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Reduced T-Dependent Humoral Immunity in CD20-Deficient Mice

Deyaa El Deen Morsy,* Ratna Sanyal,* Anne K. Zaiss,†,1 Rucha Deo,* Dan A. Muruve,† and Julie P. Deans*

CD20 is a tetraspanning membrane protein expressed on B lymphocytes. CD20 deficiency in both mice and humans has recently been shown to have deleterious effects on Ab responses to T-independent Ags; however, no effect on T-dependent immunity has been reported. In this study, we used a Cd20−/− mouse line to evaluate Ab responses to adeno-associated virus and SRBCs. The neutralizing Ab response to adeno-associated virus was significantly reduced by CD20 deficiency; both primary (IgM) and secondary (IgG1 and IgG2b) responses to SRBC were also reduced in Cd20−/− mice, and this was associated with a reduction in the number of germinal center B cells. A successful humoral response requires the integration of intracellular signaling networks that critically rely on calcium mobilization. In this article, we confirm that BCR-mediated calcium mobilization is impaired in Cd20−/− murine B cells after BCR stimulation in vitro, and further show that the reduction is due to an effect on calcium influx rather than calcium release from intracellular stores. Calcium-dependent upregulation of CD69 was impaired in Cd20−/− cells, as was upregulation of CD86. Altogether, this study demonstrates a role for CD20 in B cell activation and T-dependent humoral immunity.


Because CD20 is highly expressed on all mature B cells in both mice and humans, the lack of a demonstrated effect of CD20 deficiency on TD humoral immunity was surprising. We therefore sought to examine TD immune responses in Cd20−/− mice using the well-characterized complex Ags, adeno-associated virus (AAV) and SRBCs. We found significant reduction in humoral immunity to both Ags. The response to SRBCs was evaluated further and found to affect both primary (IgM) and secondary (IgG1 and IgG2b) Ab responses, and was associated with reduced germinal center (GC) B cell numbers. To our knowledge, this is the first report of an effect of CD20 deficiency on the humoral immune response to TD Ags.

The development of humoral immunity requires normal BCR signaling. Evidence from several laboratories, using cell lines expressing either endogenous or ectopic CD20, supported a role for CD20 in calcium signaling, potentially as a calcium channel linked to the BCR (6, 10, 11). However, previous studies examining BCR-mediated calcium mobilization in Cd20−/− primary murine B cells yielded inconsistent results (9, 12). We show in this article that calcium mobilization is indeed reduced in Cd20−/− B cells after BCR stimulation, and that this is due to reduced influx of extracellular calcium. Further, we found that calcium-dependent upregulation of CD69, as well as CD86, after BCR and/or CD40 stimulation was impaired in Cd20−/− B cells, whereas CD69 upregulation was normal in CD3-activated T cells from the same mice. Altogether, the data reported in this article show that CD20 function is necessary for optimal BCR-mediated calcium entry, B cell activation, and TD humoral immunity.

Materials and Methods

Mice

Cd20−/− mice used in this study were previously described (12) and were backcrossed onto C57BL/6 background for at least seven generations. C57BL/6 wild-type (WT) mice were purchased from Jackson Laboratories (Bar Harbor, ME) and crossed with Cd20−/− C57BL/6 mice. Heterozygous mice from the first generation were crossed and the resulting pups were genotyped to obtain homozygous Cd20−/− and WT C57BL/6 mice that were inbred to maintain colonies that were housed in a specific-
pathogen-free barrier facility at the University of Calgary. Mice were used for experiments at 2–3 mo of age. All studies were reviewed and approved by the Animal Care Committee at the University of Calgary.

**Immunization**

Mice were immunized using AAV type-2 vector (1 x 10^11 particles/mouse), provided by Dr. J.S. Bartlett, as described previously (13). Injections were done via the femoral vein (i.v.) under general anesthesia in a total volume of 100 μl sucrose vehicle (3% sucrose, 150 mM NaCl, 10 mM Tris [pH 7.4], 1 mM MgCl2). All control animals received the vehicle alone. For immunization with SRBCs, fresh SRBCs (Cedarlane, Hornby, Ontario, Canada) were washed twice in sterile PBS, pH 7.2, before use. Mice were primed by i.p. injection of 1 x 10^7, 3 x 10^7, or 1 x 10^8 SRBCs in 0.5 ml PBSt. Booster i.p. injections were conducted with the same dose for each experiment on day 21. Blood samples were collected by tail-vein puncture. Serum was obtained from blood by centrifugation at 1000 × g for 5 min and stored at −80°C.

**Anti-AAV Ab titration**

Neutralizing anti-AAV Abs were detected as described previously (13). In brief, mouse sera were serially diluted in serum-free DMEM tissue culture medium and incubated with 8 x 10^5 AAV-GFP particles for 10 min at 37°C in a total volume of 100 μl. The AAV-serum mix was then applied to wells of a 24-well plate (Thermo Scientific, Bremen, Germany) seeded with HEK293 cells that were ~90% confluent. Another 100-μl tissue culture medium containing 2 x 10^5 particles of WT Adenovirus-2 was added to each well to enhance transgene expression of the AAV-transduced cells. Cells were collected after 24–48 h, washed in PBS, and resuspended in 2% paraformaldehyde. GFP expression was quantified by flow cytometry and plotted against the serum dilution. Titers of neutralizing Abs were estimated by determining the dilution of mouse serum that inhibited 50% of AAV transduction as measured by GFP-transgene expression.

**Anti-SRBC ELISA**

SRBC ghosts were prepared as described previously (14) and aliquots stored at −80°C until needed. Flat-bottom polystyrol microtiter plates (Nunc, Rochester, NY) were coated with 100 ng SRBC preparation in 100 μl PBS well and allowed to settle for 24 h at 4°C. Plates were washed twice with STT buffer (0.2 M NaCl, 0.04 M Tris [pH 9.0]) and blocked with 1% BSA in STT buffer for 24 h at 4°C. Plates were washed twice with STT buffer, and mouse sera (1:100) were added in a total volume of 100 μl PBS/well and incubated for 1.5 h at 37°C. Plates were washed twice with STT buffer and incubated with either rat anti-mouse IgG or IgM-alkaline phosphatase (Serotec, Oxford, U.K.) or goat anti-mouse IgM-alkaline phosphatase (Serotec) for 1 h at 37°C, then washed again with STT buffer. A total of 100 μl peroxidase substrate (Serotec) was added to each well. Plates were covered and incubated for 1 h at room temperature; then 500 μl EGTA was added to each well to stop the reaction. Plates were read using SpectraMax-plus microplate reader (Molecular Devices, Sunnyvale, CA).

**H&E staining and immunohistochemistry**

For H&E staining, mouse spleens were fixed in 10% neutral buffered formalin and sections were stained using standard methods. The prepared slides were stored at room temperature. Imaging was performed using an Olympus BX51 Wide-field Microscope (Olympus).

For immunohistochemistry, fresh-frozen splenic sections were cut at 4–6 μm, air-dried at room temperature for 4–5 h, then incubated in 2% universal blocking reagent (PowerBlock, BioGenex) at room temperature for 10 min. Sections were then rinsed three times with PBS and incubated in a humidity chamber at room temperature for 30 min with PBS containing optimal concentrations of PE-labeled rat anti-mouse IgD (Acris GmbH), FITC-labeled peanut agglutinin (PNA) (Sigma), Alexa 647-labeled rat anti-mouse B220 (BD), and/or Alexa 488-labeled hamster anti-mouse CD3 (AbD Serotec). The sections were then rinsed three times with PBS at room temperature, mounted gently with Dako Fluorescence Mounting Medium, covered with coverslips, and data were acquired with an Olympus IX70 Wide-field Fluorescence microscope (Olympus) and analyzed with Velocity 6.0.1 software (PerkinElmer). A threshold level was determined, and a fixed-sized rectangular area was drawn inside each image. The mean fluorescence value for the specified area in each image (CD3, B220, PNA, and IgD) was quantified using ImageJ (National Institutes of Health, Bethesda, MD), and the ratio of the mean fluorescence values for different images was then calculated.

**Intracellular calcium measurements**

Splenic B cells were isolated by negative selection using the EasySep B cell enrichment kit (StemCell Technologies, Vancouver, BC, Canada). A second cycle of enrichment was conducted for all B cell purities <96%. A total of 1 x 10^7 B cells was incubated with 2 μM indo-1 AM (Sigma (Aldrich, St. Louis, MO) in RPMI 1640 at 37°C for 30 min. Cells were washed with RPMI 1640, stained using PE-conjugated rat anti-mouse CD19 (eBioscience, San Diego, CA), then loaded with either calcium buffer (1.5 mM CaCl2, 150 mM NaCl, 3 mM KCl, 20 mM Hepes, 10 mM d-glucose, 0.25 mM sulfinpyrazone) or EGTA buffer (25 mM EGTA, 150 mM NaCl, 3 mM KCl, 20 mM Hepes, 10 mM d-glucose, 0.25 mM sulfinpyrazone). Samples containing 2 x 10^5 B cells in 1 ml were incubated at 37°C, and basal levels of indo-1 fluorescence were acquired for 30 s before cell stimulation. Cells were then stimulated using F(ab')2 goat anti-mouse IgM (Jackson Immunoresearch Laboratories, West Grove, PA) or F(ab')2 goat anti-mouse IgG (Immunoresearch Laboratories, West Grove, PA). Thapsigargin (Calbiochem, EMD, Gibbstown, NJ) was used to induce passive calcium store release, and ionomycin (Molecular Probes) was used to assess indo-1 loading. Changes in fluorescence ratios of indo-1 emission at 405/485 nm were collected by flow cytometry using LSR II analyzer (BD). Data were analyzed using FlowJo software.

**Flow cytometry**

Purified B cells subjected to different conditions of stimulation were stained using allophycocyanin-conjugated hamster anti-mouse CD90 or PE-conjugated rat anti-mouse CD86 (eBioscience). Total splenocytes obtained from the same spleens were stimulated using purified rat anti-mouse CD3 (eBioscience), then stained with FITC-conjugated hamster anti-mouse CD3e (eBioscience) and allophycocyanin-conjugated hamster anti-mouse CD69. Some samples were treated with cyclosporin A (150 ng/ml; Sigma-Aldrich) immediately before cell stimulation. Data acquisition was performed using a Becton Dickinson FACScan cytometer (BD Biosciences). Data analysis was conducted using FlowJo software (Tree Star, San Carlos, CA).

**Statistics**

Data were statistically evaluated using the two-tailed unpaired Student t test. A p value <0.05 was considered statistically significant.

**Results**

**Impaired humoral immunity in Cd20−/− mice**

i.v. administration of AAV is known to result in TD humoral immunity, with development of neutralizing anti-AAV Abs (15–17). We injected 1 x 10^11 AAV particles i.v. into five Cd20−/− and five WT mice, and determined the levels of neutralizing Abs at days 14 and 21 postimmunization using transduction assays. The results showed that WT mice generated higher anti-AAV neutralizing Ab responses (mean ±1:11,000 on day 21 after AAV injection) compared with Cd20−/− mice (mean ±1:6000 at the same time point; Fig. 1).

![Image](http://www.jimmunol.org/)

**FIGURE 1.** Reduced neutralizing Ab response to AAV in Cd20−/− mice. Neutralizing Ab titers were measured in C57BL/6 WT and Cd20−/− mice. Five WT mice (solid boxes) and age- and sex-matched Cd20−/− mice (solid circles) were immunized with 1 x 10^11 AAV particles i.v., and sera were collected at the time points indicated. Anti-AAV titers were determined by AAV transduction assays. Each mouse is represented by a box or circle. y-Axis represents the inverse of serum dilution (*p < 0.05).
SRBCs have been extensively used to assess TD humoral immunity in rodents. To further examine the effect of CD20 deficiency on Ab responses to a TD Ag, we primed *Cd20<sup>−/−</sup>* and WT mice with 1 × 10<sup>7</sup>, 3 × 10<sup>7</sup>, or 1 × 10<sup>8</sup> SRBCs on day 0 and boosted with similar doses on day 21. Serum IgM and IgG anti-SRBC were measured by ELISA on days 0, 4, 7, and 14, and IgG anti-SRBC were additionally measured on days 21, 25, 28, and 35. Typical time-course data showed that the peak IgM and IgG responses occurred 4 d after the initial or booster injections, respectively (Fig. 2A, 2B). At these time points, the primary IgM response was significantly reduced in *Cd20<sup>−/−</sup>* mice at all doses of SRBCs (*p* < 0.05; Fig. 2C), and the secondary IgG response was significantly reduced after injection with 3 × 10<sup>7</sup> SRBCs (*p* < 0.01). IgM anti-SRBC was reduced by ~45% after immunization with 1 × 10<sup>7</sup> SRBCs, by 24% after immunization with 3 × 10<sup>7</sup> SRBCs, and by 21% after immunization with 1 × 10<sup>8</sup> SRBCs. IgG anti-SRBC was reduced by ~47% in *Cd20<sup>−/−</sup>* mice on day 25 after injection with 3 × 10<sup>7</sup> SRBC (Fig. 2D). IgG anti-SRBC

![Graphs showing immune responses to SRBCs](image-url)
responses were mostly of the IgG1 and IgG2b isotypes, and both were significantly reduced in Cd20−/− mice (Fig. 2E).

**Reduced GC B cell numbers in immunized Cd20−/− mice**

GCs develop in response to TD Ags. During the primary immune response, GCs typically appear 2–4 d after immunization, become fully developed by 7–10 d, and persist for about 3 wk. To determine whether lower anti-SRBc responses in Cd20−/− mice were associated with a detectable reduction in GC B cells, four WT and four Cd20−/− mice were immunized with 3 × 10^7 SRBCs, and spleens were collected on day 7 postimmunization. Flow cytometric analysis performed using the GC markers PNA and GL7 demonstrated significant reduction in the numbers of PNA+ or GL7+ splenic B cells in Cd20−/− mice (Fig. 3A, 3B). No significant change in the size or distribution of the red and white pulp (Fig. 3D), or in the distribution of T and B cells (Fig. 3C, left panels), was noted in Cd20−/− mice spleens as compared with WT. However, splenic sections costained with PNA and anti-IgD, a marker for non-GC B cells, showed an obvious deficiency in the gross formation of GCs, as visualized by reduced PNA staining, in two of the four Cd20−/− mice examined (Fig. 3C, right panels).

**Impaired BCR-activated calcium influx in Cd20−/− mice**

Calcium signaling resulting from BCR ligation is one of the important mechanisms initiating B cell activation, and previous work has implicated CD20 in BCR-activated calcium mobilization. To examine calcium signaling in Cd20-deficient B cells, we loaded splenic B cells from Cd20−/− and WT mice with the calcium-sensitive ratiometric dye, indo-1. After acquiring basal levels of indo-1 fluorescence emission, we stimulated the cells using F(ab')2 anti-IgM. Calcium responses were found to be lower in Cd20−/− B cells compared with WT B cells (Fig. 4A). This was a highly reproducible finding in >20 independently conducted experiments using a range of Ab concentrations from 1 to 40 μg, with the greatest differences seen at suboptimal concentrations (Supplemental Fig. 1). No difference in surface IgM expression was detected (Supplemental Fig. 2). Chelation of extracellular calcium with EGTA abolished the difference in responses between Cd20−/− and WT B cells (Fig. 4A). Cross-linking the BCR in memory B cells using F(ab')2 anti-mouse IgG also resulted in lower magnitude of calcium flux in Cd20−/− B cells (Fig. 4B). Calcium flux elicited using ionomycin was similar in Cd20−/− and WT B cells, indicating no difference in indo-1 loading (Fig. 4C). To assess whether there was a difference in the amounts of intracellular stored calcium, we treated cells with thapsigargin, a specific inhibitor of sarcoplasmic/endoplasmic reticulum calcium ATPases that maintains the steep gradient of calcium across the endoplasmic reticulum membrane. Thapsigargin treatment therefore results in the unopposed leak of calcium into the cytoplasm and depletion of the store. Calcium flux elicited using thapsigargin was similar in Cd20−/− and WT B cells, indicating no difference in the amounts of stored calcium (Fig. 4D). These findings indicate that the difference in BCR-activated calcium mobilization between Cd20−/− and WT B cells is due to calcium influx and not due to calcium release from intracellular stores.

It was previously reported that cross-linking CD19 profoundly reduced calcium mobilization in Cd20−/− B cells (9). Therefore, we were interested to determine the effect of coligating CD19 with the BCR, an experimental condition that is thought to mimic coligation of the BCR with the CD21/CD19 receptor complex by complement component C3d-opsonized Ag. We used biotin-conjugated F(ab')2 anti-IgM and anti-CD19 Abs premixed at varying ratios with a fixed molar ratio of avidin. Surprisingly, we found that, regardless of the stoichiometry of coligation, calcium mobilization was enhanced in Cd20−/− B cells when CD19 was cross-linked to the BCR (Fig. 5). No difference in surface CD19 expression was detected (Supplemental Fig. 2).

**Impaired B cell activation in Cd20−/− mice**

BCR-activated calcium influx is clearly defective in Cd20-deficient B cells. To test whether CD20 deficiency affects cellular activation, we examined upregulation of CD86 and CD69 in purified splenic B cells after stimulation with 1 μg F(ab')2 anti-IgM. As a control, we examined the expression of CD69 in T cells from the same splenic populations activated with 1 μg anti-CD3. Expression of CD69 and CD86 was similar in unstimulated WT and CD20−/− B cells (9). Therefore, any differences in T and B cell responses were mostly of the IgG1 and IgG2b isotypes, and both were significantly reduced in Cd20−/− mice (Fig. 2E).

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CD20-deficient B cells. After activation, however, expression of both markers was reduced in CD20-deficient B cells (Fig. 6A, 6B, respectively), whereas there was no difference in CD69 upregulation in activated T cells from the same WT and Cd202/2 mice (Fig. 6C). Impaired upregulation of both CD69 and CD86 in CD20-deficient B cells was also observed in the presence and absence of anti-CD40 costimulation, and at a higher concentration of anti-IgM (Fig. 6D, 6E). In T cells, CD69 upregulation requires sustained high levels of intracellular calcium achieved with anti-CD3 cross-linking (18) or with ionomycin (19). Induction of CD69 expression in ionomycin-treated T cells was prevented by inhibition of calcineurin using cyclosporin A (19). To confirm that upregulation of CD69 was calcium dependent in B cells, we incubated WT and CD20-deficient B cells with anti-IgM in the presence or absence of cyclosporin A. We found that CD69 upregulation was completely inhibited under these conditions (Fig. 6F).

**Discussion**

To evaluate TD humoral immunity in Cd20−/− mice, we used two complex particulate Ags, AAV and SRBC, for immunization. Our results show that Cd20−/− mice had reduced levels of neutralizing Abs against AAV, and reduced primary and secondary responses to SRBCs. The secondary anti-SRBC response in both WT and Cd20−/− mice was composed mainly of IgG1 and IgG2b Abs, and was associated with reduced GC B cell numbers, characteristic of TD humoral immunity. A more stringent requirement for CD20 during primary immune responses was suggested by the significant reductions in IgM anti-SRBC in Cd20−/− mice at all doses of SRBCs used for immunization, whereas IgG responses were significantly reduced only after immunization with an intermediate dose of SRBCs. A preferential requirement for CD20 during the primary immune response may reflect differences in intracellular signaling mediated through surface IgM versus surface IgG, or other requirements of naive versus memory B cells.

Uchida et al. (9) examined TD antihapten responses to DNP-KLH and 4-hydroxy-3-nitrophenylacetyl–chicken gamma globulin in Cd20−/− mice and reported no effect of CD20 deficiency on the response to either Ag. However, a trend toward lower IgM and IgG responses was apparent in the DNP-KLH–immunized Cd20−/− mice (9), and might have been evident in a larger study. Alternatively, TD humoral immunity to these small synthetic Ags may be less dependent on CD20 than complex particulate Ags, such as those used in our study.

The effect of CD20 deficiency in humans is more severe than in mice. Whereas basal levels of all serum Igs are normal in Cd20−/− mice, the case report of a Cd20−/− CVID patient described persistently low serum IgG levels over several years of testing (8). Serum IgM was at the high end of the normal range, suggesting a defect in isotype switching. Consistent with this, switched memory B cells were low in the circulation, and in vitro activation of the patient’s B cells with either TD or TI stimuli resulted in normal proliferation and IgM secretion, but absent or very reduced IgG. The response to vaccination with TI Ags was low. Although the response to one TD vaccine, tetanus toxoid, was normal, the persistently low serum IgG suggests decreased TD immune responses. These data, together with our own findings, indicate that in both mice and humans, CD20 expression is essential for optimal humoral immunity to both TI and TD Ags.
The effect of CD20 on humoral immunity is probably the result of its role in calcium mobilization. In numerous independent experiments conducted on murine Cd20−/− naive B cells, we found that calcium influx after BCR stimulation was consistently reduced. Uchida et al. (9) also reported lower calcium responses after anti-IgM stimulation, although in their study lower calcium flux was not entirely the result of reduced extracellular calcium entry and may have been due, in part, to an effect on the release of calcium from intracellular stores. Lower expression of surface IgM on splenic B cells from their Cd20−/− mice might account for this effect (9). However, we detected no difference in the level of surface IgM on splenic B cells from the Cd20−/− mouse line generated by O’Keefe et al. (12), and yet we found BCR-activated calcium influx to be consistently reduced by ~50%. Similar experiments performed by Neuberger’s group (12) showed no significant reduction in calcium mobilization using F(ab′)2 anti-IgM at either 1 or 20 μg/ml. A possible explanation may lie in the source of anti-IgM used to stimulate the cells, or perhaps in the concentration of Ab, because we found the greatest differences at intermediate suboptimal concentrations of F(ab′)2 anti-IgM (5–10 μg/ml; Supplemental Fig. 1). Alternatively, other experimental conditions, such as indo-1 dye loading, may be responsible for inconsistent results between the two studies.

In human B cells, the involvement of CD20 in BCR-activated calcium influx has been demonstrated using Ramos cells in which Cd20 was downregulated with siRNA (6). However, experiments on B cell lines derived from the Cd20−/− CVID patient showed no effect on calcium responses induced by either anti-IgM or anti-IgG (8). The manipulations required to generate these B cell lines undoubtedly altered the characteristics of the peripheral blood B cells from which they were derived (20), so it cannot be assumed that calcium signaling is not affected by CD20 deficiency in unmanipulated primary human B cells. Nevertheless, this finding shows that the requirement for CD20 in BCR-activated calcium mobilization is not absolute. The inability of Neuberger’s group to detect altered calcium mobilization in Cd20−/− murine B cells when CD19 was cross-linked to the BCR. The significance of this observation in relation to humoral immunity is unclear because we found no evidence of enhanced Ab responses to AAV or SRBCs; however, it suggests that CD20 is unlikely to function directly as a calcium channel but rather modulates calcium influx depending on the conditions of engagement.

Others have shown that phosphorylation of src-family tyrosine kinases, phospholipase Cγ-1, CD19, Btk, and MAPK, is normal in Cd20-deficient B cells (9), and that there are no apparent in vitro defects in proliferative responses to anti-IgM, LPS, or IL-4 (9, 12). However, in addition to reduced calcium influx, we also found that upregulation of the activation markers CD69 and CD86 was impaired by CD20 deficiency. In T cells, upregulation of CD69 is known to depend on sustained high levels of calcium (19), and we confirmed that this was the case for B cells in our experiments. Impairment in both CD69 and CD86 upregulation was observed after BCR cross-linking in either the presence or absence of CD20.
absence of anti-CD40. However, it was not strictly dependent on BCR stimulation because some effect was observed even when CD40 alone was cross-linked. Physical and functional links between CD40 and CD20 have been previously reported (21, 22), but because anti-CD40 is not known to activate calcium responses, the basis of the effect of CD20 deficiency on B cell activation by anti-CD40 is not clear.

In summary, data reported in this article show for the first time, to our knowledge, reduced BCR/CD40-mediated B cell activation in vitro in CD20-deficient B cells, and reduced TD humoral immunity in Cd20⁻/⁻ mice. Thus, although CD20 is a member of the membrane-spanning 4-domain A family of genes, several of which may be expressed in B cells (1, 2), its role in humoral immunity is not redundant and its absence results in decreased Ab responses in both humans and mice. The influence of CD20 is likely to be at the level of BCR-mediated signaling leading to calcium influx; however, its function is more likely to be modulatory than to direct involvement as a calcium channel. It seems likely that reduced calcium responses and suboptimal cellular activation in Cd20⁻/⁻ B cells leads to reduced humoral immunity; however, it is possible that other undiscovered effects of CD20 deficiency contribute to the phenotype observed.

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Disclosures
The authors have no financial conflicts of interest.

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