalities, organomegaly, corneal clouding, and coarse hair and facial features (1, 2).

With a combined incidence in Australia of 1:5000 births (1), the impact of LSDs on the Australian healthcare system is considerable. Early detection, paving the way for early intervention of these diseases, would provide considerable benefits to the patients and their families, with the greatest benefit coming from effective therapy.

Several treatment strategies are currently being used to treat some LSDs, including drug therapy (3, 4), bone marrow transplantation (5), and enzyme replacement therapy (4–6). In addition, gene replacement therapy is under development for many LSDs (1, 6). Studies in animal models have shown that maximum efficacy is achieved if intervention occurs before onset of major clinical pathology (7, 8).

Except for cases with a family history of the disease, the presymptomatic detection of LSDs will only be possible through a newborn screening program. Currently in most cases, a presumptive diagnosis is made after presentation of clinical symptoms and is followed by a definitive diagnosis, which is determined through a large number of enzyme assays usually requiring the use of cell culture to provide enough material for analysis. These procedures...
are expensive, time-consuming, and invasive, making them unsuitable for mass screening applications.

Screening has been defined as the application of a test to people who are as yet asymptomatic for the purpose of classifying them with respect to their likelihood of having a particular disease (9). The screening procedure itself generally indicates a high probability of disease, and those who test positive are evaluated further by subsequent, more definitive diagnostic tests or procedures. In some screening programs, both the primary or first-tier screen and the second-tier screen are performed on blood spots taken from the Guthrie card. Newborn screening for cystic fibrosis is one such program; it involves a primary screen for immunoreactive trypsin from which the top 1% are selected to undergo a secondary screen based on the detection of specific mutations in the CFTR gene (10, 11). We propose that the newborn screening program for LSDs, currently under development, follow this model. The first-tier screen will involve the identification of an “at increased risk” population based on one or more specific protein markers; this population will then be screened further by a series of second-tier assays designed to identify specific storage products in blood spots taken from the same Guthrie cards. The justification for newborn screening for LSDs was previously presented by Meikle et al. (12), who evaluated a lysosome-associated membrane protein (LAMP-1) as a general marker for LSDs.

The LAMP-1 study showed that of the LSD-affected patients tested, 68% had LAMP-1 concentrations that were increased, with 32% having LAMP-1 concentrations within the reference range. They concluded that LAMP-1 may be a useful marker but that additional markers would need to be identified for a complete LSD screen. A potential candidate for a second or alternative marker protein is the related protein LAMP-2.

LAMP-1 and LAMP-2 proteins are the most abundant proteins in the lysosomal membrane, representing ~0.1–0.2% of the total cell proteins (13). They have high (36.7%) amino acid sequence identity (14). They are structurally similar, and both consist of a large luminal domain (~350 residues), which is heavily glycosylated, a single transmembrane segment, and a short cytoplasmic tail, which contains the signal for targeting the proteins to lysosomes (14).

Despite the structural similarity between these two proteins, it has been reported that they are differentially regulated. Comparison of the genes for LAMP-1 and LAMP-2 suggests that LAMP-1 is constitutively expressed (15), whereas the expression of LAMP-2 varies greatly with cell types and developmental stage (16). The expression of LAMP-2 has been shown to be uniquely regulated at the level of transcription, with a strong promoter in the 5' flanking region of the gene and a KpnI repeat sequence upstream, which suppresses the promoter activity (17).

LAMP-2 concentrations, detected by antibody binding, increased after differentiation in mouse embryo carcinoma cells (15). The synthesis and cell surface expression of LAMP-2 also increases when rat macrophages are activated in vivo and in patients with certain autoimmune diseases (17, 18). These results indicate that the expression of LAMP-2 is regulated in a specific fashion that may be related to an increase in phagocytic or immunological responses (17).

This differential regulation may lead to LAMP-2 displaying an altered pattern of expression in LSDs in addition to that which is anticipated because of the increase in lysosomal membranes in these disorders. To determine the suitability of LAMP-2 as a screening marker for LSDs, LAMP-2 has been quantified in plasma samples from unaffected and LSD-affected individuals and compared with LAMP-1 concentrations. In addition, we have also established the distribution and health-related reference range for LAMP-1 and LAMP-2 in blood spots taken from newborns in the Australian population.

Materials and Methods

PATIENT SAMPLES

Blood spots used in this blind study were part of the routine samples collected from newborns over a 4-week period from the Newborn Screening Laboratory at the Women’s and Children’s Hospital in North Adelaide. Plasma samples used were the same as described previously (12).

POLYCLONAL ANTIBODIES

Anti-LAMP-2 polyclonal antibodies were produced as described by Dahlgren et al. (19) and were shown to have no cross-reactivity with LAMP-1. Polyclonal antibodies were purified on a 1-mL Hitrap TM Protein G column (Pharmacia Biotech). Antibody concentrations were determined by absorbance at 280 nm (absorbance = 1.4 for 1.0 g/L).

MONOCLONAL ANTIBODIES

A hybridoma cell line (clone CD3) producing monoclonal antibodies against LAMP-2 has been described previously (13). Monoclonal antibodies were produced from this cell line, using a Diacult dialysis system (Inter Med Laboratory) as described (20). Antibodies from Diacult supernatant were purified by on Protein A–Sepharose® (Pharmacia Biotech), using high salt antibody purification procedures (21). Briefly, culture supernatant was adjusted to a final concentration of 3.3 mol/L NaCl, 100 mmol/L sodium borate, pH 8.9, and 1.5 mol/L glycine and applied to a Protein A column (1 × 5 cm) at a flow rate of 0.5 mL/min. The column was washed with 5 mL of 3.0 mol/L NaCl, 50 mmol/L sodium borate, pH 8.9, and then 5 mL of 3.0 mol/L NaCl, 10 mmol/L sodium borate, pH 8.9, and the antibody was eluted with 5 mL of 0.1 mol/L H3PO4/NaOH, pH 2.5. Fractions (1 mL) were collected, and the protein content was estimated by absorbance at 280 nm. The eluate was dialyzed against cold phosphate-buffered saline for 24 h.
EUROPIUM LABELING OF MONOCLONAL ANTIBODY
Purified anti-LAMP-2 monoclonal antibody was labeled with Eu$^{3+}$, using the DELFIA® labeling kit (Wallac), and purified on a Pharmacia Superose 12 Fast Phase Liquid Chromatography column (1.5 × 30 cm) as described previously (12). The concentration of Eu$^{3+}$ conjugated to each antibody molecule was determined from protein mass and fluorescence output of the conjugate.

IMMUNOQUANTIFICATION OF LAMP-2

One-step assay. Microtiter plates (Immuno 4, Dynatech Laboratories, Inc.) were coated overnight at 4 °C with anti-LAMP-2 polyclonal antibody diluted in 0.1 mol/L NaHCO$_3$ (5 mg/L, 100 μL/well) and then prewashed (1 time) in DELFIA wash buffer. Samples were diluted in DELFIA assay buffer and applied to each well in a total volume of 100 μL containing 250 μg/L Eu$^{3+}$-labeled anti-LAMP-2 monoclonal antibody. The plates were shaken for 10 min and incubated overnight at 4 °C. The plates were then washed with DELFIA wash buffer (6 times), and 200 μL of DELFIA enhancement solution was added. The plates were shaken for 10 min at room temperature, and the fluorescence was read on a 1234 DELFIA Research Fluorometer.

Determination of LAMP-2 in blood spots was performed using the one-step method with the following modifications. Microtiter plates were coated as described above; blood spots were then added to the wells with 200 μL of assay buffer containing 250 μg/L Eu$^{3+}$-labeled anti-LAMP-2 monoclonal antibody. The plates were shaken at room temperature for 1 h before being incubated overnight at 4 °C. The plates were then washed, enhancement solution was added, and the fluorescence measured as described.

Two-step assay. Samples containing chemicals that were incompatible with the Eu$^{3+}$ label, such as EDTA or citrate, were assayed using the two-step method. Plates were coated with polyclonal antibody as described for the one-step assay; samples were diluted in DELFIA assay buffer (100 μL/well), but without the addition of Eu$^{3+}$-labeled monoclonal antibodies, and incubated overnight at 4 °C. The plates were then washed (6 times), and 100 μL of assay buffer containing 250 μg/L of Eu$^{3+}$-labeled anti-LAMP-2 monoclonal antibody was added to each well; the plates were then incubated overnight at 4 °C. After an additional wash (6 times), DELFIA enhancement solution was added, the plates were shaken for 10 min, and the fluorescence was measured. LAMP-2 concentrations were calculated using Multicalc Data Analysis software (Wallac).

PREPARATION OF CALIBRATORS
Calibrators for immunoquantification of LAMP-2 were prepared using purified LAMP-2 protein (13). The purified LAMP-2 protein was diluted in DELFIA assay buffer to obtain a final concentration of 100, 50, 25, 12.5, 6.3, and 3.1 μg/L. When plasma samples (0.5 μL) were assayed, calibrators were supplemented with 0.5 μL/well of unaffected plasma to control for the inhibitory effects of plasma on the assay. Assays for the quantification of LAMP-2 in blood spots utilized blood spot calibrators to give equivalent elution of LAMP-2 from the filter paper for all samples. Blood spot calibrators were prepared by adding 10, 5, 2.5, 1.25, 0.63, or 0.31 ng of LAMP-2 per 3.5 μL of whole blood. The blood samples with LAMP-2 added were then spotted onto filter paper cards (Guthrie cards) and allowed to dry, and 3-mm spots were punched out for the assay. Three controls containing low (3.5 ng), medium (6.0 ng), and high (13.5 ng) LAMP-2/blood spot were prepared by adding purified LAMP-2 to whole blood.

Liquid calibrators and controls were stored at 4 °C, and Guthrie cards containing calibrators or controls were stored at −20 °C in a sealed bag containing silica gel. Each microtiter plate of assays contained both calibrators and controls. Calibrators were assayed in duplicate at the beginning of each plate, and controls (low, medium, and high) were assayed singly and dispersed in different positions on the plate. All unknown plasma samples were assayed in duplicate, and unknown blood spots were assayed singly; any sample needing a repeat was assayed in duplicate. All plasma LAMP-2 results were evaluated using Multicalc Data Analysis software, and blood spot results were calculated using linear regression.

FRACTIONATION OF WHOLE BLOOD
Peripheral blood leukocytes and plasma were isolated from whole blood collected in heparin-containing tubes by the method of Kampine et al. (22); the white cell pellet was resuspended in 0.15 mol/L NaCl containing 10 mL/L Nonidet P-40 (lysis solution). Red cells isolated in the same procedure were washed twice with 0.15 mol/L NaCl before being resuspended in lysis solution. White cells in the 0.15 mol/L NaCl washes were collected by centrifugation and combined with the original white cell fraction. The supernatants were pooled with the plasma for determination of LAMP-2 protein.

SODIUM DODECYL SULFATE-GEL ELECTROPHORESIS
Purified LAMP-2 and anti-LAMP-2 monoclonal and polyclonal antibodies were analyzed by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis according to Laemmli (23) and silver stained (24).

RESULTS

IMMUNOQUANTIFICATION OF LAMP-2
Purified LAMP-2 protein was shown to be >95% pure by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and was used as a calibrator in all assays. Labeling of the purified monoclonal antibodies with Eu$^{3+}$ yielded a 76% recovery with 18 Eu$^{3+}$ atoms per antibody molecule.

Optimization of the assay conditions was performed by generating a series of calibration curves, using various
Reagent concentrations or incubation times. Factors that were optimized included the concentration of primary polyclonal antibody and secondary Eu³⁺-labeled monoclonal antibody and incubation times. In all cases, the calibration curves showed a general trend of increasing signal with increasing antibody concentration or incubation time. The final assay conditions chosen were a compromise between consumption of reagents, assay time, and signal intensity.

The immunoquantification of purified LAMP-2 by either the one-step or two-step assay gave typical calibration curves with a linear response in the lower regions but showing signs of saturation of the antibody at higher LAMP-2 concentrations (Fig. 1). However, when plasma was the source of LAMP-2 (Fig. 2) the linear response range was less than observed that for the purified LAMP-2 calibration curve. The maximum signal from the plasma calibration curve (50 000 fluorescence units; Fig 2) was also reduced compared with the LAMP-2 curve with aqueous calibrators, which is still increasing at 100 000 fluorescence units after a 2-h secondary antibody incubation (Fig. 1). When a fixed quantity (2.5 ng) of purified LAMP-2 protein was added to each well in the plasma calibration curve, there was a marked inhibition (Fig. 2), which approximately balanced the contribution of the plasma LAMP-2 and thus was dependent on plasma concentration. Similarly, when the LAMP-2 calibration curve was constructed with 0.5 μL of plasma, there was a reduction of -50% in the fluorescence signal (Fig. 3).

We hypothesized that the inhibition observed was attributable to the action of proteases present in plasma digesting the LAMP-2. This was not the case, however, because the addition of a combination of protease inhibitors (0.5 mg/L leupeptin, 1 mmol/L pepstatin, 85 mg/L phenylmethylsulfonyl fluoride, or 1 mmol/L EDTA) to the calibrators did not prevent inhibition. Addition of human albumin (50 g/L) and gamma globulin (10 g/L) to aqueous calibrators at physiological concentrations had no effect on the signal. A fractionated plasma sample was prepared that had the IgG fraction removed on a protein G column and was then bound and eluted from a Concanavalin A column, thereby removing all of the albumin present. An amount equivalent to 0.5 μL of unaffected plasma (minus IgG and albumin) was added to wells containing LAMP-2 calibrators. The result showed the same inhibitory effect on the calibration curve as was seen
with the unfractionated plasma. This inhibition phenomenon was not characterized further; however, 0.5 \( \mu \text{L} \) of plasma was added to calibrators in all subsequent assays when 0.5-\( \mu \text{L} \) plasma samples were being measured.

**LAMP-2 Concentrations in Plasma**

Plasma samples from 202 control subjects (median age, 7 years; range, 0–66 years) and 312 LSD-affected individuals, representing 25 different disorders, were measured (Table 1). The LAMP-2 concentrations in these samples showed a tight distribution in the healthy population, with a median of 1.21 mg/L and the 5th and 95th percentiles at 0.77 and 1.82 mg/L, respectively (Fig. 4). The majority of the LSD-affected individuals had LAMP-2 concentrations that were above the 95th percentile of the control population, with some individuals having up to ninefold more than the median concentration of the control population (Table 1).

The intraassay imprecision in these assays was determined by repetitive measurement (20 times) of three control samples that contained low (6.3 mg/L), medium (25 mg/L), and high (100 mg/L) concentrations of LAMP-2 and was determined to be 4%, 2%, and 8%, respectively. The interassay imprecision was determined by comparing the controls between 20 assays performed on 10 different days. The CV in this instance was 12%, 10%, and 4% for the low, medium, and high controls, respectively.

There was no significant correlation between the LAMP-2 concentrations and age in the control population. Because the same plasma samples were previously assayed for LAMP-1 (12), we were able to directly compare the LAMP-1 and LAMP-2 concentrations. We observed a statistically significant Pearson correlation coefficient \((P < 0.01, \text{two-tailed})\) between the LAMP-1 and LAMP-2 in both the control and LSD-affected plasma samples (Fig. 5). In addition, the LAMP-2 concentrations in the plasmas of the control and LSD-affected samples were approximately fourfold higher than the corresponding LAMP-1 concentrations.

### Table 1. Median LAMP-2 concentrations in plasma of patients with 25 LSDs compared with nondiseased plasma.

<table>
<thead>
<tr>
<th>Disorder</th>
<th>n(^a)</th>
<th>Age(^b)</th>
<th>LAMP-2(^c)</th>
<th>Concentration 95th percentile(^d)</th>
<th>LAMP-2/LAMP-1(^e)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>202</td>
<td>7 (0–66)</td>
<td>1.21</td>
<td></td>
<td>4.74</td>
</tr>
<tr>
<td>Fabry disease</td>
<td>23</td>
<td>27 (4–47)</td>
<td>0.98</td>
<td>0</td>
<td>3.05(^e)</td>
</tr>
<tr>
<td>Galactosialidosis</td>
<td>1</td>
<td>16</td>
<td>8.77</td>
<td>100</td>
<td>5.31</td>
</tr>
<tr>
<td>Gaucher disease</td>
<td>51</td>
<td>12 (0–68)</td>
<td>3.31</td>
<td>92</td>
<td>4.12(^e)</td>
</tr>
<tr>
<td>GM1-gangliosidosis</td>
<td>12</td>
<td>1 (0–15)</td>
<td>2.85</td>
<td>75</td>
<td>2.72(^e)</td>
</tr>
<tr>
<td>I-cell disease</td>
<td>15</td>
<td>3 (0–25)</td>
<td>5.33</td>
<td>100</td>
<td>2.83(^e)</td>
</tr>
<tr>
<td>Krabbe disease</td>
<td>12</td>
<td>0.4 (0–1)</td>
<td>1.33</td>
<td>8</td>
<td>3.45(^e)</td>
</tr>
<tr>
<td>(\alpha)-Mannosidosis</td>
<td>4</td>
<td>4 (3–15)</td>
<td>3.29</td>
<td>100</td>
<td>2.83(^e)</td>
</tr>
<tr>
<td>Metachromatic leukodystrophy</td>
<td>31</td>
<td>3 (0–30)</td>
<td>1.48</td>
<td>16</td>
<td>4.00(^e)</td>
</tr>
<tr>
<td>Mucopolysaccharidosis I</td>
<td>18</td>
<td>1 (0–29)</td>
<td>4.34</td>
<td>100</td>
<td>3.13(^e)</td>
</tr>
<tr>
<td>Mucopolysaccharidosis II</td>
<td>23</td>
<td>3 (0–11)</td>
<td>4.65</td>
<td>100</td>
<td>3.40(^e)</td>
</tr>
<tr>
<td>Mucopolysaccharidosis IIIA</td>
<td>19</td>
<td>4 (1–17)</td>
<td>4.19</td>
<td>100</td>
<td>4.03(^e)</td>
</tr>
<tr>
<td>Mucopolysaccharidosis IIIB</td>
<td>16</td>
<td>3 (2–21)</td>
<td>3.75</td>
<td>94</td>
<td>4.12(^e)</td>
</tr>
<tr>
<td>Mucopolysaccharidosis IIIC</td>
<td>3</td>
<td>11 (6–20)</td>
<td>3.10</td>
<td>100</td>
<td>4.36</td>
</tr>
<tr>
<td>Mucopolysaccharidosis IIID</td>
<td>3</td>
<td>3 (0–3)</td>
<td>3.69</td>
<td>100</td>
<td>3.27(^e)</td>
</tr>
<tr>
<td>Mucopolysaccharidosis IVA</td>
<td>17</td>
<td>3 (0–12)</td>
<td>2.88</td>
<td>88</td>
<td>4.74</td>
</tr>
<tr>
<td>Mucopolysaccharidosis VI</td>
<td>10</td>
<td>4 (0–16)</td>
<td>2.72</td>
<td>80</td>
<td>2.54(^e)</td>
</tr>
<tr>
<td>Multiple sulfatase deficiency</td>
<td>1</td>
<td>5</td>
<td>1.55</td>
<td>0</td>
<td>3.47</td>
</tr>
<tr>
<td>Niemann-Pick disease (A &amp; B)</td>
<td>10</td>
<td>22 (1–44)</td>
<td>1.36</td>
<td>20</td>
<td>3.86(^e)</td>
</tr>
<tr>
<td>Niemann-Pick disease (C)</td>
<td>10</td>
<td>12 (0–41)</td>
<td>1.32</td>
<td>10</td>
<td>3.61(^e)</td>
</tr>
<tr>
<td>Pompe disease</td>
<td>4</td>
<td>0.4 (0–1)</td>
<td>1.27</td>
<td>0</td>
<td>3.02(^e)</td>
</tr>
<tr>
<td>Sandhoff disease</td>
<td>6</td>
<td>1 (0–1)</td>
<td>1.25</td>
<td>0</td>
<td>2.21(^e)</td>
</tr>
<tr>
<td>Sialic acid storage disease</td>
<td>2</td>
<td>2 (0–3)</td>
<td>2.89</td>
<td>100</td>
<td>2.80(^e)</td>
</tr>
<tr>
<td>Tay-Sachs disease</td>
<td>17</td>
<td>1 (0–27)</td>
<td>0.90</td>
<td>0</td>
<td>2.77(^e)</td>
</tr>
<tr>
<td>Tay-Sachs disease (AB)</td>
<td>2</td>
<td>7 (6–8)</td>
<td>1.06</td>
<td>0</td>
<td>1.82(^e)</td>
</tr>
<tr>
<td>Wolman disease</td>
<td>2</td>
<td>1 (0–10)</td>
<td>1.41</td>
<td></td>
<td>3.075</td>
</tr>
</tbody>
</table>

\(^a\) n, number of individuals.

\(^b\) Median age (range) of patients in each group, in years.

\(^c\) Median LAMP-2 concentrations in plasma (mg/L).

\(^d\) Percentage of each disorder group with LAMP-2 concentrations above the 95th percentile of the control population (1.82 mg/L).

\(^e, f\) Mean values of the LAMP-2 to LAMP-1 ratio for each disorder. Significant two-tailed \(P\) values from the Mann–Whitney test comparing ratio values from each disorder group with the control group: \(^*\) \(P < 0.01\) and \(^\dagger\) \(P < 0.05\).
LAMP-2 assay and the previously established LAMP-1 assay (12). To control for the inhibitory effect of plasma on the calibration curve, blood spot calibrators for both LAMP-1 and LAMP-2 were prepared as described in Materials and Methods. A total of 1600 individual blood spots were assayed for LAMP-1 and LAMP-2. The calibration curves were subjected to linear regression analysis, and concentrations of samples were determined directly from the resulting regression line. Fig. 6 shows a calibration curve for LAMP-2 blood spots. All plates were assayed with appropriate internal controls, and the intra- and interassay variation over the 4-week period were determined as described for the plasma assays. The CV for the intraassay variation was 9%, 20%, and 16% for the low (3.5 ng/spot), medium (6.0 ng/spot), and high (13.5 ng/spot) controls. The interassay variation was 18%, 17%, and 15% for the same controls. These variations were considered acceptable when compared to current screening tests performed in the Newborn Screening Laboratory. The elution of LAMP from blood spots was also monitored over this period by regularly assaying blood spot replicates from the same Guthrie card. No change in the concentrations of LAMP eluted were observed.

Both LAMP-1 and LAMP-2 concentrations from blood spots from newborns showed characteristic skewed distributions when compared with the theoretical gaussian distribution (Fig. 7). LAMP-1 gave a median concentration of 0.33 mg/L, with the 5th and 95th percentiles at 0.20 mg/L [0.60 multiples of median (MOM)] and 0.54 mg/L (1.61 MOM), respectively. LAMP-2 showed a slightly broader distribution than LAMP-1, with a median of 1.62 mg/L and 5th and 95th percentiles of 0.95 mg/L (0.58 MOM) and 3.06 mg/L (1.88 MOM), respectively. We also compared the LAMP concentrations with age, sex, and birth weight and found no significant correlation. However, when LAMP-1 and LAMP-2 concentrations from the same blood spots were correlated, a significant Pearson correlation coefficient was obtained ($P < 0.01$, two-tailed).

Whole blood from six healthy adults were each fractionated into plasma, white cells, and red cells, and the proportion of LAMP-2 in each component was determined. Whole blood samples had an average of 2.66

---

**Fig. 4.** Box plot of LAMP-2 concentrations in plasma from control and LSD-affected individuals. LAMP-2 concentrations were determined using 0.5-μL plasma samples in the two-step assay. N, number of samples in each group. Shaded areas represent the 25th and 75th percentiles, and bars show the upper and lower limits. ◦ denotes outliers, and ✹ represents extreme outliers. GM1, GM1 gangliosidosis; MLD, metachromatic leukodystrophy; MPS, mucopolysaccharidosis; MSD, multiple sulfatase deficiency; NP, Niemann-Pick disease; SAS, sialic acid storage disease; and TSD, Tay-Sachs disease.

**Fig. 5.** Correlation of LAMP-1 and LAMP-2 from control and LSD-affected plasma samples. Pearson correlation coefficient, $P < 0.01$, two-tailed.

**Fig. 6.** Calibration curve for LAMP-2 in blood spots, using the one-step assay. Microwell plates were coated with 5 mg/L anti-LAMP-2 polyclonal antibody for 4 h at 37 °C. LAMP-2 blood spot calibrators were assayed using the one-step assay with 250 μg/L Eu$^{3+}$-labeled anti-LAMP-2 monoclonal antibody for 2 h.
mg/L LAMP-2, with the distribution being 70\% \pm 1.8\% in the plasma, 21\% \pm 0.8\% in red cells, and 9\% \pm 2.1\% in white cells.

Discussion

LSDs comprise >40 known genetically inherited diseases that, like other inborn errors of metabolism, can lead to catastrophic health problems. In the absence of a family history, the only way in which these disorders can be presymptomatically detected is by newborn screening. Screening for LSDs can be justified both in terms of benefits to the child and family affected and in terms of the cost to the healthcare system. The maximum benefits, however, will be to the affected individual because early diagnosis will enable therapy to begin before development of irreversible pathology, thus maximizing efficacy.

The lysosome-associated membrane protein LAMP-2 was evaluated in this study following the report that the related protein, LAMP-1, was increased in many, but not all, LSDs (12). Because LAMP-2 is also found principally in the lysosomal membrane, it was also expected to be increased in association with the increased lysosomal volume characteristic of LSDs (12). LAMP-1 and LAMP-2 are structurally similar proteins; however, these proteins have been reported to be differentially regulated. We therefore hypothesized that the difference in regulation between LAMP-1 and LAMP-2 may be reflected as differences in plasma concentrations in different disorders and that LAMP-2 may be a better or complementary diagnostic marker for LSDs.

It was noted previously in the LAMP-1 assay (12) that there was an interaction of LAMP-1 with plasma proteins, which caused a slight decrease in the measured concentration of exogenous LAMP-1 that was added to plasma. We investigated this effect for LAMP-2 and observed a similar but more pronounced reduction in signal of almost 50\%.

We were not able to identify the particular component causing the inhibitory effect; however, we were able to confirm that it was not because of the action of proteases or binding of the major plasma proteins, albumin and gamma globulin. Therefore, some other component of plasma is interfering with antibody binding. LAMP proteins on the plasma membrane have been previously reported to bind to endothelial cells expressing P- or E-selectins (25). Although this interaction occurs on the cell surface, both P- and E-selectins are also found in a biologically active form in circulation (26). This suggests that some of the LAMP proteins present in plasma may be interacting with the soluble selectins or possibly other, as yet unidentified, plasma proteins.

The mean concentration of LAMP-2 in unaffected plasma was 1.21 mg/L, fourfold higher than LAMP-1, which was 0.31 mg/L. Although little is known about the function of LAMP-2, it has been implicated as having a role during inflammatory responses (19), mediating lymphocyte adhesion to the vascular endothelium by interacting with P- and E-selectins (18, 26), and as protecting the lysosomal membrane from intralysosomal degradation (17).

Although we were able to detect LAMP-2 in plasma, how it is released from the cells and its form in plasma has not been determined. Jadot et al. (27) reported the presence of a soluble (not membrane-bound) pool of LAMP-2 in purified rat liver lysosomes, which suggests that LAMP-2 can exist in more than one form.

We determined that \sim 70\% of the LAMP-2 in blood is present in the plasma, suggesting that the secretion of LAMP is by a selective mechanism and not entirely attributable to the processes of cellular apoptosis or turnover. As yet, there have been no reports indicating how these membrane proteins are secreted into plasma. Aumuller et al. (28) showed by immunoelectron microscopy and Western blotting that a high amount of LAMP-2 was found in so-called prostasomes and secreted in the male genital system. Their study indicated that most of
the membrane-bound LAMP-2 is released from the secretory cells in an apocrine fashion. It is also possible that LAMP molecules may be proteolytically cleaved from the plasma membrane and released into circulation because this mechanism is known for other surface proteins, such as E-selectins (29).

To determine the potential of LAMP-2 as a screening marker for LSDs, we measured the concentrations of LAMP-2 in plasma samples from 312 affected individuals representing 25 different LSDs and compared these with 202 control samples. When considered as a group, ~66% of LSD-affected individuals had LAMP-2 plasma concentrations higher than the 95th percentile of the control population. However, the increase in LAMP-2 was only seen in specific disorders: 14 of the 25 disorder groups tested had >75% of individuals above the 95th percentile of the reference range, with 9 groups having 100% above.

The significant correlation between the amount of LAMP-1 and LAMP-2 in blood spots from newborns and plasma from both unaffected and LSD-affected individuals demonstrates that these are not independent variables and, as such, that there would be no advantage to screening for both markers. LAMP-2 is present in higher concentrations in circulation and therefore offers the advantage of easier detection. However, LAMP-1 showed an increase in 17 of the 25 disorders tested (72% of patients), whereas LAMP-2 showed an increase in only 14 of the same disorders (66% of patients). The lower concentration of detection with LAMP-2 may result from the tighter distribution in the control population, observed with LAMP-1 (12) compared with LAMP-2. The final selection between LAMP-1 and LAMP-2 will be dependent on what other diagnostic markers are identified and the overlap between the disorders that are detected with the LAMP markers.

The degree of LAMP-2 increase, observed in the different disorders, may be related to several factors, including the type of material stored, the amount of storage, and the site of storage. This has been discussed previously (12); however, it is still unknown why LAMP-1 and LAMP-2 are increased in some disorders and not others. It is probable that several factors play a role, including the tissues involved in pathology, the type of storage material, and the variability in phenotype.

The distributions of both LAMP-1 and LAMP-2 in the unpartitioned newborn population were characteristically skewed towards the high concentrations; this will produce a large number of false positives arising from the primary screen. We propose that the top 1–5% of the population would then be examined further (using the same Guthrie card) with a panel of second-tier diagnostic assays designed to detect the storage product for the particular disorder involved. This would effectively identify the affected individuals from the false positives identified in the first-tier screen. We are currently developing the second-tier screening assays for this procedure, utilizing tandem mass spectrometry.

We gratefully acknowledge the staff of the National Referral Laboratory headed by Bill Carey for supplying plasma samples from LSD-affected individuals. We also thank Elaine Ravenscroft for assistance with tissue culture and Rosemarie Gerace and Bronwen Bartlett for providing and assisting with the screening of blood spots. We are very grateful to Enzo Ranieri for his helpful discussions relating to the immunoquantification of LAMP-2 using time-resolved fluorescence. This work was supported by the University of South Australia, the Channel 7 Children’s Research Foundation, the Australian Research Council, the National Health and Medical Research Council of Australia, and the Swedish Medical Research Council (03X-07886).

References


