Comparison of radioimmunoprecipitation with luciferase immunoprecipitation for autoantibodies to GAD65 and IA-2β

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Objective: To compare the sensitivity and specificity of luciferase immunoprecipitation (LIPS) with radioimmunoprecipitation (RIP) for the measurement of autoantibodies to the type 1 diabetes (T1D) autoantigens glutamic acid decarboxylase 65 (GAD65) and IA-2ß.

Research Design And Methods: Sera from 49 T1D patients and 100 non-diabetic controls from DASP-2007 were used to screen for autoantibodies to GAD65. An additional 200 T1D patients and 200 non-diabetic controls were used to validate the GAD65 results and screen for autoantibodies to IA-2ß.

Results: LIPS showed equal sensitivity and specificity to RIP for detecting autoantibodies to GAD65 and IA-2ß. Receiver operating characteristic analysis revealed that the detection of autoantibodies to GAD65 and IA-2ß by LIPS and RIP were not statistically different.

Conclusion: The LIPS assay does not require the use of radioisotopes or in vitro transcription/translation and is a practical alternative at the clinical level for the RIP assay.
Autoantibodies to GAD65, IA-2 and IA-2β are major diagnostic and predictive markers in T1D (1, 2). Autoantibodies to these proteins appear years before the development of clinical disease and in combination with certain HLA haplotypes, are being used to enter subjects into therapeutic intervention trials (3). The RIP assay has been used extensively to detect these autoantibodies. Recently, we showed that LIPS displayed equal sensitivity and specificity to RIP for detecting IA-2 autoantibodies (4). The present experiments were initiated to see whether LIPS could be used to measure autoantibodies to GAD65 and IA-2β with a sensitivity and specificity equal to that of RIP.

RESEARCH DESIGN AND METHODS

For the LIPS assay, full-length GAD65 or the intracellular portion of IA-2β (aa 662-1033) (5) was cloned into the pREN2 vector downstream of the Renilla luciferase reporter and extracts were prepared from transfected Cos1 cells as described (4-6). For the RIP assay, GAD65 and IA-2β were cloned into pTNT and pGBK7 vectors, respectively, and the [35S] methionine-labelled proteins were produced by in vitro transcription/translation (7). Autoantibodies to GAD65 and IA-2β were detected by liquid phase immunoprecipitation using 1.0 × 10^7 light units (LU) of cell extracts in LIPS and approximately 40,000 counts per minute (cpm) of radio labeled protein for RIP.

Sera from 100 controls and 49 T1D patients was obtained from the 2007 Diabetes Antibody Standardization Program (DASP) (8) and used to measure autoantibodies to GAD65. In the 2007 serum exchange, sensitivity and specificity for autoantibodies to GAD65 were 82% and 96%, respectively. Controls from the DASP included some samples with high levels of islet autoantibodies, presumably because they were from subjects who were at high risk of developing T1D. Neither the DASP patients with T1D nor the controls are representative of the T1D population or the general public. Additional sera from 200 age-matched non-diabetes controls and 200 T1D (Malmö Diabetes Study) (9) were used to validate the GAD65 findings and measure autoantibodies to IA-2β. A serum was positive if the precipitated cpm or LU exceeded the mean + 3 SD of the controls. MedCalc Software (Mariakerke, Belgium) was used for statistical analyses. Signal-to-noise ratios of autoantibodies for RIP and LIPS in the T1D samples were determined as described (10).

RESULTS

Anti-GAD65 autoantibodies determined by LIPS showed that only 3 of the 100 non-diabetic controls were positive (Fig. 1A). In contrast, 77.6% (38/49) of the sera from patients with T1D were positive by LIPS with a specificity of 97%. In RIP, 5 of the 100 non-diabetic controls were autoantibody positive, whereas 77.6% (38/49) of the sera from subjects with T1D were autoantibody positive (Fig. 1B), with a specificity of 95%. The coefficient of variation (CV) for duplicate samples was 13.5% for LIPS and 3.1% for RIP (Supplemental Fig. 1A). Comparison of the assays revealed a high coefficient of determination with an \( R^2 \) of 0.778 (Supplemental Fig. 1C). Receiver operating characteristic (ROC) analysis showed that the area under the curves for autoantibodies to GAD65 by LIPS and RIP were not statistically different (\( p=0.592 \)) (Fig. 1E). Validation studies for GAD65 autoantibodies with 200 T1D sera revealed 53.5% positivity by LIPS and 49.3% positivity by RIP (data not shown). LIPS profiling of IA-2β autoantibodies revealed that none of the 200 non-diabetic control sera were positive (Fig. 1C). In contrast, 62.5% (125/200) of T1D subjects were autoantibody positive with a specificity of 100%. In RIP, 3 of 200 non-diabetic control sera were positive, whereas 51.0% (102/200) of the sera from subjects with T1D were autoantibody positive (Fig. 1D) with a 97.5% specificity. The CV for duplicate samples was 10.9% for LIPS and 6.8% for RIP (Supplemental Fig. 1B). Comparison of the assays revealed a high coefficient of
determination with an $R^2$ of 0.904 (Supplemental Fig. 1D). ROC analysis showed that the area under the curves for autoantibodies to IA-2$\beta$ by LIPS and RIP were not statistically different ($p=0.062$) (Fig. 1F). However, the signal-to-noise ratio for detecting autoantibodies to GAD65 and IA2-\(\beta\) was higher as determined by LIPS than by RIP (Fig. 1A-D and Supplemental Fig 1E and 1F).

**CONCLUSION**

In non-diabetic subjects, the presence of autoantibodies to more than one of the major diabetes-associated autoantigens is a better predictor of the development of clinical diabetes than the presence of any single autoantibody (3). Initially, autoantibodies directed against islet cells (ICA) were detected by immunofluorescence. In recent years, the ICA technique has been replaced by the quantitative RIP assay. In the present paper on autoantibodies to GAD65 and IA-2\(\beta\) and in our recent paper on autoantibodies to IA-2 (4), we showed that the liquid phase LIPS assay is equal in sensitivity and specificity to the liquid phase RIP assay, that the two assays have a high correlation coefficient, and that by ROC analysis the areas under the curves are not statistically different.

Although further documentation is needed, our current study shows that the sensitivity for detecting autoantibodies to IA-2\(\beta\) by LIPS is not only equal to that of RIP, but may be slightly higher (62.5% vs. 51.0%). Also, the signal-to-noise ratio is higher for LIPS compared to RIP, but the clinical significance of the very high autoantibody-positive sera detected in LIPS is not known at this time. Moreover, we have no concrete evidence that the LIPS assay is more reliable or sensitive than the RIP assay for discriminating borderline autoantibody positive and negative sera. It is the failure to reproducibly distinguish between positive and negative signals at the borderline that is responsible for much of the variation in routine autoantibody assays.

Although LIPS and RIP appear to be equal in sensitivity and specificity, LIPS has the advantage for a clinical laboratory of not requiring the use of radioisotopes; avoids the time and expense of *in vitro* transcription/translation; and offers the potential of detecting mammalian cell post-translational modifications which would not be found in a bacterial expression systems or by *in vitro* transcription/translation. Use of a mixture of *Renilla* luciferase-tagged antigen with firefly luciferase-tagged antigen may also allow detection of autoantibodies to two different autoantigens at the same time.

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**Legend to Figures**

**Figure 1.** GAD65 autoantibodies as determined by LIPS (A) and RIP (B) and IA-2\(\beta\) autoantibodies as determined by LIPS (C) and RIP (D). Dotted lines represent 3 SD above the mean of the non-diabetic control sera. (E) ROC analysis showing the area under the curve for GAD autoantibodies by LIPS (0.929; 95% CI 0.875-0.964) and by RIP (0.941; 95% CI 0.891-0.973). There was no statistical difference ($p=0.592$). (F) ROC analysis showing the area under the curve for IA-2\(\beta\) autoantibodies by LIPS (0.844; 95% CI 0.804–0.879) and by RIP (0.807; 95% CI 0.763–0.849). There was no statistical difference ($p=0.062$).
REFERENCES