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Immunoproteomic Profiling of Antiviral Antibodies in New-Onset Type 1 Diabetes Using Protein Arrays

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The rapid rise in the incidence of type 1 diabetes (T1D) suggests the involvement of environmental factors including viral infections. We evaluated the association between viral infections and T1D by profiling antiviral antibodies using a high-throughput immunoproteomics approach in patients with new-onset T1D. We constructed a viral protein array comprising the complete proteomes of seven viruses associated with T1D and open reading frames from other common viruses. Antibody responses to 646 viral antigens were assessed in 42 patients with T1D and 42 age- and sex-matched healthy control subjects (mean age 12.7 years, 50% males). Prevalence of antiviral antibodies agreed with known infection rates for the corresponding virus based on epidemiological studies. Antibody responses to Epstein-Barr virus (EBV) were significantly higher in case than control subjects (odds ratio 6.6; 95% CI 2.0–25.7), whereas the other viruses showed no differences. The EBV and T1D association was significant in both sex and age subgroups (≤12 and >12 years), and there was a trend toward early EBV infections among the case subjects. These results suggest a potential role for EBV in T1D development. We believe our innovative immunoproteomics platform is useful for understanding the role of viral infections in T1D and other disorders where associations between viral infection and disease are unclear.

Type 1 diabetes (T1D) is a chronic heterogeneous disease characterized by the progressive autoimmune destruction of pancreatic β-cells. The incidence of T1D is rising by an average of 3–5% in recent years, which cannot be fully explained by genetic predisposition alone (1). Moreover, the concordance rate for developing T1D among monozygotic twins is ∼66%, lower than that for type 2 diabetes (2). Hence, it is likely that environmental factors play a significant role during T1D development (3). Among various environmental factors considered relevant to T1D are those of nutrition and psychosocial factors; yet, viral infections have attracted particular interest (4,5).

Although there are a number of studies indicating viral effects on T1D pathogenesis, the exact mechanistic explanations for how viruses contribute to T1D etiology are still unknown. Viral infection or presence may act as a longitudinal factor during the induction of a single islet antibody, the simulation from a single islet antibody to multiple islet antibodies, or the progression from β-cell autoimmunity to clinical onset of T1D (6). Several studies reported that both the initial development of autoantibodies (AAbs) and the progression to multiple AAbs occurred at an early age. Subsequently, individuals progress to clinical T1D at different paces during which viral infections may act as an accelerator (7,8). For example, enterovirus infection was shown to increase progression to clinical onset in the Diabetes and Autoimmunity Study in the Young (DAISY) study (9). As the complex role of viral infections in T1D remains elusive, it would be valuable to address this important scientific question by assessing immune responses to many viruses and their antigens using many samples collected longitudinally from birth to disease onset.

Many viruses have been implicated in T1D in both animal models and humans with varying levels of evidence.

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Historically, the prevalence of viral infections in T1D was explored either by genomic approaches (which work if the viral nucleic acids remain present at the time of assay) or immunological approaches that only evaluated one viral protein or one type of virus at a time (10,11). Viral DNA or mRNA were detected by PCR or in situ hybridization in a relatively low-throughput manner (12,13). At the protein level, immunohistochemical staining and electron microscopy have been used to stain and observe viral proteins (14,15). Both in situ hybridization and immunohistochemical require the use of pancreatic sections from rare pancreatic tissue followed by tedious sample processing procedures. Many serological studies investigated the presence of antibodies to viruses. M-antibody ELISA has been a classic way to profile immunoglobulin (Ig)M antibodies in T1D patients (11). The plaque assay, which measures the presence of neutralization antibodies against the whole virus, is another method to profile serological antibodies to specific viral serotypes (16,17). The complement fixation test uses complement activation and the lysis of red blood cells to indicate the presence of certain viruses (10). Recent advances in next-generation sequencing technology have opened new venues for studying the role of viral infection in T1D development (18).

Despite these efforts, we still do not have a clear understanding of the association between viral infections and T1D development. A lack of quantitative and high-throughput technologies has limited the ability to study the role of viral infections in this disease comprehensively. Conflicting reports have stemmed from observations based on limited sample sizes (4). Previous studies focusing on a single viral protein or a single viral species have failed to provide a complete picture of infection history and their antibody responses at the systems level. Protein microarrays provide an ideal tool for multiplexed screening of specific antibodies in sera against thousands of different viral proteins printed on a standard microscope slide.

The aim of this study was to assess the prevalence of antiviral antibodies to 646 viral proteins from 23 T1D-related and other common viruses in patients with new-onset T1D and age- and sex-matched healthy control subjects. By examining antibody responses to hundreds of individual viral antigens at the proteome level, we hope to provide a complete picture of infection at a dimension never achieved before. Antibody-positive rates of studied viruses were determined and compared between T1D case and control subjects. Specific antibody responses on the array were validated by a confirmatory ELISA. The past onset nature of T1D samples may prevent us from drawing conclusions of whether the virus is pathological in inducing β-cell autoimmunity or accelerating clinical T1D. Nonetheless, this is the first comprehensive study of antibody response to a large number of viral species at the individual viral protein level in T1D. We believe the successful application of our platform to a large number of samples collected longitudinally before or after seroconversion will provide a better understanding of viral infection and T1D development.

RESEARCH DESIGN AND METHODS

Serum Samples

All samples were collected at clinics overseen by the University of Florida, with subjects emanating from that geographic region. They were collected with written informed consent and with the approval of institutional review boards at the University of Florida. Serum samples were obtained from patients with new-onset T1D within 3 months of diagnosis. Control samples were prepared in a fashion identical to that for the T1D samples and were selected to be matched with regard to age, sex, time of sampling, and geographic area. Control subjects were relatives of case subjects. Control samples were tested to be negative for major known T1D AAbs (islet antigen 2 [IA-2] antibody [IA-2A], GAD antibody [GADA], and zinc transport 8 antibody [ZnT8A]). Peripheral blood samples were drawn from the antecubital vein, and serum was prepared and stored as aliquots at −80°C. All individuals were free of other autoimmune diseases at the time of collection. The sample information is shown in Table 1.

Selection of Viral Strains

Based on literature mining, we identified viruses from seven different genera that have been implicated in T1D, which include enterovirus (coxsackievirus B [CVB]4), human endogenous retrovirus K (IDDMK1, 2–22 strain), rubivirus (rubella virus), rubulavirus (mumps virus [MuV]), rotavirus (rotavirus A), cytomegalovirus (HCMV), and lymphocryptovirus (Epstein-Barr virus [EBV]). These viruses encompassed a variety of genome types (ssRNA+, ssRNA−, dsRNA, and dsDNA). Selection of viral strain from each genus was based on their relevance to T1D as well as the availability of viral genome template (10,12,13,19–22). We also obtained additional viral genes from other common viruses to further enrich our collection (Table 2).

Viral Gene Cloning

All viral genes were first cloned into the pDONR221 Gateway compatible donor vector (Life Technologies, Carlsbad, CA). Different cloning approaches were used based on the available resources (viral genome type, gene

Table 1—Characteristics of patients with new-onset T1D and healthy control subjects

	Patients with new-onset T1D (n = 42)	Healthy control subjects (n = 42)
Age		
Median	11.5	12
Range	4–31	4–31
Male sex, n (%)	21 (50)	21 (50)
AAb status, n (%)		
GADA positive	33 (78.6)	0
IA-2A positive	18 (54.5)	0
ZnT8A positive	20 (47.6)	0

Table 2—Characteristics of viruses

Virus species	Abbreviation	Family	Genome	ORF clones	% of complete ORFeome
Human cytomegalovirus*	HCMV/HHV-5	*Herpesvirinae*	dsDNA	164	100
Epstein-Barr virus *	EBV/HHV-4	*Herpesvirinae*	dsDNA	85	100
Coxsackievirus B *	CVB	*Picornaviridae*	ssRNA+	12	100
Rubella virus*	RUBA	*Togaviridae*	ssRNA+	6	100
Mumps virus*	MuV	*Paramyxoviridae*	ssRNA−	9	100
Human endogenous retrovirus K*	HERK	*Retroviridea*	ssRNA−	4	100
Rotaviruses*	RV	*Reoviridae*	dsRNA	12	100
Hepatitis B virus	HBV	*Herpesviridae*	dsDNA	10	100
Human papillomavirus 16	HPV16	*Papillomaviridae*	dsDNA	8	100
Human papillomavirus 18	HPV18	*Papillomaviridae*	dsDNA	8	100
Chikungunya virus	CHIKV	*Togaviridae*	ssRNA+	9	100
Semliki Forest virus	SFV	*Togaviridae*	ssRNA+	9	100
Sindbis virus	SINV	*Togaviridae*	ssRNA+	9	100
Influenza A virus (H1N1)	n/a	*Orthomyxoviridae*	ssRNA−	10	100
Influenza A virus (H3N2)	n/a	*Orthomyxoviridae*	ssRNA−	10	100
Varicella-zoster virus	VZV	*Herpeviridae*	dsDNA	68	93.1
Simian virus 40	SV40	*Polyomaviridae*	dsDNA	6	85.7
Vaccinia virus	VACV	*Poxviridae*	dsDNA	167	74.9
Yellow fever virus	YF	*Flaviviridae*	ssRNA+	11	71.4
Measles virus, vaccine strain	MeV, vaccine	*Paramyxoviridae*	ssRNA−	5	62.5
Measles virus, WT strain	MeV, WT	*Paramyxoviridae*	ssRNA−	5	62.5
Adenovirus	n/a	*Adenoviridae*	dsDNA	16	42.1
Tioman virus	n/a	*Paramyxoviridae*	ssRNA−	3	37.5

*The seven viruses epidemiologically associated with T1D. n/a, not applicable.

characteristics, etc.). Viral genes were directly amplified by PCR if the viral DNA or cDNA templates were available. Viruses of ssRNA+ genome type, such as coxsackievirus, were first reverse transcribed into cDNA before PCR amplification. Some viral genes are not continuous across the viral genome, for example, UL89 from HCMV. Each gene fragment was amplified separately and then fused together by PCR elongation to obtain full-length viral genes. Genes without available templates, such as the superantigen gene from the IDDMK1, 2–22 strain, were produced using de novo gene synthesis. We are also grateful for the many Gateway-compatible viral entry clones obtained from the scientific community (Fig. 1). Viral genes in the pDONR221 vector were transferred to a T7 promoter–based in vitro expression vector pANT7_cGST by LR reaction (23). All viral genes cloned in this vector have a COOH-terminal glutathione *S*-transferase (GST) fusion tag in frame with the protein. The sequence verified clones were stored in and are available from the plasmid repository (http://dnasu.org/DNASU/) (24).

Nucleic Acid Programmable Protein Array Production

With use of the standard protocol (25,26), 1,200 ng/μL plasmid DNA was coprinted with rabbit anti-GST polyclonal antibody (GE Healthcare, Pittsburgh, PA), BSA, and bissulfosuccinimidyl suberate protein cross-linker (Thermo Fisher Scientific, Waltham, MA) as printing mix using Genetix QArrayer (Genetix, New Milton, U.K.). Each viral gene was printed in duplicate on each slide. Human IgG, IgA, and IgM were printed to show successful detection by secondary antibodies. Plasmid encoding a known T1D autoantigen (IA-2 protein) was printed as a positive control for T1D samples. Empty spots, spots printed with printing buffer without plasmid DNA, spots printed with printing buffer with plasmid encoding a hemagglutinin-fusion protein and anti-GST capture antibody were negative controls. Hemagglutinin-fusion protein can be expressed but not captured by anti-GST capture antibody served as a negative control for nonspecific capturing. Nucleic acid programmable protein array (NAPPA) has been successfully applied in AAb biomarker discovery and protein posttranslational modifications studies (27,28).

Array Quality Assessment

As previously described (29,30), plasmid DNA on the array was stained by a Quant-iT PicoGreen dsDNA Assay kit (Life technologies, CA) to verify successful printing. For protein display, groups of four slides were blocked in 30 mL SuperBlock Blocking Buffer (Thermo Fisher Scientific) at room temperature (RT) for 1 h on the shaker. Then slides were rinsed five times with deionized (DI)

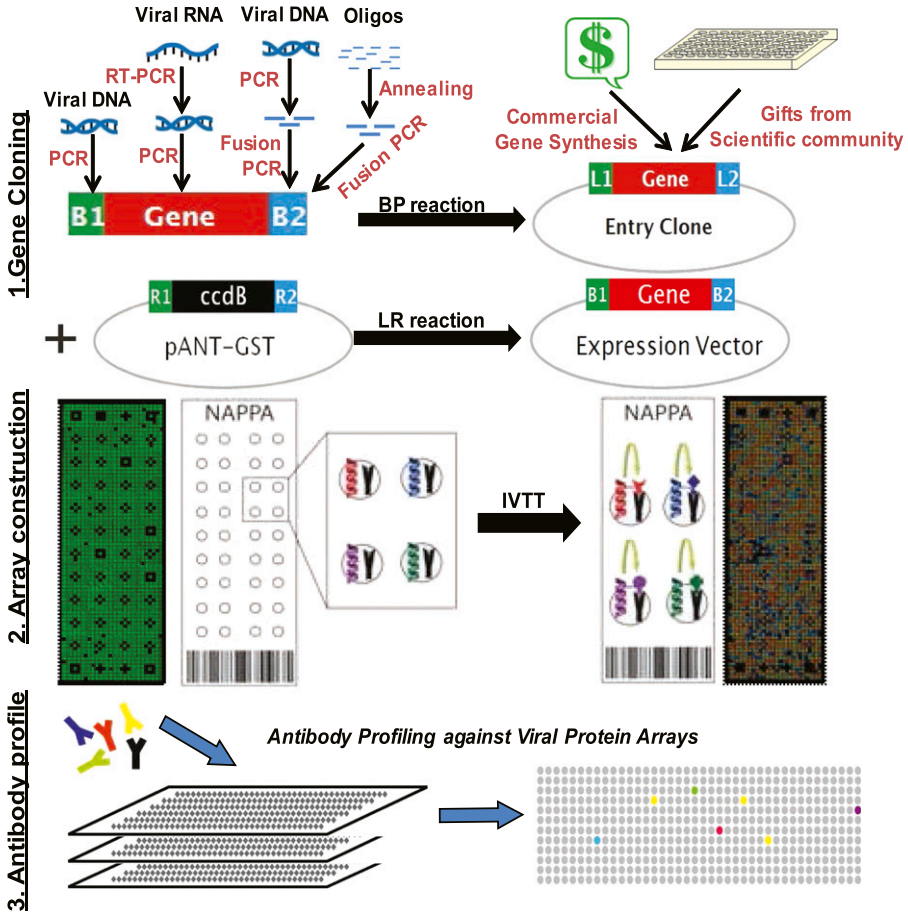

Figure 1—Study design. *1*) Gene cloning: various approaches were used to capture viral genes into protein array compatible expression vector. *2*) Array construction: protein arrays were constructed as previous described and quality assured. *3*) Antibody profiling: antibodies to viral proteins were profiled between new-onset T1D case and healthy control subjects. IVTT, in vitro transcription and translation.

water and placed in a metal slide rack (Amazon, Seattle, WA) for drying by centrifugation at 1,000 rpm for 3 min at RT. A 160 μL human HeLa cell lysate–based protein expression system (Thermo Fisher Scientific) was injected into HybriWell (Grace Bio-Labs, Bend, OR) sealed slides and incubated in the oven (EchoTherm, Carlsbad, CA) at 30°C for 1.5 h for protein expression and 15°C for 30 min for protein capture. Viral proteins were produced with a COOH-terminal GST fusion tag and captured in situ by coprinted anti-GST capture antibody on the array. The use of a tag at the COOH terminus ensures that captured proteins have been fully translated. Each slide was incubated with 5% milk PBS with 0.2% Tween-20, 3 mL of 1:200 diluted anti-GST monoclonal antibody (Cell Signaling Technology, MA), and 3 mL of 1:500 diluted horseradish peroxidase–labeled goat anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 h at RT on the shaker, respectively. Slides were washed three times with 5% milk PBST, with 5 min each time in between. Finally, slides were washed with PBST and rinsed with deionized water. Each slide was incubated with 500 μL 1:50 diluted tyramide signal amplification buffer (PerkinElmer, MA) to generate fluorescent signals. Then slides were washed, dried, and scanned by Tecan scanner (Tecan Group LTD, Männedorf, Switzerland).

Antiviral Antibody Profiling

Antiviral antibodies were profiled on the HS 4800 Pro hybridization station (Tecan, Männedorf, Switzerland). Proteins were expressed as previously described. Expressed slides were placed in the hybridization chambers and programmed with 1 h of blocking with 5% milk PBST and 16 h of incubation with 160 μL of 1:50 diluted serum at 4°C followed by 1 h of detection with 160 μL of 1:500 diluted Alexa Fluor 647 goat anti-human IgG or 160 μL 1:300 diluted Alexa Fluor 647 goat anti-human IgA and DyLight 549–conjugated goat anti-human IgM (Jackson ImmunoResearch Laboratories) at RT. Slides were washed, dried, and scanned by Tecan scanner. A pooled sample was prepared by mixing equal volumes of individual samples. The pooled sample was run on every serum screening day to show day-to-day technical reproducibility.

Strong antibody responses resulted in saturated signals of the local spot with diffusion to the neighboring spots.

Serum dilution was determined by the optimal sensitivity with minimum diffusion. The presence of diffusion was defined as a ring. To determine the signal intensities for the rings, we quantified the median intensity in between the ring spot and the neighboring spot as the raw ring intensity. We further quantified the median intensity in between the ring spot and the second closest spot (excluding the spot area) as the background ring intensity. The net ring intensity was calculated by subtracting the background ring intensity from the raw ring intensity. The data were only extracted for the spots with rings as ring positive net intensity data. The maximum of the ring positive net intensity for duplicates of each viral antigen was used in the quantitative analysis.

Rapid Antigenic Protein In Situ Display ELISA

Ninety-six-well ELISA plates (Corning Life Sciences, Salt Lake City, UT) were coated with 50 μL of 10 ng/mL anti-GST antibody (GE Healthcare) in coating buffer (0.5 mol/L carbonate bicarbonate buffer, pH 9.6) overnight at 4°C. On the next day, coated plates were washed five times with 100 μL PBST and blocked with 100 μL of 5% milk PBST for 1.5 h. Meanwhile, 40 ng/μL viral protein encoding plasmid was expressed in the human HeLa cell lysate-based protein expression system at 30°C for 1.5 h in the oven. Viral antigen was diluted in milk PBST at 1:200. Diluted antigen (50 μL) was captured in each well at RT for 1 h on a shaker at 500 rpm. Plates were washed five times with PBST. Each well was incubated with 50 μL of 1:1,000 diluted serum at RT for 1 h, washed again, and incubated with 50 μL of 1:10,000 diluted horseradish peroxidase–labeled anti-human secondary antibody (Jackson ImmunoResearch Laboratories) at RT for 1 h. Plates were incubated on a shaker at 500 rpm. Finally, the plates were washed and incubated with 50 μL 1-Step Ultra TMB ELISA Substrate for 10 min (Thermo Fisher Scientific) for detection and 50 μL of 2 mol/L sulfuric acid to stop the reaction. OD_{450} was measured by Envision Multilabel Reader (PerkinElmer, Waltham, MA).

Statistical Analysis

Sample information is presented as proportions and medians and ranges. A positive antibody response to a viral antigen was defined by the presence of a ring in at least one of the replicates of that protein. An individual was considered to have a positive antibody response to a virus if he or she had an antibody response to at least one viral antigen from the viral proteome. Odds ratios (ORs) and exact 95% CIs were calculated for viruses and T1D association using conditional likelihood estimation and Fisher exact test (31). Exact Wilcoxon rank sum tests were applied to the maximum of the ring positive net intensity data to determine the significance of antibodies to individual viral proteins between cases and control subjects. The Benjamini-Hochberg procedure was used to adjust *P* values to account for multiple testing. These tests were performed using the coin package in R 3.0.3. Exploratory subgroup analyses of the association between antibody positivity to viral proteins and the three known T1D autoantigens (GADA, IA-2A, or ZnT8A), sex, and age subgroups (≤12 and >12 years) were performed. *P* values <0.05 were considered significant. Heatmaps were generated in MultiExperiment Viewer, version 4.9 (http://www.tm4.org/mev.html). Graphs and plots were generated in GraphPad Prism 6.

RESULTS

Viral Protein Array Production and Quality Assessment

We have established a high-throughput pipeline (Fig. 1) and cloned 292 open reading frames (ORFs) from seven viruses reported to be associated with T1D and 354 ORFs from other common viruses into our protein array–compatible expression vector (Table 2). Among these, for 15 out of 23 viral strains we obtained 100% ORFs from the viral genome. The high coverage of viral ORFs and the diversity of viral strains would help us to fulfill the goal of a systematic survey of antiviral antibodies in patients with new-onset T1D.

For confirmation of printing quality, plasmids on the array were stained by PicoGreen that showed uniform DNA staining across the slide (Supplementary Fig. 1). The fluorescent signals of protein display were pseudocolored with a rainbow color scheme. The correlation coefficient of signal intensities of protein display as detected by the anti-GST tag antibody between two slides was >0.92 (Supplementary Fig. 1).

Profiling of Antiviral Antibodies

Quality-assured slides were challenged with serum samples from 42 patients with new-onset T1D and 42 age- and sex-matched healthy control subjects, with median ages of 11.5 and 12 years, respectively. IgG was profiled on a set of 84 slides, and IgA and IgM were profiled simultaneously on another slide set. Arrays were regularly probed with the pooled sample to assess intra- and interarray reproducibility (see RESEARCH DESIGN AND METHODS). The interspot correlation coefficients for IgG and IgA profiling of duplicate spots on the same array were 0.94 and 0.91, respectively (Supplementary Fig. 2). The interarray correlation coefficients of IgG, IgA, and IgM profiling of duplicate arrays on different days ranged from 0.92 to 0.94 (Fig. 2). The high interspot precision within slides and day-to-day reproducibility on different slides demonstrated the robustness of our serum screening practice and good control of assay quality.

We used rapid antigenic protein in situ display (RAPID) ELISA to confirm the antibody reactivity observed on the viral protein arrays. RAPID ELISA is an established in-house–developed immunoassay. Like NAPPA and several well-established clinical assays for T1D AAbs, it relies on a cell-free system to produce antigens. RAPID ELISA uses the same viral antigen-encoding plasmids used on the arrays. The intra-assay and interassay reproducibility is typically 0.99 (Supplementary Fig. 3). The ease in developing a RAPID ELISA to any antigen in the collection allowed us to confirm the performance of a smaller number of candidate antigens with many samples reproducibly, quickly, and affordably. RAPID ELISA data were consistent

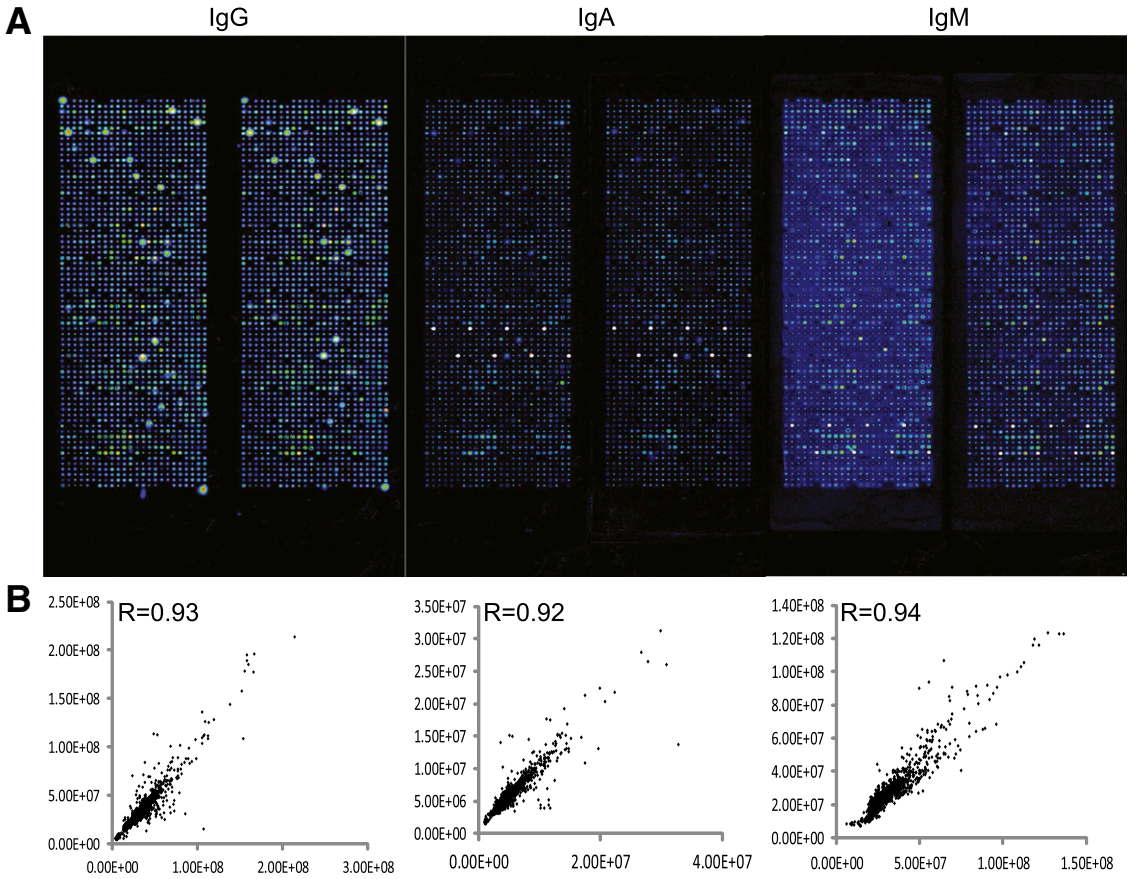

Figure 2—Reproducibility of antiviral antibody profiling. *A*: Representative images of IgG, IgA, and IgM responses of a pooled sample on two slides from two serum screening days. *B*: Scatter plot and correlation coefficients of IgG, IgA, and IgM reactivity of a pooled sample on two slides from two serum screening days.

with clinical radioimmunoprecipitation assay and the luciferase immunoprecipitation systems assay data (data not shown). Given its easy adaptability, high reproducibility, and comparability with established clinical assays, it was chosen as a confirmatory platform to validate the signals on the array. The ring positive net intensities of two EBV antigens, BFRF3 and BLRF2, obtained on the viral protein arrays agreed well with ELISA, further proving the validity of our array platform in profiling antiviral antibodies (Supplementary Fig. 4).

Differential Antiviral Antibody Response on the Arrays

A protein array approach enables the assessment of different antibody reactivity patterns for all proteins from the same virus. Heatmaps of IgG and IgA reactivity to viral proteins from the seven viruses previously associated with T1D were generated using the ring positive net intensities (Fig. 3). IgG antibody response to VP1 from CVB, nucleoprotein from MuV, and influenza A virus were detected in almost every individual in both case and control groups, which suggests they are immunodominant antigens from those viruses during infection. IgA reactivity was generally a subset of the IgG-responsive antigens albeit weaker than IgG reactivity for the same viral protein; however, VP3 and VP4 from CVB were both more reactive for IgA response than IgG. Although our secondary antibodies clearly detected both IgA and IgM control spots simultaneously on our arrays (Supplementary Fig. 5), we did not observe any positive serological IgM reactivity. This may suggest that none of the individuals had active infections at the time of blood sampling and possibly low IgM concentration in the serum.

Higher Frequency of Antibody Response to EBV in T1D Patients

We compared the humoral immune responses for each virus between the case and the control groups. As a sensitive criterion, a positive antibody response to a virus in each subject was defined by a positive antibody response to at least one viral antigen from the viral proteome. By this criterion, the prevalence of viral antibody responses obtained on the array is shown in Table 3. The historical rates of infection determined by epidemiology studies in which sera were sampled are shown in Supplementary Table 1. These two agreed well. For example, in our study subjects, the prevalence of HPV infection was low while the frequencies of influenza A viruses were high, which was in agreement with epidemiological data (Supplementary Table 1).

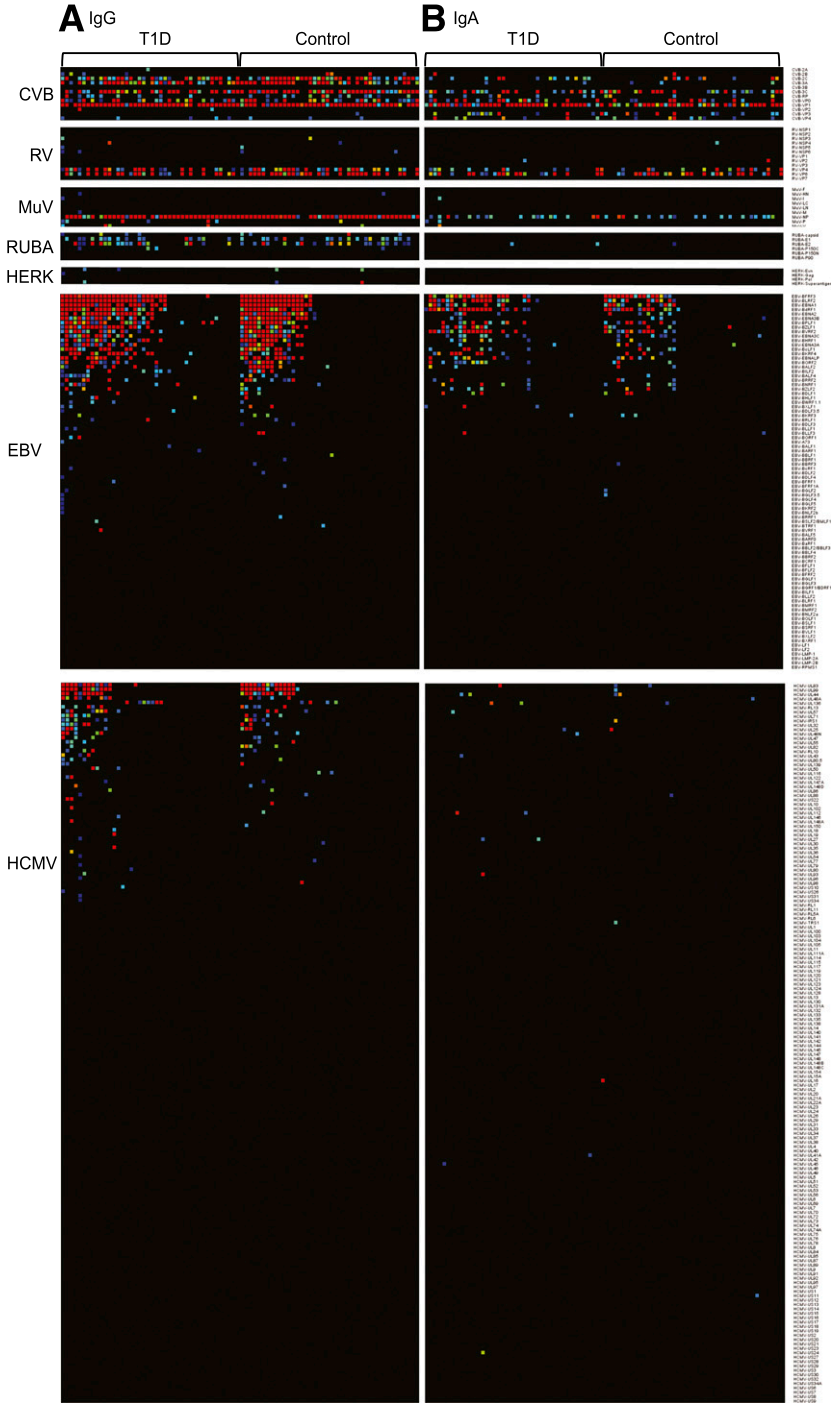

Figure 3—Heat maps of IgG and IgA reactivity to viral proteins from the seven viruses previously associated with T1D. HERK, human endogenous retrovirus K; RUBA, rubella virus; RV, rotavirus.

Notably, we found that antibody responses to EBV antigens were more frequent in patients with new-onset T1D than in healthy control subjects (88% vs. 52%; OR 6.6, 95% CI 2.0–25.7; *P* value 0.018), whereas none of the other viruses showed differences between the two groups (Table 3). Responses against EBV were higher in case than in control subjects among both sexes and in both commonly used age subgroups (≥12 and <12 years) (Supplementary Table 2). We detected many immunodominant EBV proteins based on antibody-mediated immunity such as BFRF3 and BLRF2 (viral capsid antigen [VCA]), BZLF1 (early antigen [EA]), and EBNA1 (nuclear antigen). On average, each case had antibody responses to 8 EBV proteins whereas each control had antibody responses to 6 EBV proteins.

We then assessed the association of antibody responses to individual viral proteins with subgroups of patients with

Table 3—ORs for the association of antibody responses to viruses between T1D case and healthy control subjects

Virus/protein controls	T1D (*n*)	T1D (%)	HC (*n*)	HC (%)	OR	95% CI	*P*
HCMV*	24	57.1	23	54.8	1.1	0.4–2.8	1.000
EBV*	37	88.1	22	52.4	6.6	2.0–25.7	0.018
CVB*	42	100.0	42	100.0	0.0	0.0 to Inf	1.000
RUBA*	22	52.4	25	59.5	0.8	0.3–1.9	1.000
MuV*	41	97.6	40	95.2	2.0	0.1–123.8	1.000
HERK*	3	7.1	2	4.8	1.5	0.2–19.3	1.000
RV*	27	64.3	32	76.2	0.6	0.2–1.6	1.000
HBV	1	2.4	0	0.0	Inf	0.0 to Inf	1.000
HPV16	2	4.8	1	2.4	2.0	0.1–123.8	1.000
HPV18	4	9.5	0	0.0	Inf	0.7 to Inf	1.000
CHIKV	1	2.4	0	0.0	Inf	0.0 to Inf	1.000
SFV	1	2.4	0	0.0	Inf	0.0 to Inf	1.000
SINV	0	0.0	0	0.0	0.0	0.0 to Inf	1.000
Influenza A virus (H1N1)	41	97.6	42	100.0	0.0	0.0–39.0	1.000
Influenza A virus (H3N2)	37	88.1	40	95.2	0.4	0.0–2.5	1.000
VZV	31	73.8	27	64.3	1.6	0.6–4.5	1.000
SV40	1	2.4	0	0.0	Inf	0.0 to Inf	1.000
VACV	1	2.4	2	4.8	0.5	0.0–9.8	1.000
YF	0	0.0	0	0.0	0.0	0.0 to Inf	1.000
MeV, vaccine strain	14	33.3	14	33.3	1.0	0.4–2.7	1.000
MeV, WT strain	31	73.8	35	83.3	0.6	0.2–1.8	1.000
Adenovirus	34	81.0	36	85.7	0.7	0.2–2.6	1.000
Tioman virus	0	0.0	0	0.0	0.0	0.0 to Inf	1.000
IA-2[p]	16	38.1	0	0	52.924	3.1–919.6	0.006
GST control[n]	0	0.0	0	0.0	0.0	0.0 to Inf	1.000

T1D, subjects with new-onset T1D; HC, health control subjects. Expansions for all abbreviations can be found in Table 1. Inf, infinite. *The seven viruses epidemiologically associated with T1D. [p]IA-2 protein used as a positive control. [n]Plasmid expressing only the GST-tag protein as a negative control.

T1D. IgG antibody response to IA-2 was statistically significantly higher in patients with T1D ($P = 0.006$), confirming the detection of known T1D AAbs on our protein array platform. Although no antibody to a single protein alone was a sufficient predictor, we found that a more sensitive viral response criterion could be defined as a positive antibody response to at least one viral antigen from the viral proteome. Next, we tested the association of antibodies against each viral protein against the responses to the three known T1D autoantigens (IA-2, GAD65, and ZnT8). No antibody responses to individual viral proteins exhibited statistically significant associations with any of the three known T1D autoantigens or sex. Only one protein, IgG antibody response to the NP protein from influenza A virus (H1N1), was significantly ($P = 0.007$) higher in one age subgroup (>12 years) compared with the age ≤12 years subgroup of patients with T1D (data not shown).

DISCUSSION

To our knowledge, this is the first study to examine antiviral antibodies to individual viral proteins at the proteomic level in new-onset T1D patients. We accomplished this by using an innovative and flexible protein array platform, resulting in a study with several unique strengths including the ability to display virtually any viral protein, proteins produced just-in-time for study, and proteins produced using human ribosomes and chaperone proteins. A key finding here was a higher EBV-specific immune response in T1D.

By examining antibody responses to hundreds of individual viral antigens, we analyzed the role of viral infections in T1D development at a dimension never achieved before. Previously, publications focused on the detection of antibodies to whole viruses or a limited number of viral proteins (10,11). Such studies might underrepresent the prevalence of viral infections because some patients may not produce antibodies that respond to the tested antigens. Even some commercial ELISA kits cannot achieve 100% sensitivity in detecting viral infections (32). Indeed, in our study, responses to viral antigens were quite heterogeneous from individual to individual. The examination of all proteins for each virus

affords the opportunity to look for associations not only with viral infection but also with immune responses to specific viral antigens. By focusing on the host immune response, we avoided missing events due to the transient nature of detecting viral DNA/RNA, which may only be present briefly during infection (18).

High-throughput and low sample consumption are great advantages of our array platform over conventional one-antigen-at-a-time immunoassays. With use of our platform, only 3.2 μL sera were needed to probe thousands of antigens on the slide. On average, each antigen consumes as little as 0.002 μL of serum sample, which is significantly less than individual assays such as enzyme immunoassay and radioimmunoprecipitation assay, which consume several microliters per antigen per assay.

Our array platform enables the multiplexed detection of different immunoglobulin classes and the flexibility to include any genes of interest into the study design. Typically, IgG is considered the most common and durable response, providing information of historical infections. IgA associates with mucosal immunity and IgM correlates with acute infection. It will be useful to consider expanding the content of these viral protein arrays. The emergence of next-generation sequencing techniques provides powerful opportunities to uncover new viral/microbiome strains that associate with T1D (18,33). Any proteins with known gene sequences can be easily included on this array platform to assess host immunoreactivity. Therefore, it is straightforward to incorporate metagenomics findings of T1D-relevant viruses/pathogens into the design of our viral/pathogen protein arrays to study patient responses. This combination of antibody immunoprofiles and viral metagenomics in T1D will lead to a better understanding of viral infections in T1D.

The prevalence of subjects with positive antibody responses to the studied viruses (Table 3) agreed well with the infection rate of the corresponding viruses from epidemiological studies in the U.S. (34–41) (Supplementary Table 1), validating the ability of the array platform to assess viral infection history. In addition to infections, antibody responses to viruses may also depend on the vaccine history, vaccine efficacy, antibody specificity, and detection methods (42,43). Unfortunately, the vaccination histories for these de-identified samples were not available.

Our results clearly demonstrate the value of a systems approach to assess the relationship between infections and chronic disease. We found a significant association between antibody responses to EBV and patients with new-onset T1D (88% vs. 52%; OR 6.6, 95% CI 2.0–25.7; *P* value 0.018). Responses to single EBV proteins were not predictors of disease status because different individuals responded to different proteins. This illustrates the heterogeneity of responses and further emphasizes the importance of a systematic evaluation that examines every sample against all viral proteins. Similarly, immunoassays based on the whole viral particles only detect responses against viral capsid antigens.

The relationships among age, EBV infection, and T1D are interesting. By dividing our subjects into two commonly used age-groups for such studies (≥12 and <12 years), we observed that there was a statistically higher prevalence of EBV infection among the T1D cases for both subgroups. However, as nearly all adults eventually develop antibody responses to EBV (41), we wondered if there were a relationship between age and the occurrence of antibodies to EBV antigens in patients compared with control subjects. To address this, we binned the individuals into different age-groups (4–7, 8–11, 12–15, and >15 years) and evaluated the response rate to at least one EBV protein. These subgroups were too small to evaluate statistically; however, as shown in Supplementary Fig. 6, for individuals <15 years, patients with T1D trended toward a greater likelihood of having been infected with EBV at a younger age than control subjects. This may suggest the possibility that an early age of EBV infection might contribute to the risk of developing T1D, although we cannot rule out the possibility that individuals with high T1D risk might be more prone to EBV infection at an earlier age. This question could be best addressed with a longitudinal study of individual's serum responses before and after development of T1D.

Connections between EBV and T1D date back to 1974 (44). Earlier studies focused on detecting low-grade temporary viral infections using either PCR or serological antibody responses in studies with a small sample size (45,46). Elliott and Pilcher reported that EBV infection was not associated with islet cell and insulin AAb seroconversion (46). Hyöty et al. (47) profiled antibody levels to VCA and EA using a commercial ELISA kit, which examined responses against the combined protein antigen and found lower VCA IgG class antibody levels in T1D patients. This apparent difference from our findings may arise from differences in the methods for determining viral infections. Our study profiled antibodies to all 85 EBV proteins tested individually, whereas the earlier studies focused on antibody responses to the combination of only several EBV proteins. Notably, if we had considered responses to the several proteins from VCA or EA from our data, none would have reached significance (Supplementary Table 3). There were also important differences in the sample source: our samples were obtained in U.S. compared with Finland for the previous study. Viral infections are both seasonal and regional. Differences of viral infections in the general population may be different based on sample locations.

The mechanism through which EBV might contribute to the pathogenesis of T1D remains uncertain. However, several possible scenarios can be envisioned. First, EBV may be spread from circulating infected B cells to pancreatic tissue, resulting in local antiviral immune responses that damage β-cells. Second, EBV infection induces the release of cytokines, which promotes the maturation of immune cells to enhance their cytotoxicity (48,49). Third, EBV infection may trigger a cross-reactive autoimmune response

through molecular mimicry of viral antigens and host proteins (50). These hypotheses need further evaluation. Recent evidence showed that viral infection may play a role in accelerating the progression from β-cell autoimmunity to clinical T1D (9). However, it is also possible the higher EBV responses may be a consequence of T1D but not a cause (51). The antibody responses detected in our work reflect past infections. As the samples used in our study were from patients with T1D postonset, investigation of longitudinal samples is needed to determine whether infection is important during seroconversion or during progression to clinical diagnosis of T1D. The successful application of this multiplexed viral proteome platform would facilitate this investigation.

Historically, coxsackievirus has been the most frequently cited T1D-related virus. We selected the E2 strain of CVB in our study because it was first isolated from the pancreas of a child with diabetic ketoacidosis (19). However, we did not find a difference in the apparent history of infection (OR 1, *P* value 1.000), which is consistent with the recent work by Oikarinen et al., who used a neutralizing antibody assay to investigate 41 different enterovirus serotypes (16). The other five viruses previously reported to be related with T1D also did not show prevalence difference between case and control subjects in our study.

Despite its virtues, the current study has some limitations. We did not have HLA data on our samples; however, an association between specific HLA genotypes and antiviral antibody responses in T1D has not been clearly established. Conflicting conclusions were drawn from studies on the same virus (52–55). The association between HLA genotypes and the humoral immune response is likely to be virus specific. The findings reported here specifically relate to early seroconversion of children to responsiveness to EBV. The overwhelming majority of adults display antibody responses to EBV (>90%) (41,56,57), making it unlikely that responses to EBV are significantly restricted by HLA. In this study, we found a higher rate of antiviral antibodies to EBV in young T1D patients compared with the children of similar ages, which suggests a potential role of EBV infection in T1D development.

To make the best possible comparisons with these samples, for which there were no HLA data available, we carefully matched our case and control subjects for age, sex, and time and geographic area of sampling. Furthermore, the control subjects were relatives of case subjects, which would not guarantee HLA matching but would ensure that the control subjects came from the same HLA pool and would have similar risk of T1D (11,58). This strategy has been used successfully to detect other associations between viruses and T1D, even in the absence of HLA matching (16).

In summary, we successfully demonstrated the use of our viral protein array platform in profiling antiviral antibodies and completed a preliminary study that found a potential link between EBV infection at an early age and T1D development. The success of this work established the utility of a flexible high-throughput multiplexed platform to profile a large number of longitudinal samples including time points surrounding "seroconversion" and clinical diagnosis. A comprehensive understanding of antibody responses to thousands of viral antigens in longitudinal samples and samples from HLA-matched healthy control subjects would greatly improve our knowledge of the role of viral infection in T1D development.

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Author Contributions. X.B. collected viral genes, performed serum screening, and wrote the manuscript. G.W. was responsible for the accuracy of data analysis. A.D., A.T., J.S., and X.Y. helped with viral gene cloning. J.W. helped with RAPID ELISA experiments. C.W., D.S., and M.A. provided serum samples and valuable discussions. J.Q. and J.L. contributed to experiment design, data generation, and analysis and reviewed and edited the manuscript. X.B. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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