

A New Luminescence Assay for Autoantibodies to Mammalian Cell-Prepared Insulinoma-Associated Protein 2

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OBJECTIVE — Insulinoma-associated protein 2 (IA-2) is a major autoantigen in type 1 diabetes, and IA-2 autoantibodies are routinely detected by a liquid-phase radioimmunoprecipitation assay. The present experiments were initiated to develop a new assay that does not require the use of radioisotopes or autoantigens prepared in bacteria or by in vitro transcription/translation.

RESEARCH DESIGN AND METHODS — IA-2 luciferase fusion protein was expressed in mammalian cells and assayed for autoantibodies by liquid-phase luciferase immunoprecipitation.

RESULTS — Our study showed that there was no significant difference between the luciferase immunoprecipitation and the radioimmunoprecipitation assays in sensitivity and specificity, and comparison of the two assays revealed a high correlation coefficient ($R^2 = 0.805$).

CONCLUSIONS — The luciferase system offers a robust, inexpensive, nonradioactive method for the detection of autoantibodies to mammalian cell-prepared IA-2 and could be of practical value at the clinical level.

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Insulinoma-associated protein 2 (IA-2) is a major autoantigen in type 1 diabetes (1). IA-2 autoantibodies appear years before the development of clinical disease and, in combination with autoantibodies to GAD and insulin, are used as predictive markers (2). The liquid-phase radioimmunoprecipitation (RIP) assay is a highly sensitive and reproducible assay for IA-2 autoantibodies. Luminescence, using recombinantly expressed proteins fused to luciferase (3–5), is an alternative to the radioactive assay. In the present ar-

ticle, we describe a liquid-phase luminescence immunoprecipitation system (LIPS) assay for IA-2 autoantibodies, in which we used IA-2 luciferase fusion protein expressed in mammalian cells rather than IA-2–radiolabeled protein produced by in vitro transcription/translation. Our study showed that LIPS offers a highly sensitive, robust, nonradioactive method for the detection of IA-2 autoantibodies in type 1 diabetes.

RESEARCH DESIGN AND

METHODS — In the LIPS assay, IA-2 (amino acids 601–979) was cloned into the pREN2 mammalian expression vector downstream of the Renilla luciferase (Ruc) reporter (3–5). Monkey kidney cells (Cos1) in a 100 mm² dish were transfected, and 48 h later, the cells were sonicated, clarified by centrifugation, and the extract (enough for ~1,500 assays in duplicate) used without further purification. Total luciferase activity in the extract was measured in a luminometer and adjusted so that each reaction contained $\sim 1.0 \times 10^7$ light-forming units per 0.1 μ l of extract. The extract was then incubated with 1.0 μ l of sera for 1 h at room temperature in a total volume of 100 μ l, transferred to 96-well filtration plates containing 7 μ l of 30% suspension of protein A/G beads (Pierce Biochem), and incubated for an additional hour. The A/G beads with the captured antibody–luciferase labeled IA-2 complexes were washed using a BioMek-FX workstation, Renilla luciferase substrate (Promega, Madison, WI) was added, and the light-forming units were determined in a Microplate Luminometer (Berthold Technologies, Bad Wildbad, Germany).

In the RIP assay, performed as previously described (6), the same intracellular portion of IA-2 used in the LIPS assay was cloned into a pGBKT7 vector (Clontech, Mountain View, CA), and the IA-2 protein was produced by in vitro transcription/translation in the presence of ³⁵S-methionine. IA-2 autoantibodies were detected by liquid-phase radioimmunoprecipitation using $\sim 20,000$ cpm of radiolabeled IA-2.

A total of 100 sera from patients with newly diagnosed type 1 diabetes who had been previously screened for IA-2 autoantibodies by RIP were selected on the basis of the counts precipitated, from very high to moderate and low to negative. A total of 100 sera from age-matched nondiabetic individuals served as the control (6). A serum was scored as positive if the precipitated cpm exceeded the mean + 3 SD of the control subjects.

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P.D.B. has filed a patent application based on LIPS technology.

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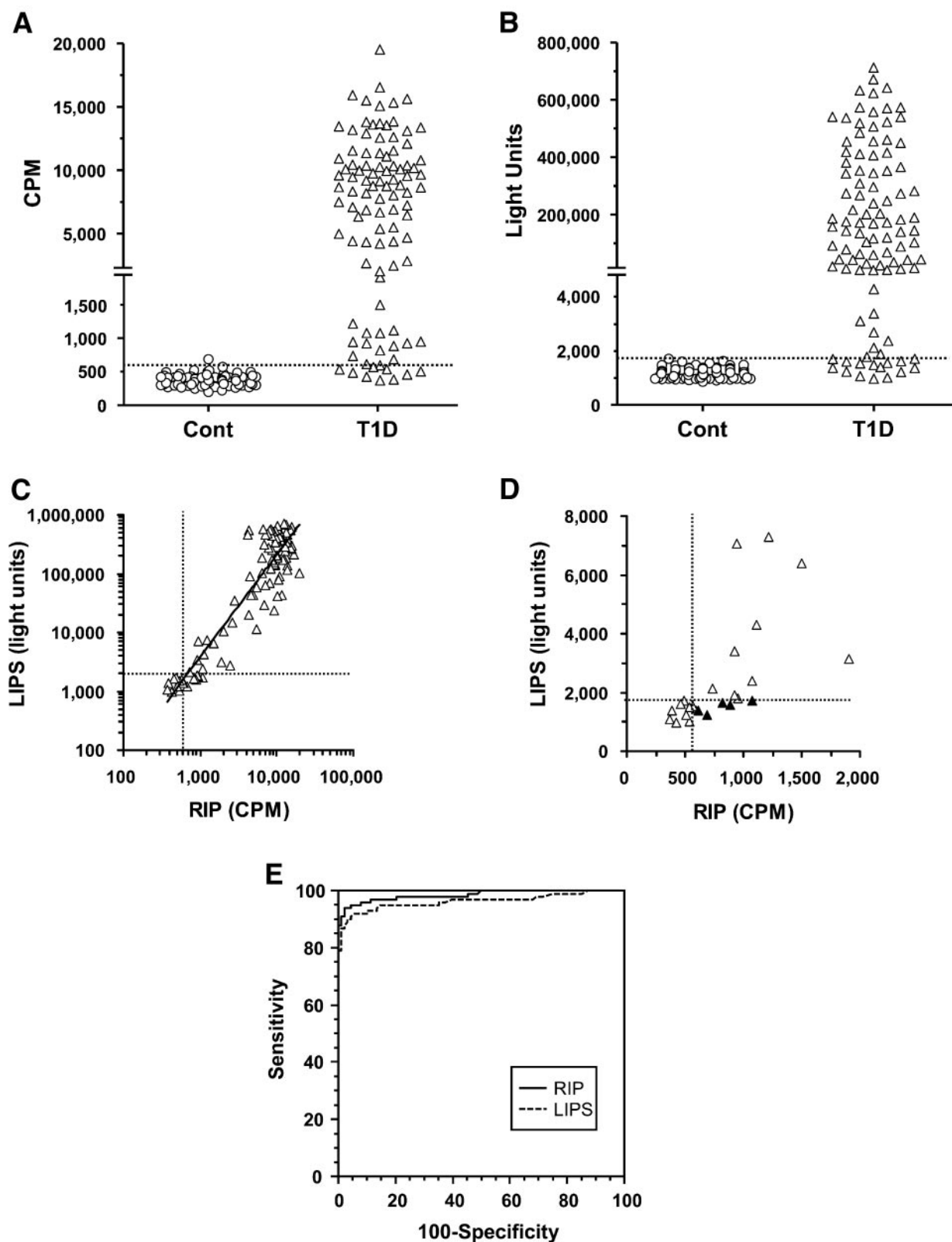


Figure 1—IA-2 autoantibodies as determined by RIP (A) and LIPS (B). C: Correlation between RIP and LIPS evaluated by the coefficient of determination ($R^2 = 0.805$). D: Comparison by RIP and LIPS of 25 sera at the borderline of positivity for IA-2 autoantibodies. Closed triangles represent the 5 sera that were determined to be positive by RIP but negative by LIPS. Dotted lines represent 3 SD above the mean of control subject sera. E: Receiver operating characteristic analysis showed that the area under the curve for IA-2 by RIP was 0.985 (95% CI 0.956–0.997) and by LIPS was 0.963 (95% CI 0.925–0.985). There was no statistical difference ($P = 0.120$).

RESULTS—Sera from diabetic ($n = 100$) and control subjects ($n = 100$) were screened for IA-2 autoantibodies by RIP assay. Only one of the control subject sera fell outside the 3 SD range, whereas 90% of the diabetic subject sera exceeded the 3 SD range of the control subject sera (Fig. 1A). The coefficient of variation (CV) for duplicate samples of the diabetic subject sera was 5.0%.

The same sera were screened for IA-2 autoantibodies by LIPS assay. None of the control subject sera fell outside the 3 SD range, whereas 85% of the diabetic subject sera exceeded the 3 SD range of the control subject sera (Fig. 1B). The CV of the diabetic subject sera was 9.3%.

Comparison of the RIP and LIPS assays revealed a high correlation, with a 0.805 coefficient of determination (R^2) (Fig. 1C). Evaluation of the 5 diabetic subject sera that were negative by the LIPS assay but positive by the RIP assay showed that 3 of the 5 negative sera were at the very borderline of positivity (Fig. 1D).

Receiver operating characteristic analysis showed that the areas under the curves for IA-2 by RIP and LIPS were not statistically different ($P = 0.120$) (Fig. 1E).

CONCLUSIONS—From these experiments, we concluded that there was no significant difference in sensitivity or specificity between the LIPS and RIP assays. However, the LIPS assay offers several potential advantages. First, because it uses luminescence rather than radioactivity, it lends itself better to most clinical laboratories and can be easily automated.

Second, because the preparation of the protein does not require in vitro transcription/translation, the protein can be prepared at a fraction of the cost of the RIP assay. Third, because the recombinant protein is fused with a luminescence marker, it does not need to be externally labeled or laboriously separated from the other proteins in the mammalian cell lysate, thereby eliminating the purification steps required for both bacteria-prepared proteins (7) used in solid-phase enzyme-linked immunosorbent assays. Fourth, because the proteins are made in mammalian cells, they may undergo a variety of processing and posttranslational modifications that do not occur when they are prepared via in vitro transcription/translation and, therefore, may more truly reflect the natural state of the autoantigen. It will be of interest to see if this increases detection and/or sensitivity when other autoantigens, prepared in mammalian cells, are screened for autoantibodies.

Our findings suggest that the LIPS assay may be of practical value at the clinical level for the detection of autoantibodies not only for diabetes, but also for other autoimmune diseases. However, as with all new assays, LIPS requires validation with a large number of sera and comparison with RIP in a Diabetes Autoantibody Standardization Program workshop.

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