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Successful Construction and Massive Expression of a Novel Anti-CD19 Human-Mouse Chimeric Antibody Hm2E8b

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CD19 antigen is a major target for human B cell malignancies. Many studies have shown that the antibodies recognizing this antigen hold clinical therapeutic potential, while CD19 antibody of mouse origin requires genetic engineering to reduce the potential side effects of the antibody for their clinical use. There are many clones of CD19 antibodies available with different subclasses of immunoglobulin. IgM type antibody holds a high affinity and high complement activating capacities facilitating the targeting efficacy when it is used in targeting therapy. However, engineering the murine IgM antibody into a functional humanized antibody remains a challenge. The aim of this study was to construct a chimeric antibody composed of a CD19 specific murine IgM antibody 2E8 single-chain antibody fragment (scFv) and human IgG1 Fc region, which was named 2E8scFv-Fc or Hm2E8b. The function and the biological activities of this engineered antibody were characterized using a variety of approaches including cellular, immunological, flow cytometric, and molecular biological approaches. After switching from IgM- to IgG-like type antibody, Hm2E8b retained full antigen-binding activity to membrane CD19 antigen as its parental antibody 2E8, and the immune effector function analysis revealed that it could mediate complement-dependent cytotoxicity (CDC) to kill the target cells via IgG1 Fc domain. The yield of the engineered antibody Hm2E8b in the supernatant was 13.3 μg/mL expressed and secreted in the CHO cell system, which reached the secretory quantity of a regular mouse hybridoma cells. Our conclusion is that the IgM type of CD19 mouse antibody can be successfully engineered into an IgG1 type human-mouse chimeric antibody with similar affinity and biological activity. The yield of the Hm2E8b expression and secretion in CHO cell system was adequate to facilitate further development for therapeutic purpose.

Introduction

Immunotherapy using monoclonal antibodies (MAbs) is an effective and safe method for the treatment of human lymphoid malignancies.[1] In the last decade, CD20 is the major target for the B cell diseases. Many non-Hodgkin's lymphomas (NHLs) and some B cell leukemias have been successfully treated by combining chemotherapy with rituximab, a chimeric anti-CD20 antibody. However, some B cell tumors lack CD20 expression or lose it during the course of rituximab treatment,[2,3] which results in the poor response to rituximab in some patients; or in some cases, patients gradually lose responsiveness during continuous administration and end up in relapse.[4] Therefore, it is necessary to develop novel antibodies that recognize target proteins exclusively expressed on the malignant cells.

CD19 is a 95 kDa transmembrane glycoprotein and member of the Ig superfamily.[5] It is B lineage specific and is expressed on most B cells from the earliest stages of B progenitor development through the terminal differentiation into plasma cells.[6] As compared to CD20, CD19 is expressed on most acute lymphoblastic leukemias (ALL), chronic lymphocytic leukemia (CLL), and lymphomas of B lineage.[7] CD19 is rarely lost during the process of neoplastic transformation and is not expressed on normal hematopoietic stem cells or on normal tissues outside the B lineage. CD19 is not shed into circulation, therefore there is no soluble CD19 to compete for the binding of CD19-specific antibody to cell surface antigen. Several CD19-specific antibodies have been evaluated for the treatment of B lineage malignancies *in vitro* in both mouse models and clinical trials, including unconjugated antibodies,[8,9] antibody-drug conjugated,[10,11] and bispecific antibodies targeting CD19 and CD3.[12,13] Anti-CD19 MAbs can induce growth arrest or death of tumor cells, recruit effector cells, reverse P-gp-mediated multi-drug resistance, and deliver organic compounds, toxins, and radioisotopes to target

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cells.[14–17] Despite recent clinical studies with anti-CD19 antibodies demonstrating encouraging results, challenges remain in optimizing anti-CD19 antibodies to achieve improved outcome.

Zhejiang Children's Hospital (ZCH)-4-2E8 (2E8), a murine IgM-type anti-CD19 antibody, was obtained in our laboratory previously. We demonstrated that 2E8 and antibody norcantharidin conjugated immunotoxin (2E8-NCTD) could specifically target the CD19 expressing B lineage leukemia cells.[18–21] However, as 2E8 is a murine MAb, it is immunogenic and does not mediate effector function in humans due to the murine origin of its constant region. In our previous study, a chimeric antibody Hm2E8 containing the murine antibody 2E8 variable domains and human IgG1 constant domains was constructed. However, the chimeric antibody Hm2E8 was only expressed in the cytoplasm of sf9 cells and lost antigen binding activity (unpublished data), which may be attributed to the possibility that the human IgG1κ leader used for Hm2E8 expression did not favor correct remodeling and secretion of murine IgM-type antibodies in the sf9 insect system, resulting in the absence of functional antibodies in the supernatant. In the present study, we amplified the 2E8 signal peptides from the parental IgM antibody 2E8 secreting hybridoma cell line by 5′RACE and connected the V_H and V_L domains by a short peptide linker to form a single-chain Fv (scFv) antibody fragment, which was then fused with the Fc (hinge, CH2, CH3) domains of human IgG1 to form human-mouse chimeric antibody 2E8scFv-Fc (Hm2E8b). The results revealed that the 2E8scFv-Fc fusion protein retained the specific antigen-binding affinity of the parental antibody 2E8 and was abundant and well secreted into supernatants.

Material and Methods

Construction of expression vector

The RNA was extracted from the 2E8 hybridoma cells and reverse transcribed into the cDNA by 5′RACE (Takara, Otsu, Shiga, Japan). The variable region genes of 2E8 were cloned into the pGEM-T easy vector (Promega, Madison, WI) and sequenced, and then the signal peptides were predicted by Signal P3.0 software. The V_H containing signal peptide of 2E8V_H and V_L were ligated with the linker peptide $(Gly_4Ser)_3$ by splice overlap extension. The resulting PCR product was then digested with BamH I and EcoR I (Promega) and inserted into the same sites of pHMCH3 expression vector (constructed in our lab previously) containing hinge, CH2, CH3 of human IgG1, which was named pHMCH3-Hm2E8b (Fig. 1).

Transfection into CHO cells

CHO cells were grown in RPMI-1640 medium with 10% newborn calf serum at 37°C in a humidified atmosphere of 5% CO_2 and were split when they reached 70–80% confluency by trypsinization. CHO cells were plated at a density of 1×10^5 cells/well in 24-well plates 24 h before transfection without the addition of penicillin and streptomycin. CHO cells were transiently transfected with either purified pHMCH3-Hm2E8b or pHMCH3 vector using Lipofectamine™ 2000 Reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol. Positive clones were selected in the presence of 600 μg/mL G418 (BBI, Madison, WI), and single cell clones were isolated by limiting dilution. The highest antibody-producing clone was chosen after flow cytometric analysis for further experimentation.

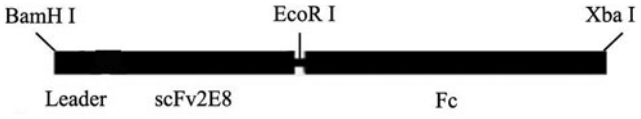

FIG. 1. Details of cloning sites of pHMCH3-Hm2E8b.

Purification of Hm2E8b antibody

The supernatant was harvested and concentrated 20-fold with an Amicon ultra-15 centrifugal filter unit (Millipore, Darmstadt, Germany), then purified by a Bio-scale™ Mini Affi-prep Protein A cartridge (Bio-Rad, Hercules, CA). The eluted fractions were again concentrated five-fold with the Amicon centrifugal filter (Millipore).

Western blot analysis

Purified Hm2E8b was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), according to the method described by Sambrook and colleagues,[22] with 12% separation polyacrylamide gel and 5% condensed gel. The gel was then transferred onto the PVDF membrane. After blocking with milk for 2 h, the transferred membrane was incubated with 1:1000 dilution of HRP-conjugated goat anti-human IgG1 Fc antibody (Sigma, St. Louis, MO) for 2 h. Specific binding was detected with ECL Western blotting detection reagents (Bio-Rad).

Binding activity of Hm2E8b detected by flow cytometric analysis

Briefly, 5×10^5 Nalm-6 cells were added to each tube and incubated with 0.5 μg of the purified Hm2E8b for 30 min on ice. After washing three times with phosphate-buffered saline (PBS), the cells were stained with fluorescein isothiocyanate (FITC) labeled goat anti-human IgG Fc antibody (KPL, Gaithersburg, MD) for 30 min on ice in the dark, followed by another three rinses with PBS. The fluorescent signals were detected by a FACSCalibur™ flow cytometer (Becton-Dickinson, San Jose, CA) using Cellquest software.

The specificity of its binding to the CD19 antigen was further assessed by blocking test to its parental antibody 2E8. For this purpose, 5 μg of purified Hm2E8b was added to 5×10^5 cells and incubated for 30 min on ice. After washing three times with PBS, the cells were further incubated with parental antibody 2E8-FITC for 30 min on ice in the dark. The cells were washed and analyzed by flow cytometry.

Competitive inhibition assay

Nalm-6 cells were incubated on ice with the 2E8-FITC at a fixed sub-saturated concentration of 0.5 μg/mL either alone or in the presence of varying concentrations of either the Hm2E8b or the parental 2E8 antibody as inhibitors. After washing, the cells were analyzed by flow cytometry. Relative inhibition of binding by 2E8-FITC was calculated as follows: % of inhibition = [(% of positive cells without inhibitor – % of positive cells with inhibitor)/(% of positive cells without inhibitor)] × 100%. The experiment was performed three times and the mean values were calculated. IC_{50} values (concentration of inhibitor producing 50% inhibition of binding by

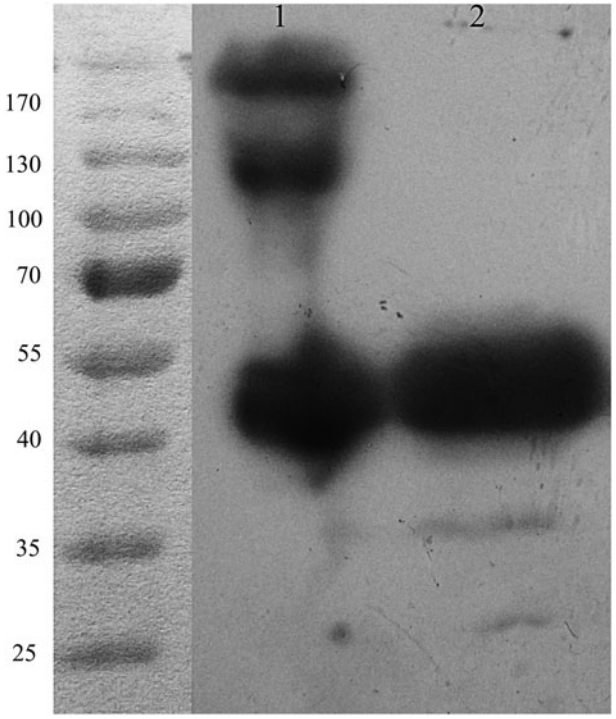

FIG. 2. Western blot analysis of Hm2E8b. Protein ladder is shown in the left lane while Hm2E8b was run under non-reducing (lane 1) and reducing (lane 2) conditions detected with HRP-conjugated goat anti-human IgG1 Fc antibody.

2E8-FITC) were calculated using a sigmoidal dose-response curve fit.

Complement-dependent cytotoxicity assay

CDC assay was performed as described previously.[23] Briefly, CD19 positive human leukemia cell line Nalm-6 cells were used as target. Nalm-6 cells were washed and suspended in serum-free RPMI-1640 at a concentration of 1×10^6 cells/mL. Fifty μL (5×10^4) of the cell suspension were added to each well of a 96-well, flat-bottom plate and 25 μL different concentrations of the Hm2E8b and 2E8 antibodies were added per well. Cultures were performed in triplicate and plates were incubated for 1 h. As a source of complement, 25 μL normal human serum prepared from healthy volunteers was added and incubated at 37°C for 2 h. Wells omitting antibodies while retaining the same amount of culture medium and complement added or omitting Nalm-6 cells while retaining the same amount of both antibodies and complement were set as the negative controls. Ten μL of 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-8) were added to each well and incubated for an additional 4 h at 37°C. The absorbance (A_{450}–A_{650}) of the formazan dye produced by metabolically active cells of each well was detected on a GeneQuant II (Biotech, Piscataway, NJ). Cytotoxicity was calculated according to the following formula:

$$\%\ \text{of cytotoxicity} = 100\times(\text{E}-\text{S})/(\text{M}-\text{S})$$

where E is the absorbance of experimental well, S is that in the absence of MAb (cells were incubated with medium and complement alone), and M is that of antibody and complement in the absence of target cells.

Results

Construction, expression, and purification of Hm2E8b

Hm2E8b was constructed by joining the murine scFv2E8 with the human IgG1-Fc fragment at the hinge region (Fig. 1). CHO cells were stably transfected with pHMCH3-Hm2E8b by Lipofectamine 2000 Reagent (Invitrogen) and selected with G418. Hm2E8b was successfully purified from culture medium by affinity chromatography using A-Sepharose. The yield of the purified antibody was at approximately 2 mg/mL in 1 mL after concentration from 150 mL of supernatant, which represented at least 13.3 μg/mL in unconcentrated culture supernatant. Western blot analysis showed that under the reducing condition only one band at 50 kDa was observed, which was in close agreement with the predicted molecular weight of 54kDa (Fig. 2, lane 2). Under non-reducing conditions, the purified protein showed three bands on the gel with molecular weights of 50, 130, and 200 kDa, respectively (Fig. 2, lane 1).

Binding activity of Hm2E8b

The specificity of Hm2E8b to recognize membrane CD19 antigen was examined by flow cytometry using a human pre-B leukemia cell line Nalm-6, which has been previously characterized to express CD19 on the cell membrane. As shown in Figure 3, Hm2E8b bound to Nalm-6 cells with

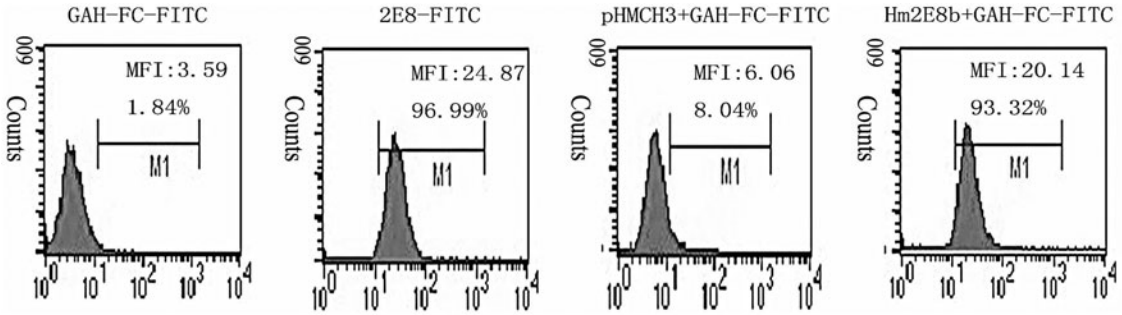

FIG. 3. Flow cytometry analysis of Hm2E8b antibody. Four groups including background control (GAH-Fc-FITC), positive control (2E8-FITC), culture supernatant from CHO-pHMCH3 and culture supernatant from CHO-Hm2E8b. Two parameters as percentage of positive cells (%Gate) and mean fluorescence intensity (Geo mean) were used to assess the binding ability of the Hm2E8b antibody.

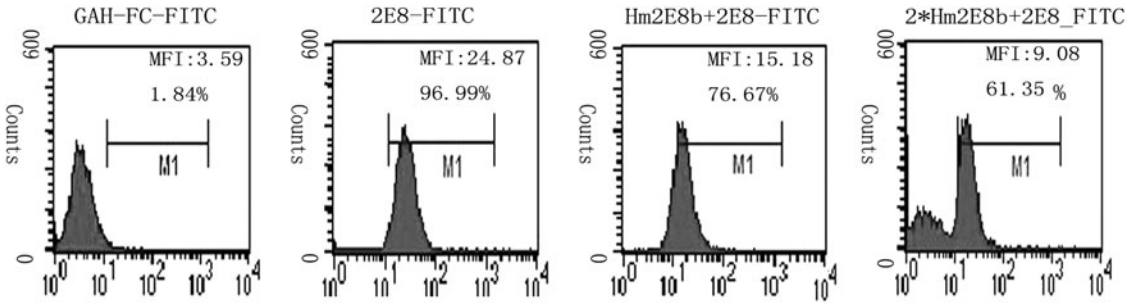

FIG. 4. Blocking test of Hm2E8b antibody to 2E8-FITC. Binding activity of parental 2E8 antibody was partially blocked by chimeric antibody Hm2E8b. The panel includes four groups from left to right, representing background control (GAH-Fc-FITC), positive control (2E8-FITC), Hm2E8b + 2E8-FITC, incubated with Hm2E8b twice + 2E8-FITC. Two parameters as percentage of positive cells (%Gate) and mean fluorescence intensity (Geo mean) were used to assess the binding ability of the Hm2E8b antibody.

comparable capacity to its parental murine antibody 2E8. The blocking test using our 2E8 MAb confirmed the above results (Fig. 4), which indicated that the engineered chimeric antibody Hm2E8b retained full antigen-binding activity as its parental murine antibody 2E8.

Relative affinity was assessed by competitive inhibition assay of the ability of the Hm2E8b or the 2E8 to compete with 2E8-FITC for binding to Nalm-6 cells. As shown in Figure 5, both Hm2E8b and 2E8 competitively inhibited the binding of 2E8-FITC in a concentration-dependent manner. To compare the affinity of Hm2E8b and that of parental 2E8, the IC_{50} values were determined. The IC_{50} values for the Hm2E8b and 2E8 were approximately 68 and 15 μg/mL, respectively. Thus the concentration of Hm2E8b (a monomer) to achieve half-maximum inhibition was slightly higher than that of 2E8 (a pentamer), indicating reduced binding affinity in comparison to its parental IgM antibody 2E8.

Killing target cells by complement-dependent cytotoxicity

While negative controls showed very limited killing cytotoxicity, both the murine IgM antibody 2E8 and the chimeric antibody Hm2E8b could kill CD19 positive Nalm-6 cells by CDC (Fig. 6) in a dose-dependent manner. The LD_{50} values of 2E8 and Hm2E8b to Nalm-6 cells were 25 and 52 μg/mL, respectively.

Discussion

Antibody therapy has proven to be one of the most promising therapies for hematopoietic malignancies. CD19 has been identified as an excellent target for B lineage malignancy targeting. 2E8 is a novel murine IgM antibody that can specifically target the CD19 expressing B lineage leukemia cells. Unfortunately, murine antibody cannot be used in therapy due to the human anti-mouse antibody (HAMA) responses in clinical patients.[24] This obstacle can be overcome by antibody

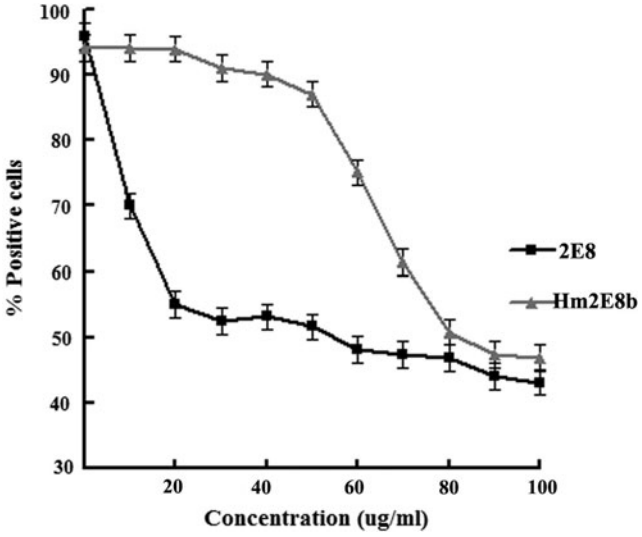

FIG. 5. Competitive inhibition assay of Hm2E8b antibody. Decrease of 2E8-FITC binding with increasing concentrations of inhibitors Hm2E8b and 2E8 was monitored by flow cytometry. Approximately 4.5-times greater concentrations of Hm2E8b than that of the parental 2E8 antibody were needed to achieve half-maximum inhibition.

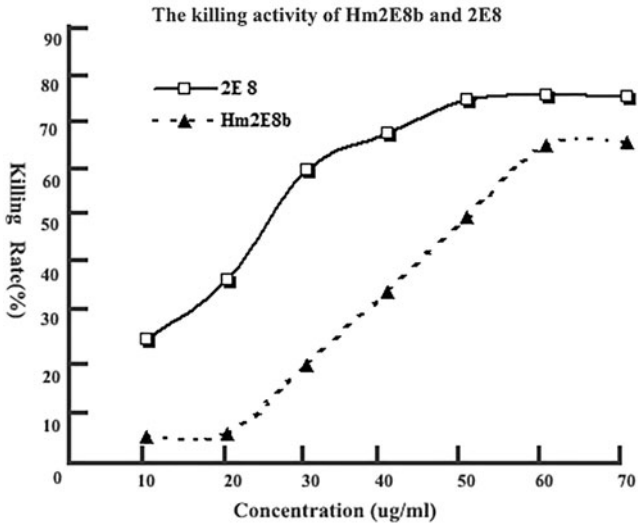

FIG. 6. Complement-dependent cytotoxicity of Hm2E8b antibody. $CD19^+$ B lineage leukemia cell line Nalm-6 was used as target cell. Results showed that Hm2E8b and 2E8 could kill Nalm-6 cells effectively in a dose-dependent manner.

engineering or humanization to maximally reduce the antigenicity. On the other hand, how to humanize a murine IgM antibody into a functional antibody is still a critical issue. In 1994, Zebedee and colleagues generated a mouse-human IgG1 type chimeric antibody (ch2D10) by connecting the variable regions of a murine IgM antibody (m2D10) to the constant regions of a human IgG1. However the engineered antibody had significantly reduced binding affinity in comparison to its parental murine antibody.[25] Wang and colleagues constructed a similar chimeric antibody (C1-28), which lost its binding activity.[26] The loss of binding activity was considered to be a consequence of the fact that the Fv domain was apt to dissociate due to the change of constant region; the problem was resolved by fusing a scFv of 1-28 with the human IgG1 constant region to form a new chimeric antibody 5S, which was expressed in the 293T human embryonic kidney cell culture and yielded a supernatant of approximately 50 μg/mL IgG antibody. Most importantly, with this effort, the chimeric antibody retained the binding activity of its murine counterpart.[27]

In the present study, we have successfully constructed a novel chimeric antibody Hm2E8b by linking the anti-CD19 mouse IgM scFv2E8 to human IgG1 Fc region. Because the Fc region of human IgG1 contains a hinge region, Fc fusions can offer disulfide bonds for dimerization. The Western blot analysis has shown that the molecular weight of the homodimer was not strictly twice that of the monomer, which is possibly caused by extensive glycosylation of the antibody in CHO cells and/or a result of denaturation during electrophoresis.[28] The scFv-Fc Hm2E8b could also self-assemble into multimers; the cross-pairing of the variable regions may be the basis of the multimerization of the scFv-Fc constructs.[29] Hm2E8b has retained nearly full antigen-binding activity of its parental murine antibody 2E8.

Although the binding effects of the engineered Hm2E8b were reduced significantly after the IgM-type antibody was engineered into a IgG-type antibody, it is reasonable that the engineered IgG type chimeric antibody is only a monomer while the original IgM type parental antibody was a pentamer. So we considered that the antibody subtype switch most probably would not hamper the clinical application if it is used in patients in the future.

Another important issue for successful antibody engineering is the yields of the antibody expression after engineering and transfection in host cells. Using our pHMCH3 vector system, the yield of our Hm2E8b reached 13.3 μg/mL after several purification/concentration steps, which is comparable to the productive amount of a regular mouse hybridoma cell line in culture media. Mass production of Hm2E8b could be achieved by using a bioreactor with continuous perfusion, which can yield 1 g/L of chimeric antibodies.[30]

Taken together, our results have shown that by switching from an IgM- to IgG-like antibody is a viable option in the humanization of murine IgM antibody. The engineered antibody Hm2E8b presents a potent biological activity of recognition and kill of CD19 positive human leukemia cells, which warrants the further development of this antibody for clinical testing.

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Author Disclosure Statement

The authors have no financial interests to disclose.

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