

Association Between Rotavirus Infection and Pancreatic Islet Autoimmunity in Children at Risk of Developing Type 1 Diabetes

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Pancreatic islet autoimmunity leading to type 1 diabetes could be triggered by viruses in genetically susceptible individuals. Rotavirus (RV), the most common cause of childhood gastroenteritis, contains peptide sequences highly similar to T-cell epitopes in the islet autoantigens GAD and tyrosine phosphatase IA-2 (IA-2), suggesting T-cells to RV could trigger islet autoimmunity by molecular mimicry. We therefore sought an association between RV infection and islet autoantibody markers in children at risk for diabetes who were followed from birth. There was a specific and highly significant association between RV seroconversion and increases in any of these antibodies: 86% of antibodies to IA-2, 62% to insulin, and 50% to GAD first appeared or increased with increases in RV IgG or IgA. RV infection may therefore trigger or exacerbate islet autoimmunity in genetically susceptible children. *Diabetes* 49:1319–1324, 2000

Type 1 diabetes is an autoimmune disease that results from the destruction of pancreatic islet β -cells in genetically predisposed individuals. A large proportion of the lifetime risk of type 1 diabetes is attributed to environmental agents (1,2), but the only virus shown unequivocally to be responsible for clinical disease is rubella following infection in utero of infants bearing the HLA haplotype B8-(DR3-DQ2) (3). Circulating insulin

autoantibodies (IAA), GAD65 antibodies (GADAb), and tyrosine phosphatase IA-2 autoantibodies (IA-2Ab) are markers of islet autoimmunity that predict the T-cell-mediated destruction of β -cells (4–6). Peptides in these islet autoantigens that are recognized by T-cells may provide clues to environmental agents that trigger or exacerbate islet autoimmunity through the mechanism of molecular mimicry (7,8).

Recently, we identified T-cell epitope peptides in tyrosine phosphatase IA-2 (IA-2) restricted by HLA-DR4 in individuals at risk for type 1 diabetes (9,10). The dominant epitope (amino acid [aa] 805–820) contains a core 9-aa sequence (aa 41–49) within virus protein (VP)7 of rotavirus (RV) serotype G3 and to a lesser extent in the G1 and G2 serotypes. Because the T-cell contact residues in these similar IA-2 and RV VP7 sequences appear to be identical (9), the potential exists for molecular mimicry. Furthermore, just NH_2 -terminal of this region in VP7 is a 12-aa sequence (11) strongly similar to a sequence in GAD65 (aa 117–128) (9) that is a T-cell epitope in HLA-DR4 transgenic mice (12) and DR4-DQ8 homozygous at-risk humans (13). All human RV serotypes in the GenBank database contain the GAD-related sequence. VP7, the major outer capsid protein of RV, is an important determinant of virulence and induces virus-neutralizing antibodies (14). However, elimination of RV infection is predominantly due to T-cells (15). The CD4 T-cell epitopes in VP7 are unknown, but in C57/Bl 6 and BALB/c mice, CD8 T-cell epitopes (15,16) map adjacent to the IA-2- and GAD-like sequences, confirming that this signal sequence is a strongly immunogenic region. We suggested, therefore, that RV infection might simultaneously activate T-cells cross-reactive to 2 islet autoantigens. To identify a possible link between RV and islet autoimmunity, we looked for an association between RV infection and islet antibodies (Ab) in a population of genetically susceptible children.

RESEARCH DESIGN AND METHODS

Subjects. In the Australian BabyDiab Study, 360 children with a parent or sibling with type 1 diabetes had serum assayed every 6 months from birth for IAA, GADAb, and IA-2Ab. High-risk children who developed diabetes ($n = 5$, 2 male, mean age 29.5 ± 10.4 months) or had at least 2 islet Ab or 1 islet antibody detected on at least 2 occasions ($n = 19$, 11 male) were studied for evidence of RV infections. All these high-risk children were positive for the HLA class II type 1 diabetes susceptibility allele DR4, and 38% had the DR3 susceptibility allele. To compare the incidence of RV infections, these children were matched for age and HLA class II with a further 17 (9 male) unrelated children from the study who had no detectable islet Ab over the same period. To determine intrafamilial transmission of RV, 3 siblings (2 male) of the high-risk children and 10 siblings (7 male) of the lower-risk children were also studied. The studies were approved by Human Ethics Committees.

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Additional information can be found in an online appendix at www.diabetes.org/diabetes/appendix.asp.

aa, amino acid; Ab, antibodies; ANA, antibodies to nuclei; CBV, Coxsackie B virus; CBVM, IgM antibodies to the Coxsackie B virus; CV, coefficient of variation; EIA, enzyme immunoassay; GADAb, GAD antibodies; IA-2, tyrosine phosphatase IA-2; IA-2Ab, IA-2 antibodies; IAA, insulin autoantibodies; OR, odds ratio; ROC, receiver operator curve; RV, rotavirus; RVA, IgA antibodies to RV; RVG, IgG antibodies to RV; TPOAb, thyroid peroxidase antibodies; VP, virus protein.

Laboratory methods. Antibody assays were performed in duplicate and repeated if duplicates differed from each other by >15%.

IAA. IAA were assayed by polyethylene glycol precipitation of bound ¹²⁵I-labeled insulin (17), and results were expressed as percent of total counts. The control range <5.5% (mean + 2 SD) was derived from 190 healthy children (mean age 9.7 years, range 4.9–15.5). The maximum interassay coefficient of variation (CV) within the range of increased values was 16%.

GADAb and IA-2Ab. GADAb and IA-2Ab were each assayed by protein-A sepharose precipitation of ³⁵S-labeled methionine recombinant human proteins generated by *in vitro* transcription/translation (18,19). The control range for GADAb (<5 U) was below the 98.5th percentile and was derived by receiver operator curve (ROC) analysis of 246 control subjects and 135 newly diagnosed patients, 103 (76%) of whom were positive. The maximum interassay CV was 12%. The control range for IA-2Ab (<3 U) was below the 97th percentile and was derived by ROC analysis of 145 control subjects and 94 newly diagnosed patients, 72 (78%) of whom were positive. The maximum interassay CV was 20%. All 3 autoantibody assays have sensitivities and specificities of 100% in International Workshops and Proficiency Tests (18,19).

Rotavirus IgA and IgG Ab. RV Ab were measured initially at a serum dilution of 1:100 and then at 1:500 as necessary by direct enzyme immunoassay (EIA) (14,20). A human serum pool standard arbitrarily assigned to contain 20,000 U IgG antibodies to RV (RVG)/ml and 30,000 U IgA antibodies to RV (RVA)/ml was titrated in doubling dilutions on EIA plates (Maxisorp, Nunc, Roskilde, Denmark) and used to determine units per milliliter of RVG and RVA in test sera. The cutoff for RVA (241 U) was the mean + 2 SD of 25 cord sera from at-risk children. Because all cord sera have maternal RVG, the cutoff for RVG (550 U) was the mean + 2 SD of the lower value of paired sera collected from the same 25 children at 6 and 12 months of age, to allow for decay of transplacentally acquired RVG. The specificity of the assays has been established (14,20). The maximum interassay CV for both RVA and RVG was 20%.

Coxsackie B IgM Ab. IgM antibodies to the Coxsackie B virus (CBVM) were measured by EIA (21) in 41 paired consecutive 6-month serum samples from high-risk children, for which the second sample exhibited a significant increase in RVA or RVG and in 1 or more islet Ab. The assay detects homotypic responses in young children that become heterotypic, i.e., against multiple serotypes of the Coxsackie B virus (CBV), with increasing age (21). Sera were tested at optimal dilution (1:400) against pooled antigens from CBV serotypes 4 and 5, and positives were retested against individual antigens. The cutoff was the mean + 3 SD of 10 known negative serum samples per tray. Specificity was demonstrated by absence of CBVM in sera positive for Ab to Epstein-Barr, measles, mumps and hepatitis A viruses, and *Mycoplasma pneumoniae*, and for the rheumatoid factor.

Thyroid peroxidase antibodies. Thyroid peroxidase antibodies (TPOAb) were measured with the ELI Test anti-TPO kit (Henning, Berlin).

Anti-nuclear antibodies. Antibodies to nuclei (ANA) were measured with the HEP 2000 Fluorescent ANA-Ro Test System (Immunoconcepts, Sacramento, CA).

HLA typing. HLA-DR alleles were assigned by oligotyping using the 11th International Histocompatibility Workshop protocol. DNA extracted from 2 ml cord or peripheral blood was amplified using polymerase chain reaction with primers for DRB1. Alleles were assigned on the basis of the hybridization patterns obtained.

Statistical analysis. As per routine practice in virological assays (20), an antibody increase ≥ 2 interassay CVs in consecutive 6-month samples was considered to be significant. Thus, for RVA or RVG, an increase of $\geq 40\%$ was considered to be significant and indicative of infection during the preceding 6-month period. A significant increase in either RVA or RVG was taken to indicate RV infection because children at risk for type 1 diabetes may have impaired IgA responses (22,23), and RVG may not increase immediately if already high (24). Because an IgM response is usually transient after infection, any increase in CBVM above the mean + 3 SD of controls was taken to indicate infection. Significant increases in islet Ab were as follows: IAA $\geq 32\%$, GADAb $\geq 24\%$, and IA-2Ab $\geq 68\%$. To permit comparison between consecutive samples, undetectable Ab were given a score of 0.1. A significant increase was scored as 1; no significant increase was scored as 0 (Fig. 1; see RESULTS). At each time point, the data were reduced to a single comparison between islet autoimmunity (represented by any islet antibody) and RV Ab (represented by RVA or RVG). Concordance (1,1 or 0,0) or discordance (1,0 or 0,1) was then determined; for example, the results for child 1, sample 6 (Fig. 1) of IAA 0, GADAb 1, IA-2Ab 0, RVA 0, and RVG 1, would represent concordance (1,1), whereas the results for child 1, sample 4 of IAA 0, GADAb 0, IA-2Ab 0, RVA 0, and RVG 1, would represent discordance (0,1). The total numbers of concordant and discordant samples were then analyzed by the χ^2 test with Yates' correction to determine if there was an association between increased levels of islet autoimmunity and RV seroconversion. To further ensure statistical stringency, we subjected the single comparison results to a permutation analysis (25). For each child, the odds ratio (OR) was calculated from the concordant and discordant results as $(1,1 \times 0,0)/(1,0 \times 0,1)$, and the mean \log_{10} OR for all 24 high-risk children was then determined. The distribution of the mean log ORs expected if there was no association (null hypothesis) was derived by 1,000 permutations that shuffled the positions of the scores of 1 and 0 for RV Ab in each child, calculating the mean log OR at each permutation. The distribution of the expected mean log ORs was then used to determine the probability of the association of the observed mean log OR (25).

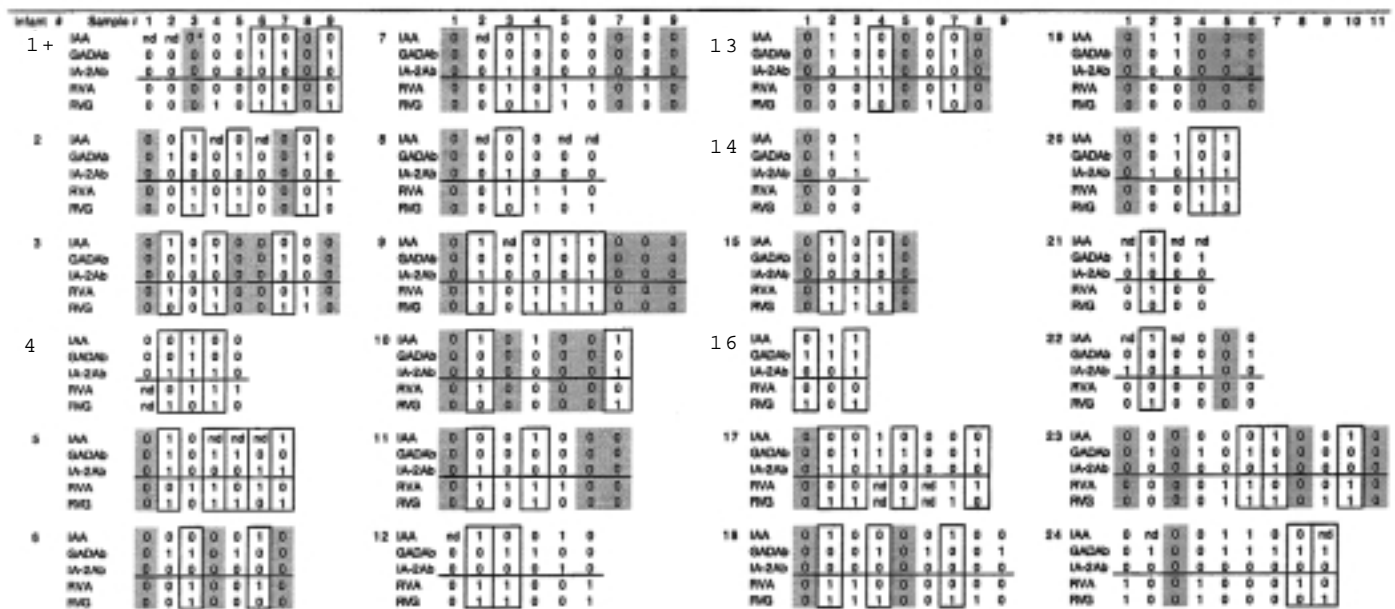


FIG. 1. Serial (6-month) islet and RV antibodies in high-risk children. Original data from which this figure was derived are available on request or at www.diabetes.org/diabetes/appendix.asp. Bolded numbers represent children who developed diabetes. 1, Significant increase; 0, no significant increase. Boxed areas indicate concordance of any islet Ab with any RV Ab. Shaded areas indicate concordance of no islet Ab with no RV Ab. nd, not done/insufficient sample.

RESULTS

The RV seroconversion rate in both high-risk and matched islet antibody-negative lower-risk children averaged 0.9 per year. To examine intrafamilial transmission, levels of RV Ab were determined in samples collected over the same 6-month period ($n = 54$) from 13 sibling pairs. Of the 28 RV antibody seroconversions detected, 15 occurred concurrently in the siblings, giving a concurrent infection rate for at-risk children of 54%. There was a trend for a higher concurrent infection rate in the 3 high-risk sibling pairs (5/6, 83%) than in the lower-risk sibling pairs (10/22, 45%).

In the high-risk children, IAA, GADAb, and IA-2Ab increased with repeated RV infections (Fig. 1). The original data set is available on the *Diabetes* website (at www.diabetes.org/diabetes/appendix.asp) or from the authors on request. It was clear that not all islet Ab appeared in all children. IAA first appeared with an increase in RV Ab in 13 of 21 children (62%), GADAb in 10 of 20 (50%), and IA-2Ab in 12 of 14 (86%). A representative example of RV antibody and islet antibody changes in a sibling pair is shown in Fig. 2. The χ^2 analysis of the concordance/discordance between significant increases in islet Ab and RV Ab (Fig. 1, boxed/unboxed results, respectively) revealed that an increase in any islet antibody was significantly associated with an increase in RVA or RVG during the same 6-month period ($n = 54/152$, $\chi^2_{yc} 15.5$, $P < 0.0001$, relative risk 2.1). When increases only above the

normal range of islet Ab were scored as 1, the association between islet Ab and RV Ab was still significant ($n = 35/152$, $\chi^2_{yc} 8.0$, $P < 0.005$, relative risk 1.9).

To guard against the possibility that the χ^2 test was biased by a high frequency of analyzed events per subject, a more stringent permutation analysis was also applied (Fig. 3). The association between significant increases in levels of islet Ab and RV Ab in serial samples from each of the 24 high-risk children expressed as the mean observed OR was 7.91, or 0.898 as the mean \log_{10} OR. The expected mean \log_{10} ORs from 1,000 permutations of 1 (significant increase) and 0 (no increase) were normally distributed between -0.8 and 1.0 (Fig. 3). Consequently, the observed association between islet Ab and RV Ab (mean \log_{10} OR = 0.898) was significant ($P < 0.02$).

Increases in islet antibody levels with RV infection were not associated with concurrent CBV4 or CBV5 infection. Of 41 paired sera that exhibited concordant increases in levels of islet Ab and RV Ab over 6 months, only 2, with increases in levels of GADAb, were also positive for CBVM (one each of serotypes 4 and 5). TPOAb level increased in only one paired serum with concurrent increases in RV antibody and islet antibody levels. ANA were not detected in any of these sera.

To determine if there was cross-reactivity between islet antigens and RV at the antibody level, 4 sera, each containing GADAb, IA-2Ab, and both RVA and RVG, were absorbed overnight at 4°C on high-titer RV precoated to EIA plates

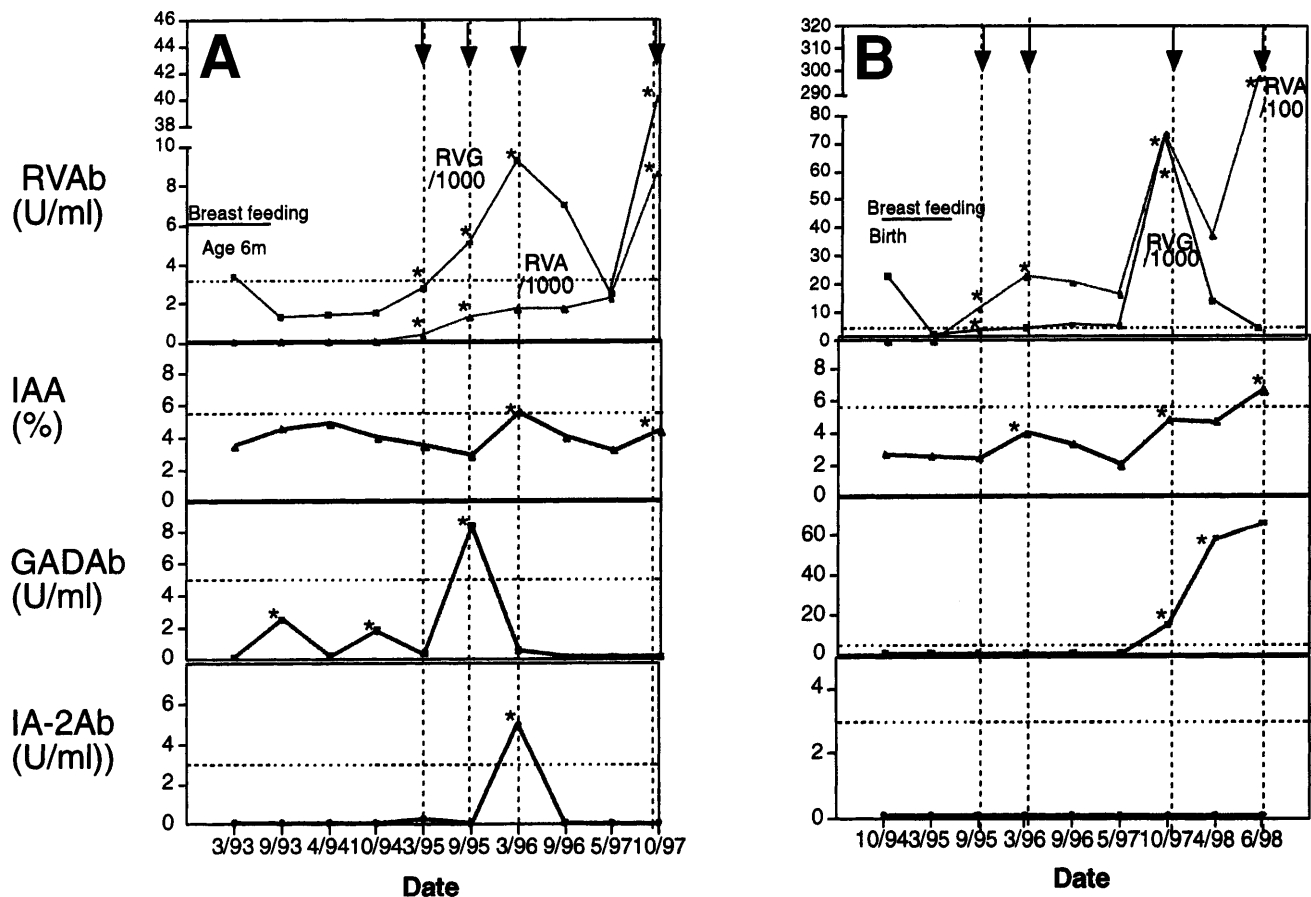


FIG. 2. RV Ab and islet Ab measured every 6 months over 5 years in 2 siblings at risk for type 1 diabetes. A: Sibling 1, female, HLA-DR3,4; B: sibling 2, male, HLA-DR1,4. Arrows indicate RV infections; horizontal dotted lines indicate antibody cutoffs. *Rise in Ab $> 2 \times$ CV of assay.

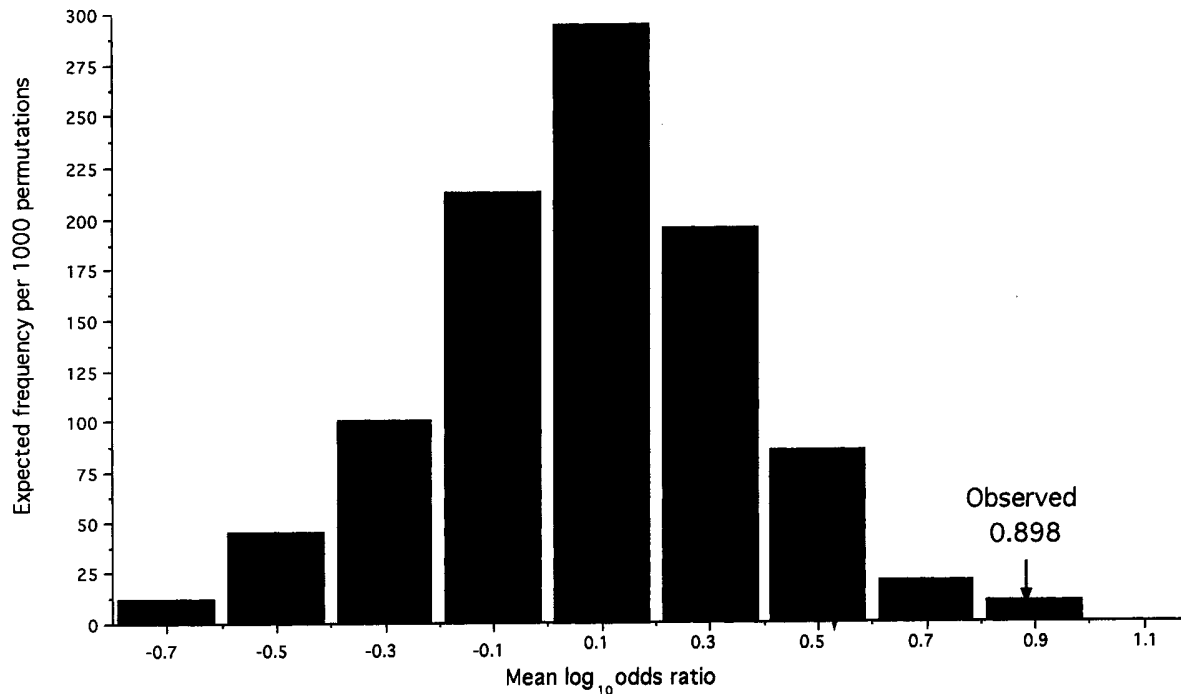


FIG. 3. Distribution of the expected mean \log_{10} ORs from 1,000 permutations of the positions of the scores of 1 (significant increase) and 0 (no significant increase) for any islet Ab with RV Ab in each child. The arrow indicates the observed mean \log_{10} OR.

and then retested in parallel with unadsorbed sera. RVA and RVG decreased by a mean of 57 and 40%, respectively, whereas GADAb (7%) and IA-2Ab (0%) were unchanged.

DISCUSSION

Serologically defined RV infection was significantly associated with an increase in islet antibody levels, most strongly with IA-2Ab, then with IAA and GADAb. The RV seroconversion rate in both type 1 diabetic high-risk and lower-risk groups of children was similar to that generally reported in children (0.8/year) (14). In addition, the rate of concurrent RV infection in the sibling pairs was the same as that (55%) reported for intrafamilial transmission in families without type 1 diabetes (24,26). Thus, at-risk children in these type 1 diabetes families did not appear overall to be infected by RV with increased frequency. In the high-risk siblings, the rate of transmission of RV was possibly higher than that in those at lower risk. However, because the rate of infection by RV was no greater in the high-risk group of children than in the HLA- and age-matched lower-risk groups of children, a further factor (either genetic or co-acquired) probably accounts for the observed association between RV infection and islet Ab in the high-risk children.

We found no evidence that infection with enteroviruses CBV4 or CBV5, previously implicated in type 1 diabetes (27,28), could account for the association of increased levels of islet Ab with RV infection. Furthermore, islet antibody increases did not appear to reflect a general autoantibody response, because increases in TPOAb or ANA were not detected. There was no apparent cross-reactivity between islet antigens and RV at the antibody level. This result is not unexpected even if there was molecular mimicry (9,10) between GAD or IA-2 and RV because mimicry is based on the similarity of linear epitopes recognized by T-cells, whereas B-cells gener-

ally recognize conformational epitopes. Moreover, the immunodominant antibody epitopes in the RV strain (SA11) used in the RV antibody assays are on the major inner capsid protein VP6, not VP7, and shared VP7 antibody epitopes are not in the region of T-cell epitope sequence similarities (29,30).

RV, a double-stranded RNA virus, is the major cause of gastroenteritis in early childhood, with multiple serotypes causing regular winter outbreaks until herd immunity is almost complete by age 5 years (14,24). During this study, the prevailing RV serotype detected in children with severe gastroenteritis was G1. Strains of the G3 serotype comprised up to 5% (E. Palombo, unpublished data). Almost half of RV infections are asymptomatic (31). Infection is particularly prevalent in day-care centers, in which early-age attendance has been associated with an increased risk for type 1 diabetes (32). We suggest therefore that the increase in type 1 diabetes incidence over the last decade, predominantly in the 0- to 4-year age-group (33,34), could be due to increasing attendance at day-care centers where young children are exposed to RV.

Whereas the clinical association found here between islet Ab and RV Ab is consistent with an etiologic role for RV in type 1 diabetes, it does not establish causality. RV infection could be coincident with other unrecognized infections or events. Nevertheless, RV infection appears to satisfy at least 6 of the 9 criteria for causality proposed by Hill (35) and discussed by Rothman (36), i.e., statistical strength, specificity of observations, temporality, coherence, analogy, and plausibility. Statistical strength and specificity of observations are reported here and temporality is supported by the occurrence of RV infections before both islet autoimmunity and diabetes, coherence by the increased incidence of type 1 diabetes in 0- to 4-year-old children (33,34), and analogy with rubella virus causation of type 1 diabetes (3). Plausibility is supported by our finding of peptide sequence similarities

between the immunogenic RV VP7 protein and T-cell epitopes in IA-2 and GAD (9), having the potential for molecular mimicry and immune cross-reactivity.

If an alternate mechanism was direct infection of pancreatic islets by RV, plausibility is also supported by our finding that reovirus (from the same Reoviridae family as RV) can infect human islets (37), by reports of pancreatitis associated with RV infection (38,39), and by the fact that a product of the endocrine pancreas, trypsin, renders RV infectious (40). The association of IAA, as well as IA-2 and GADAb, with RV infection could be a consequence of β -cell destruction in susceptible individuals, whether secondary to molecular mimicry, direct infection, or direct pancreatic infection followed by mimicry.

Our findings suggest that RV infection may trigger or exacerbate pancreatic islet autoimmunity on the HLA-DR4 background. These findings have implications for the development of safe RV vaccines to protect against islet autoimmunity and prevent type 1 diabetes.

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