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Evidence for Molecular Mimicry between Human T Cell Epitopes in Rotavirus and Pancreatic Islet Autoantigens

Margo C. Honeyman,* Natalie L. Stone,* Ben A. Falk, † Gerald Nepom, † and Leonard C. Harrison*

In type 1 diabetes, autoreactive T cells mediate destruction of insulin-producing pancreatic β cells. The major genetic contribution to the lifetime risk of type 1 diabetes comes from HLA genes in the MHC that encode proteins that present antigenic peptides to T cells (1). Several lines of evidence demonstrate that genetic susceptibility to type 1 diabetes is strongly modified by environmental factors. Thus, the disease is discordant in ~50% of identical twins (2), its incidence has risen progressively over the last 40 y, especially in younger children (3) concomitant with a decreased contribution of high-risk HLA genes (4), and circumstantial evidence implicates environmental agents including viruses (5).

Circulating autoantibody markers of pancreatic islet autoimmunity are directed against four defined autoantigens: (pro)insulin, the Mr 65,000 isof orm of glutamic acid decarboxylase (GAD65), tyrosine phosphatase-like insulinoma Ag 2 (IA2) (6), and zinc transporter 8 (7). In at-risk children followed from birth in the Australian BabyDiab Study, we documented a temporal association between rotavirus (RV) infection and the first appearance of, or an increase in, insulin autoantibodies and autoantibodies to GAD65 or IA2 (8). RVs, dsRNA viruses of the family Reoviridae, are the major cause of gastroenteritis in infants worldwide, being transmitted by fecal-oral contamination and activated in the small intestine by pancreas-derived trypsin. RV infection has been associated with pancreatitis (9–11), and we demonstrated that RV infects β cells (12).

We observed that the strongly immunogenic VP7 protein of RV contains a peptide sequence (aa40–52) highly similar to one in IA2 (aa805–817), as well as a contiguous sequence (aa16–28) highly similar to one in GAD65 (aa115–128) (13) (Fig. 1). Both the IA2 and GAD65 sequences are T cell epitopes restricted by HLA-DR4 (DRB1*0401) (14). Subsequently, the IA2 epitope was shown to be processed naturally by APCs in vitro (15). The GAD65 sequence was also found to be a CD4+ T cell epitope in HLA-DRB1*0401 transgenic mice (16, 17). Recently, these IA2 and GAD65 sequences were shown to encompass dominant HLA-A2–restricted epitopes for CD8+ T cells in individuals with recent-onset type 1 diabetes (18, 19). Collectively, these findings support the hypothesis that molecular mimicry between immunogenic peptides in RV-VP7 and similar peptides in IA2 and GAD65 is a mechanism by which islet autoimmunity may be triggered or exacerbated. To obtain further evidence for this hypothesis, we first determined if the similar RV-VP7, IA2 and GAD65 peptides bound to HLA class II molecules associated with type 1 diabetes. We then measured T cell proliferation to these peptides in islet Ab-positive individuals at risk for type 1 diabetes and in HLA-similar controls. Finally, we asked if T cells expanded to the IA2 epitope could be restimulated by the similar peptide in RV-VP7 and if T cell clones generated to the IA2-like epitope in RV-VP7 cross-reacted with the IA2 epitope itself.

Materials and Methods

Subjects
To identify T cell epitopes restricted by type 1 diabetes-associated HLA haplotypes, heparinized venous blood was obtained from three individuals who were homozygous for HLA-DRB1*0301-DQB1*0201 (two at risk for type 1 diabetes, one healthy) and from three individuals homozygous for HLA-DRB1*0401-DQB1*0203 (two at risk for type 1 diabetes, one healthy). The individuals at risk had Abs to the islet Ags GAD65 and IA2 and were pre-diabetic, developing clinical type 1 diabetes within 2 y of testing. To confirm
these epitopes in a range of subjects at risk for type 1 diabetes, blood was obtained from 52 predominantly HLA-heterozygous islet autoantibody-positive, first-degree relatives of people with type 1 diabetes (27 males, 25 females; median age 12 y, range 4–57 y) and from 27 of their islet autoantibody-negative healthy siblings (11 males, 16 females; median age 12 y, range 5–55 y). HLA phenotypes for these subjects are shown in Table II. To determine if T cells expanded to the IA2 epitope would respond by expression of the proinflammatory cytokine IFN-γ after restimulation by the similar RV-VP7 peptide, blood was obtained from a 19-year-old male with type 1 diabetes at risk with female Abs to GAD65 and IA2 and the highest-risk HLA genotype (A1,2; B8,44; DRB1*0301, *0401; DQ2,8). To determine if CD4+ T cells cloned to the IA2-like RV-VP7 peptide could recognize the IA2 epitope, blood was obtained from a 21-year-old male with the intermediate-risk HLA genotype (A2,25; B39,44; DRB1*0401, *1501; DQ1,8) and autoantibodies to insulin, GAD65, and IA2.

All blood samples were collected between 8:30 and 10:00 AM. The study was approved by the Melbourne Health Human Research Ethics Committee and conducted with informed consent.

HLA typing

Subjects were typed for type 1 diabetes susceptibility HLA class II haplotypes DR4-DQ8 and DR3-DQ2 by sequence-specific oligotyping, following the International Histocompatibility Workshop protocol. HLA class I alleles were typed by the standard microlymphocytotoxicity method for all recognized alleles.

Islet autoantibody assays

Abs to islet Ags were measured in internationally standardized assays (e.g., see Ref. 20), by immunoprecipitation of [35S]methionine-labeled recombinant IA2 or GAD65 or by precipitation of [125I]insulin.

Peptides

Previously defined T cell epitope peptides in IA2 805–817 (VIVMLTQYKVSKFD) and similar sequences in VP7 of the human G V g3 serotype strain P. RV-VP7 40–52 (IVISSLPLNAQN) and 16–28 (VILNNYVKLSSLTR), respectively, were synthesized by Fmoc chemistry (Auspep, Melbourne, Australia) and determined to be >95% pure by HPLC and mass spectrometry. Peptides were dissolved in 40% acetonitrile/acetic acid to a 2 mg/ml stock solution and aliquots stored at −80°C. The stock solution was diluted in RPMI 1640 medium to 200 μg/ml and 10 μl dispensed into six replicate wells of 96-well round-bottomed trays (Linbro Scientific, Hamden, CT), stored at −80°C, and thawed on ice immediately before T cell assays. The endotoxin concentration of peptide stock solutions measured by the Liposan lysate assay (BioWhittaker, Walkersville, MD) was <5 ng/ml.

HLA-DQ binding

The binding affinities of IA2 805–817, GAD65 115–127 (MNILLQYVVKSFD) and similar sequences in VP7 of the human G V g3 serotype strain P. RV-VP7 40–52 (IVISSLPLNAQN) and 16–28 (VILNNYVKLSSLTR), respectively, were synthesized by Fmoc chemistry (Auspep, Melbourne, Australia) and determined to be >95% pure by HPLC and mass spectrometry. Peptides were dissolved in 40% acetonitrile/acetic acid to a 2 mg/ml stock solution and aliquots stored at −80°C. The stock solution was diluted in RPMI 1640 medium to 200 μg/ml and 10 μl dispensed into six replicate wells of 96-well round-bottomed trays (Linbro Scientific, Hamden, CT), stored at −80°C, and thawed on ice immediately before T cell assays. The endotoxin concentration of peptide stock solutions measured by the Liposan lysate assay (BioWhittaker, Walkersville, MD) was <5 ng/ml.

HLA-DR binding

The binding affinities of IA2 805–817, GAD65 115–127, and VP7 40–52 and 16–28 peptides to type 1 diabetes susceptibility HLA alleles DRB1*0401, *0404, and *0301, as well as to DRB4*0101 on the type 1 diabetes protective allele DRB1*1501, were measured by a direct-competition ELISA as previously described (21). Binding affinity was expressed as IC50, the concentration of peptide (μM) that inhibited binding of labeled indicator peptide (GAD65 555–567) by 50% and denoted as very strong (∼<0.01 μM), strong (0.01 ≤ 0.1), moderate (> 0.1 ≤ 1.0), or weak (> 1).

T cell proliferation assay

PBMCs obtained by Ficoll-Hypaque gradient centrifugation of sodium heparinized blood were added at 2 × 105 cells in 200 μl to U-bottomed wells of 96-well plates (Linbro Scientific) in RPMI 1640 (Life Technologies, Melbourne, Australia) containing 10% pooled human serum and 25 μg/ml (final concentration) of each peptide, in replicates of six, and incubated for 6 d in 5% CO2 in air at 37°C. To optimize reproducibility, blood was drawn at the same time of day (8:30–10:00 AM). PBMCs were separated within 15 min of blood sampling, and T cell responses to similar peptides were measured in the one plate. The outer wells of the plates were not used but contained sterile water only, and each plate was placed within a humidified container in the incubator. Proliferation was measured by uptake of [3H]thymidine (ICN, Sydney, Australia) added at 37 kBq for 7 h before harvesting and liquid scintillation counting. The T cell response was defined as the stimulation index, the median counts per minute with test Ag/without Ag. The cutoff for a significant stimulation index was defined as ≥ 2 SD above the mean + 3 SD of the basal (without Ag) for all subjects (1.59). The reproducibility of M cells in complete IMDM was tested by repeat assays weekly for 4 wk in three subjects; the intra-assay coefficient of variation ranged from 13.1% to 18.9% and the inter-assay coefficient of variation from 14.2 to 26.2.

Peptide crossover restimulation IFN-γ assay

To investigate cross-reactivity between IA2 and RV-VP7 epitopes, PBMCs were incubated for 7 d with and without IA2 805–817, and then restimulated for 24 h with IA2 805–817 or PBS (CFSEdim) over 7 d that was IFN-γ+ Ag/without Ag. The cutoff for a significant stimulation index was defined as $>$ 1.59. IFN-γ+ Ag/without Ag produces a stimulation index = 2. The cutoff for a significant stimulation index was defined as $>$ 1.59. IFN-γ+ Ag/without Ag produces a stimulation index = 2.

**Table I.** Sequence-similar peptides in the islet autoantigens IA2 and GAD65 and rotavirus VP7 (serotype G3, human strain P). An X denotes an anchor residue for binding to the indicated HLA molecule. Identical residues are in black and similar residues in gray.
as above, without PHA. Three of 15 clones that expanded the most were tested and confirmed for monoclonality by RT-PCR of the TCR clonotype as previously described (23). Cloned cells were taken 4 d after addition of cytokines, washed in PBS then IMDM, and resuspended in complete IMDM. Cells were added in triplicate at $2 \times 10^4$ well to a 96-well U-bottomed plate, each well containing $2 \times 10^5$ PBMCs from an HLA-DR-DQ–matched donor as APCs and RV-VP7 40–52 or IA2 805–817 peptides, ranging in final concentration from 1–25 μg/ml. After incubation for 2 d in 5% CO$_2$ in air at 37°C, cells were pulsed with [$^3$H]thymidine as above, harvested 16 h later, and counted.

### Statistical analysis

The medians of subject groups were compared overall with the Kruskal-Wallis test and then for pairs of groups with Dunn’s posttest. Fisher’s exact tests were used to compare the frequencies of T cell responses, and a Bonferroni correction was made for multiple comparisons. Correlation was determined by Spearman rank-log test. Statistics were performed with GraphPad Prism software (version 3 for Macintosh, GraphPad, San Diego, CA).

### Results

#### Islet autoantigen and similar RV peptides bind to HLA-DR molecules associated with type 1 diabetes

IA2 805–817 and GAD65 115–127, and the similar RV-VP7 peptides (Fig. 1), were bound to HLA class II molecules encoded by alleles DRB1*0401, DRB1*0404, and DRB4*0101 (also present on DRB1*04 haplotypes) for type 1 diabetes susceptibility, and to the protective allele DRB1*1501, but not to the susceptibility allele DRB1*0301 (Fig. 2, Table I). IA2 805–817 and RV-VP7 40–52 peptides had similar affinities for DRB1*0401 (weak), DRB4*0101 (moderate), and DRB1*0404 (strong and very strong). GAD65 115–127 and RV-VP7 16–28 peptides had similar affinities for DRB1*0401 (moderate) and DRB1*0404 (strong and very strong), with strong and moderate affinities, respectively, for DRB4*0101. IA2 805–817, GAD65 115–127, and RV-VP7 16–28 each bound strongly to the protective allele DRB1*1501, but RV-VP7 40–52 did not bind. Thus, overall, the RV-VP7 peptides had similar or stronger affinities for binding to HLA-DR4 susceptibility alleles than the respective sequence-similar IA2 and GAD65 epitopes.

#### RV and autoantigen-derived peptides elicit similar frequencies of T cell proliferative responses

T cell proliferative responses to IA2 805–817 and GAD65 115–127, and to the similar peptides in RV-VP7, are shown and summarized in Fig. 3. A substantial proportion of both islet autoimmune and control subjects, whose distribution of HLA-DR phenotypes is shown (Table II), responded to each peptide. Overall, the proportions of subjects responding or the mean responses did not differ between the groups, with the exception of a higher proportion of islet autoimmune responders to RV-VP7 40–52 (84% versus 60%; $p < 0.025$, Fisher’s exact test).

### Table I. Binding of IA2, GAD65m, and RV-VP7 peptides to HLA-DR alleles

<table>
<thead>
<tr>
<th>Peptide</th>
<th>DRB1*0401</th>
<th>DRB1*0404</th>
<th>DRB1*0301</th>
<th>DRB4*0101</th>
<th>DRB1*1501</th>
</tr>
</thead>
<tbody>
<tr>
<td>IA2 805–817</td>
<td>2.04</td>
<td>0.020</td>
<td>&gt;10</td>
<td>0.25</td>
<td>0.041</td>
</tr>
<tr>
<td>RV-VP7 40–52</td>
<td>1.07</td>
<td>0.0085</td>
<td>&gt;10</td>
<td>0.25</td>
<td>&gt;10</td>
</tr>
<tr>
<td>GAD65 115–127</td>
<td>0.18</td>
<td>0.022</td>
<td>&gt;10</td>
<td>0.18</td>
<td>0.012</td>
</tr>
<tr>
<td>RV-VP7 16–28</td>
<td>0.28</td>
<td>0.0010</td>
<td>&gt;10</td>
<td>0.72</td>
<td>0.022</td>
</tr>
<tr>
<td>RV-VP7 12–24</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>&gt;10</td>
</tr>
<tr>
<td>RV-VP7 22–36</td>
<td>&gt;10</td>
<td>0.28</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>&gt;10</td>
</tr>
<tr>
<td>RV-VP7 28–40</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>4.2</td>
<td>0.53</td>
</tr>
<tr>
<td>Indicator peptide</td>
<td>0.020</td>
<td>0.0073</td>
<td>0.90</td>
<td>0.015</td>
<td>0.077</td>
</tr>
</tbody>
</table>

Binding is expressed as IC$_{50}$ value, the concentration of peptide (μM) that inhibited binding of the indicator peptide (GAD65 555–567 or MBP 84–102 for DRB1*1501) by 50%.
not tested (29). In the autoimmune diabetes-prone NOD mouse includes this motif bound very weakly to type 1 diabetes susceptibility HLA molecules; binding of the GAD65 sequence was found, but CBV infection of NOD mice had no effect on T cell reactivity to the GAD65 peptide or on diabetes incidence (30), T cell cross-reactivity between the P2-C and GAD65 sequences was found, but CBV infection of NOD mice had no effect on T cell reactivity to the GAD65 peptide or on diabetes incidence (27). In type 1 diabetes, enteric viruses such as Coxsackie B viruses (CBVs) and RVs have been proposed as potential etiological agents (13, 25–28). The P2-C protein of CBVs shares amino acid sequence similarities in viral and self proteins to elicit cross-reactive T cell responses has been hypothesized as a mechanism for breaking immune tolerance leading to autoimmune disease (24). In humans with type 1 diabetes, CD4+ HLA-A2–restricted T cell lines activated to GAD65 258–266 showed no proliferation to CBV P2-C (aa33–52) that includes this motif bound very weakly to type 1 diabetes susceptibility HLA molecules; binding of the GAD65 sequence was not tested (29). In the autoimmune diabetes-prone NOD mouse (30), T cell cross-reactivity between the P2-C and GAD65 sequences was found, but CBV infection of NOD mice had no effect on T cell reactivity to the GAD65 peptide or on diabetes incidence (27). In humans with type 1 diabetes, CD4+ T cell clones generated to GAD65 258–266 showed no proliferation to CBV P2-C 35–43 (31), and three CD4+ T cell lines to CBV P2-C 35–43 did not proliferate to GAD65 peptides containing the PEVKEK motif (32). Furthermore, CD8+ HLA-A2–restricted T cell lines activated to secrete IFN-γ by CBV 35–43 did not respond to GAD65 258–266 and were not cytotoxic to target cells pulsed with the peptide (33). A positive correlation was found between T cell proliferation to CBV 35–43 and GAD65 258–266, both in individuals with recent-onset type 1 diabetes and in controls (34), but the responders also had high responses to tetanus toxoid, and the results initially with an irrelevant peptide (scrambled GAD65 115–127) were 8 and 7 (Fig. 5C, 5D) and without restimulation 17 and 6 (Fig. 5E, 5F), respectively. Thus, CD4+ T cells stimulated to divide by IA2 805–817 could be restimulated to express IFN-γ to the same extent by either IA2 805–817 or RV-VP7 40–52.

**RV-VP7 and IA2 epitopes are recognized by the same TCR**

Eleven clones were generated to the RV-VP7 40–52 epitope and tested for cross-reactivity with the IA2 805–817 epitope. Of these, two, JC1B2 and JC1B5, expressing a single Vβ 13 TCR, were restimulated to divide to the same or greater extent by IA2 805–817 (Fig. 6A, 6B). Proliferation of clone JC2B3 to RV-VP7 40–52 was inhibited 93% and 42%, respectively, by mAbs to HLA-DR and -DQ (Fig. 6C), consistent with HLA-DR restriction of the response.

**Discussion**

Molecular mimicry between nonself and self at the level of primary amino acid sequence similarities in viral and self proteins to elicit cross-reactive T cell responses has been hypothesized as a mechanism for breaking immune tolerance leading to autoimmune disease (24). In type 1 diabetes, enteric viruses such as Coxsackie B viruses (CBVs) and RVs have been proposed as potential etiological agents (13, 25–28). The P2-C protein of CBVs shares amino acid sequence similarities in viral and self proteins to elicit cross-reactive T cell responses has been hypothesized as a mechanism for breaking immune tolerance leading to autoimmune disease (24). In type 1 diabetes, enteric viruses such as Coxsackie B viruses (CBVs) and RVs have been proposed as potential etiological agents (13, 25–28). The P2-C protein of CBVs shares amino acid sequence similarities in viral and self proteins to elicit cross-reactive T cell responses has been hypothesized as a mechanism for breaking immune tolerance leading to autoimmune disease (24). In type 1 diabetes, enteric viruses such as Coxsackie B viruses (CBVs) and RVs have been proposed as potential etiological agents (13, 25–28). The P2-C protein of CBVs shares amino acid sequence similarities in viral and self proteins to elicit cross-reactive T cell responses has been hypothesized as a mechanism for breaking immune tolerance leading to autoimmune disease (24). In type 1 diabetes, enteric viruses such as Coxsackie B viruses (CBVs) and RVs have been proposed as potential etiological agents (13, 25–28). The P2-C protein of CBVs shares amino acid sequence similarities in viral and self proteins to elicit cross-reactive T cell responses has been hypothesized as a mechanism for breaking immune tolerance leading to autoimmune disease (24). In type 1 diabetes, enteric viruses such as Coxsackie B viruses (CBVs) and RVs have been proposed as potential etiological agents (13, 25–28). The P2-C protein of CBVs shares amino acid sequence similarities in viral and self proteins to elicit cross-reactive T cell responses has been hypothesized as a mechanism for breaking immune tolerance leading to autoimmune disease (24). In type 1 diabetes, enteric viruses such as Coxsackie B viruses (CBVs) and RVs have been proposed as potential etiological agents (13, 25–28).
were attributed to general T cell hyperreactivity rather than molecular mimicry. Other viruses have also been investigated for evidence of mimicry in type 1 diabetes. CD8\(^+\) T cell clones to GAD65 peptides, generated from subjects with type 1 diabetes, were cytotoxic in response to rubella virus peptides with weak sequence similarities to the GAD65 peptides (35). CD4\(^+\) T cell clones to multiple GAD65 peptides, generated from a patient with stiff-person syndrome, proliferated to a naturally processed, DR3-binding peptide from CMV (36), but the relevance of this finding to type 1 diabetes is unclear. Thus, the evidence for molecular mimicry as a potential mechanism of islet autoimmunity in type 1 diabetes is negative or at best circumstantial.

We suggest that the minimum criteria for mimicry at the T cell level are that a peptide from a candidate environmental agent...
should elicit T cell responses in the context of a disease susceptibility HLA molecule similarly to the autoepitope peptide and that the one TCR recognizes both peptides. We found that peptides in RV-VP7 with sequence similarity to epitopes in the islet Ags IA2 and GAD65 bound to HLA-DR4 molecules that confer susceptibility to type 1 diabetes and elicited proliferation of T cells from islet-autoimmune and healthy individuals expressing these HLA molecules. Parenthetically, this is the first report identifying RV T cell epitopes in humans. To address the more stringent criterion for mimicry, namely that one TCR recognizes both the self- and nonself peptide, we first showed that T cells from PBMCs that proliferated to IA2 805–817 could be restimulated to express IFN-γ by both IA2 805–817 and RV-VP7 40–52. That recognition could occur via the one TCR was confirmed by showing that two T cell clones generated to RV-VP7 40–52 also proliferated in response to IA2 805–817 presented in the context of DRB1*0401. These findings meet the minimum criteria for molecular mimicry.

Stratification of responses by HLA revealed that both RV-VP7 epitopes were T cell epitopes not only in DRB1*0401 individuals, but also in some with DRB1*0301 without DRB1*0401/4, despite the lack of binding of peptides to DRB1*0301. This suggests that peptide presentation could also occur by HLA alleles at other loci on the same genotype, such as HLA-DRB3, DQ2, or -DP. HLA-DRB1*1501 is protective in type 1 diabetes, but the mechanism of this effect remains unexplained. Interestingly, both autoantigen peptides and RV-VP7 16–28 bound strongly to DRB1*1501, but RV-VP7 40–52, similar to IA2 805–817, did not bind. Therefore, if mimicry between RV-VP7 40–52 and IA2 805–817 is involved in promoting islet autoimmunity, it may be less likely to occur in DRB1*1501 individuals. To examine this possibility, T cell responses to RV-VP7 40–52 and IA2 805–817 could be measured in subjects who are DRB1*0101 but not DRB1*0401. In the current study, this was precluded by HLA matching.

A role for molecular mimicry implies a higher frequency and/or increased magnitude of T cell responses to the environmental epitope in islet-autoimmune subjects than controls; however, this was the case only for RV VP7 40–52. On the other hand, the relatively high frequency of T cell responses in controls and islet-autoimmune subjects may not be unexpected given that the controls were HLA similar. Together with lack of correlation between T cell responses to peptides and betas and tetanus toxoid, this indicates that the islet-autoimmune subjects did not have a general increase in T cell reactivity. Future studies to determine the functional properties of T cells that respond to RV-VP7 and autoepitope peptides might reveal differences between islet-autoimmune and control subjects. There is no evidence for more frequent or more persistent RV infection in islet-autoimmune subjects, based on our previous studies of islet-autoimmune and healthy children (8, 37). Mimicry to a ubiquitous agent like RV, if it contributes to disease pathogenesis, does not have to be disease-specific and would most likely synergize with other mechanisms such as direct viral damage to the target tissue, being a contributory but not sufficient condition. Martiniuzzi et al. (19) reported that IA2 805–813 was a subdominant HLA-A2-restricted CD8+ T cell epitope in patients with recent-onset type 1 diabetes, becoming the dominant epitope after several months, and that GAD65 114–123 was the dominant HLA-A2–restricted epitope at onset but was subdominant at follow-up (19). These findings strengthen the case for mimicry between the RV-VP7 and IA2 and GAD65 peptides. We suggest that these IA2 and GAD65 peptides could be composites for both CD4+ and CD8+ T cells restricted by HLA class II and class I molecules, respectively. Although direct, unequivocal proof for molecular mimicry in human autoimmune disease is probably unattainable, our findings support the hypothesis that mimicry with RV may contribute to the pathogenesis of type 1 diabetes.

Acknowledgments

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Disclosures

The authors have no financial conflicts of interest.

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