Bovine hemoglobin (glutamer-250, Hemopure)-specific immunoglobulin G antibody cross-reacts with human hemoglobin but does not lyse red blood cells in vitro

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BACKGROUND: Bovine hemoglobin (Hb)-based oxygen carrier (HbOC-201; Hb glutamer-250, Hemopure, Biopure Corp.) is a blood replacement and augmentation drug that increases oxygen-carrying capacity of circulating blood in patients with anemia and acute blood loss. The objective of this study was to assess the biologic significance (cross-reactivity, hemolysis) of humoral immune responses in humans receiving repetitive HbOC-201 administrations.

STUDY DESIGN AND METHODS: Serum samples containing immunoglobulin G (IgG) anti-HbOC-201 (n = 146) or no antibody (n = 16) were collected from subjects receiving HbOC-201 in clinical studies. IgG anti-HbOC-201 levels were quantified and the extent of cross-reactivity to human hemoglobin (HuHb) was assessed in direct-binding and competitive-inhibition immunoassays. Serum samples containing the highest levels of IgG anti-HbOC-201 were studied in a complement-mediated hemolysis assay for their ability to lyse human red cells (RBCs).

RESULTS: The IgG anti-HbOC-201 levels in the antibody-positive serum samples ranged from 0.7 to 86.8 μg per mL. Of the 146 IgG anti-HbOC-201–positive serum samples, 88.4 percent contained IgG antibodies whose binding to solid-phase HbOC-201 was competitively inhibited by incubation with soluble HuHb (11.6% [<20% inhibition]; 63% [20%-80% inhibition]; and 25.4% [>81% inhibition]). Direct-binding analysis to solid-phase HuHb confirmed that 74 percent contained IgG antibodies reactive with HuHb. Dichotomous competitive inhibition and direct-binding IgG anti-HuHb data correlated significantly ($r^2 = 0.77$, $p < 0.001$). Serum samples with the highest levels of IgG anti-HuHb, as identified from clinical studies, did not lyse human RBCs in the presence of exogenous complement or induce the direct sensitization of RBCs with human IgG or complement.

CONCLUSION: These analyses indicate that HbOC-201 administration elicits IgG antibodies in humans that react with bovine and HuHb, but do not cause hemolysis in vitro.

A number of hemoglobin (Hb)-based oxygen carriers (HbOCs) have been developed for use in humans and animals as substitutes for stored red blood cells (RBCs).[1,2] HbOC-201, for instance, is a gluteraldehyde-polymerized bovine Hb whose efficacy has been demonstrated in human clinical studies involving single and multiple administrations.[2-4] In vitro and in vivo studies have shown that HbOC-201 binds and off-loads oxygen more efficiently than human RBC Hb, with the consequence that it can increase the efficiency of RBC oxygen transport when introduced into the circulation.[5] In previous work,[6] we have demonstrated that administration of an analogous polymerized bovine Hb product (HbOC-301, oxyglobin), which has been licensed for use in the treatment of anemia in dogs, induces HbOC-specific immunoglobulin G (IgG) antibody responses within months following administration. These antibodies, however, have not been shown to be associated with tissue immunopathology or interference of oxygen binding to HbOC-301. In this report, we investigate the immunogenicity of HbOC-201 in patients receiving multiple infusions of HbOC-201 as part of clinical trials. More specifically, we examine the ability of human IgG anti-

ABBREVIATIONS: HbOC(s) = Hb-based oxygen carrier(s); HuHb = human hemoglobin; SPRIA = solid-phase radioimmunoassay.

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HbOC-201 in serum to cross-react in vitro with human hemoglobin (HuHb) or lyse intact human RBCs. The study shows that although the administration of HbOC-201 induces specific IgG antibodies and some cross-react to varying degrees with HuHb, these antibodies do not appear to facilitate complement mediated lysis of human RBCs.

MATERIALS AND METHODS

Study design

Serum samples (n = 168) were collected from subjects participating in human clinical trials sponsored by Biopure Corp., Cambridge, MA, involving the administration of either single or multiple escalating doses of HbOC-201. These studies included eight Phase I and II clinical trials involving HbOC-201 administration during prostatectomy, open repair of fractures and hip replacement, cardiopulmonary bypass, acute vasoocclusive studies, and surgery involving allogeneic transfusions, abdominal aortic reconstruction, and liver resection and two Phase III clinical trials involving noncardiac and orthopedic surgery. Serum samples from all subjects in these clinical studies were considered for inclusion; however, 146 with the highest levels of IgG anti-HbOC-201 in their study groups were selected. Moreover, 16 serum samples from subjects who produced no detectable antibody were selected as negative controls. Human IgG anti-HbOC-201 was reanalyzed by solid-phase radioimmunoassay (SPRIA; see below). Test serum samples were then evaluated for IgG antibody reactivity to HuHb using a direct-binding and indirect competitive-inhibition modifications of the SPRIA. Finally, a subset of 30 serum samples were selected from the 168 study serum samples for a human RBC lysis study. These included 10 serum samples from each of three groups containing: 1) both HuHb- and HbOC-201-reactive IgG antibody, 2) only HbOC-201-reactive IgG antibody, and 3) no HuHb- or HbOC-201-reactive antibody (negative controls).

HbOC-201- and HuHb-specific IgG antibody analyses

The 168 test serum samples were analyzed for IgG antibodies specific for HbOC-201 and HuHb with modifications of the assay design reported for the measurement of canine HbOC-301-specific IgG antibody.[6]

Direct-binding assays

In the direct-binding SPRIA, HbOC-201 and recombinant HuHb (both provided by Biopure Corp.) were individually coupled at 1 mg per mL of packed cyanogen bromide–activated Sepharose 4B-CL beads (Pharmacia Fine Chemicals, Kalamazoo, MI).[7,8] With the previously reported SPRIA format,[9] all serum samples were adsorbed overnight against uncoupled Sepharose (0.1-0.5 mL of 5% vol/vol) to remove naturally occurring carbohydrate-specific IgG antibodies.[10] The adsorbed serum was then diluted to 1:50 or greater for analysis. Dilutions of the reference or test serum samples (0.1 mL) were incubated with equal volumes of buffer or homologous hemoglobin (1 mg/mL) in separate tubes for 2 hours at room temperature. The mixtures were then pipetted into their respective duplicate 12×75-mm test tubes containing washed HbOC-201-sorbent or HuHb-sorbent with 0.5 mL (1% v/v) and rotated overnight (16-18 hr at 23°C). After four buffer washes with phosphate-buffered saline containing 1 percent bovine serum albumin, 0.05 percent Tween 20, and 0.01 percent sodium azide, ^{125}I-streptococcal protein G[11] (instead of staphylococcal protein A; 150,000 cpm/tube) was added to each tube to detect bound human IgG antibodies. After an overnight rotation at room temperature, and four buffer washes to remove unbound ^{125}I-protein G, bound radioactivity was detected in a gamma counter (Cobra IV, Packard Instruments, Meriden, CT) to statistical accuracy. The quantity of human IgG antibodies reactive with the solid-phase antigen (HbOC-201 or HuHb) was determined by interpolation of counts per minute response data from an IgG anti-HbOC-201 reference curve constructed with 10 serial dilutions of a high-titered serum (working range, 9-2200 ng/mL). The IgG anti-HbOC-201 reference serum was cross-standardized in micrograms per milliliter of IgG antibody as previously discussed.[12]

The analytical sensitivity of the IgG anti-HbOC-201 assay was 0.5 µg per mL with undiluted serum as determined based on a mean + 3SD response produced by non-HbOC-201–exposed "negative" serum samples. Specificity of the IgG anti-HbOC-201 antibody assay was confirmed by incubating each serum with HbOC-201 or buffer and computing the degree of homologous antigen inhibition. The criterion for a positive IgG antibody measurement was a net IgG anti-HbOC-201,

[sham buffer (uninhibited) – homologous inhibition antibody level],

of greater than 0.5 mg per mL with a greater than 80 percent homologous antigen inhibition. Assays in this study achieved intraassay and interassay coefficients of variation of less than 15 and less than 20 percent, respectively. In-house quality control (2SD and 3SD) ranges were established for internal quality control serum samples that were run in each IgG anti-HbOC-201 assay.

Competitive-inhibition assays

The extent of HbOC-201-specific IgG antibody cross-reactivity with HuHb was also assessed in a competitive-inhibition format of the SPRIA.[7] Agarose-preadsorbed serum samples were first incubated in separate tubes with

equal volumes of either buffer (sham control) or 1 mg per mL HbOC-201 or HuHb for 2 hours at 23°C. Incubation of serum with the homologous and heterologous Hb was performed with each serum sample in each assay to verify specific binding (homologous Hb condition) and permit the computation of cross-reactivity (heterologous Hb condition). These serum-hemoglobin mixtures were then analyzed in the direct-binding SPRIA described above using both HbOC-201-sorbent or HuHb-sorbent to detect HbOC-201 and HuHb-reactive IgG antibody, respectively. The percentage of cross-reactivity of IgG anti-HbOC-201 for HuHb was determined by examining the extent of inhibition of the IgG antibody binding to the HbOC-201-sorbent after treatment with soluble HuHb. The measure of cross-reactivity was computed as a percentage of competitive inhibition:

$$[\{([IgG_{buffer}]-[IgG_{Hb}])/[IgE_{buffer}]\}\times 100].$$

Nondetectable cross-reactivity was indicated by less than 20 percent cross-inhibition of IgG anti-HbOC-201 binding to solid-phase HbOC-201 by incubation with soluble HuHb. Partial and essentially complete inhibition were identified by 21 to 80 and greater than 81 percent cross-inhibition, respectively.

Human RBC lysis studies

The ability of Hb-reactive IgG antibody to facilitate complement-mediated human RBC lysis was assessed with 30 serum samples with standard methods: 10 serum samples containing the highest concomitant IgG anti-HuHb and IgG anti-HbOC-201 levels, 10 serum samples containing the high IgG anti-HbOC-201 levels and no IgG anti-HuHb, and 10 serum samples containing no Hb-reactive IgG antibody. A 5 percent suspension of human RBCs from a group O RBC subject was incubated for 60 minutes at 37°C with gentle mixing with 0.1 mL of fresh human serum as a complement source and 0.1 mL of the 30 test serum samples or standard hemolyzing Lewis A antibody as a positive control and normal human serum as a negative control.[13] After incubation and centrifugation, we examined the supernatants visually for free Hb and also measured free Hb spectrophotometrically, scanning at wavelengths of 380, 415, and 470 nm using a scanning spectrophotometer (Beckman-Coulter, Fullerton, CA). To an aliquot of the washed RBC suspension, polyspecific anti-human antiserum (Immucor, Norcross, GA) was added and examined for agglutination both macroscopically and microscopically.

Computations and statistical analyses

Kruskal-Wallis one-way analysis of variance on ranks was used to examine differences between the median IgG anti-HbOC-201 levels in the serum samples that contained no HuHb cross-reactive antibody (Fig. 2, Column B; and Table 1, Row 2) versus those that contained antibody that was partially or completely cross-reactive with HuHb (Fig. 2, Columns C and D; Table 1, Rows 3 and 4). All statistical analyses were performed with computer software (SigmaStat Version 3.1, Systat Software, Inc., San Jose, CA; http://www.systat.com/).

RESULTS

IgG anti-HbOC-201 levels

IgG anti-HbOC-201 levels in the 146 antibody-positive specimens ranged from 0.7 to 86.8 μg per mL whereas the 16 antibody-negative serum samples remained negative (<0.5 μg/mL) upon reanalysis (Fig. 1). A significant correlation ($r^2 = 0.77$, $p < 0.001$) between the initial IgG anti-HbOC-201 measurement obtained at the time of the initial clinical study (data not shown) and those obtained just before performing the in vitro cross-reactivity study (Fig. 1) support the reproducibility of the SPRIA and the stability of the antibody in the serum specimen after 2 to 4 years of storage at –70°C. The lack of correlation between select measurements performed 4 years apart may have resulted from variance caused by minor losses in immunoreactivity due to serum storage or changes in the reference serum used to calibrate the assay.

TABLE 1. Competitive cross-inhibition of IgG anti-HbOC-201 binding to solid-phase HbOC-201 by incubation with soluble HuHb

Category	Inhibition range (%)	Number*	Percent of IgG anti-HbOC-201-positive test serum samples	IgG anti-bovine Hb in specimen (μg/mL)†
No inhibition, Column A‡	<20	16	NA	<1 (<1), no antibody
No inhibition, Column B‡	≤20	17	11.6	1.2-86.8 (12.6), HbOC-201-specific IgG antibody
Partial inhibition, Column C‡	21-80	92	63	1.0-66.0 (9.2)
Complete inhibition, Column D‡	>81	37	25.4	1.0-53.3 (11.2), HbOC-201- and HuHb-specific IgG antibody

* A total of 146 human IgG anti-bovine Hb (HbOC-201) antibody–positive specimens were analyzed in this study.
† Data are reported as range (mean).
‡ The different rows refer to the columns of data displayed in Fig. 2.

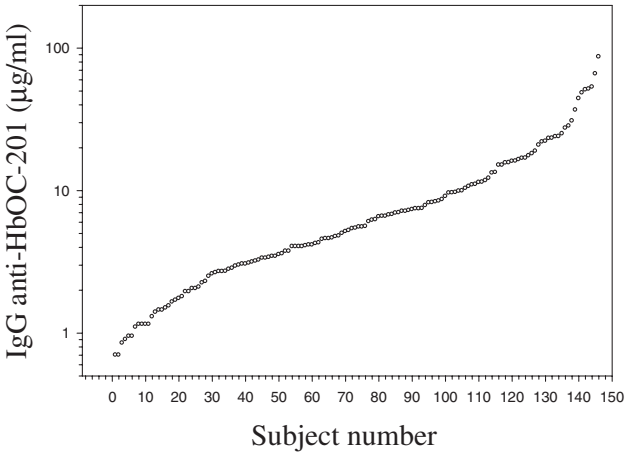

Fig. 1. Distribution of the IgG anti-HbOC-201 levels (µg/mL; range, 0.7-86.8 µg/mL) as measured in the 146 IgG anti-HbOC-201–positive test serum samples. The criterion for a positive IgG antibody measurement is a net IgG anti-HbOC-201, [sham buffer (uninhibited) – homologous inhibition antibody level], of greater than 0.5 µg per mL with greater than 80 percent homologous antigen inhibition.

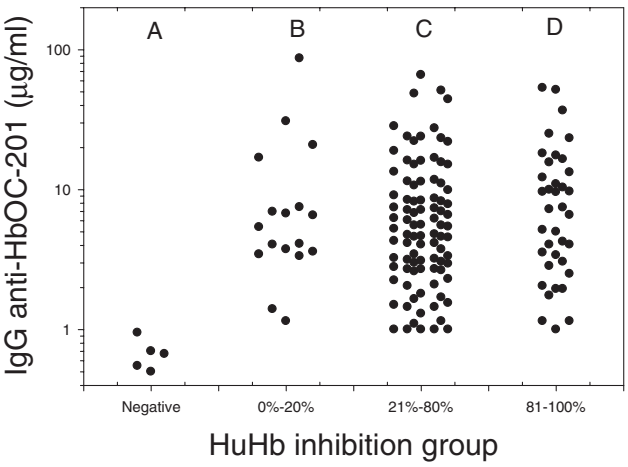

Fig. 2. IgG anti-HbOC-201 levels in serum samples that were competitively inhibited from binding to solid-phase HbOC-201 by incubation of the serum samples with HuHb. Serum samples containing no IgG anti-HbOC-201 (Column A) remained negative (<1 µg/mL) after incubation with HuHb. Serum samples in Column B contained HbOC-201-reactive IgG antibody but HuHb incubation produced negligible inhibition, suggesting no detectable HuHb–cross-reactive IgG antibody. Serum samples in Column C displayed partial inhibition after incubation with HuHb, indicating some HuHb–cross-reactive antibody. The serum samples displayed in Column D contained IgG anti-HbOC-201 that bound to determinants on the bovine Hb that were essentially completely cross-reactive with those on HuHb. These data indicate the extensive heterogeneity of the IgG antibody response for HbOC-201.

Cross-reactivity of IgG anti-HbOC-201 with HuHb

Figure 2 and Table 1 display the extent to which the binding of IgG anti-HbOC-201 to HbOC-201-solid phase could be inhibited by incubation of serum with HuHb. Serum samples containing no IgG anti-HbOC-201 (Fig. 2, Column A) remained negative (<1 µg/mL) after incubation with HuHb. Serum samples containing HbOC-201-reactive IgG antibody (1.2-85.7 mg/mL) but no HuHb-induced inhibition (Fig. 2, Column B; Table 1, Row 2) displayed exclusive reactivity for epitopes on HbOC-201. In contrast, serum samples in Fig. 2, Column D (Table 1, Row 4) contained IgG anti-HbOC-201 (1.0-52.3 mg/mL), which bound determinants on HbOC-201 that were highly cross-reactive with HuHb.

For confirmation of IgG anti-HbOC-201 cross-reactivity with HuHb, the test serum samples were reanalyzed in the direct-binding immunoassay with HuHb-sorbent. Of the 144 human IgG anti-bovine hemoglobin (HbOC-201) antibody–positive serum samples available in sufficient quantities to perform the analysis, 74 percent (107) contained IgG antibodies that bound directly to solid-phase HuHb. The HuHb-reactive IgG antibody levels ranged from 1 to 68.7 µg per mL. When dichotomous, positive or negative, cross-reactivity results from the competitive inhibition and direct-binding immunoassays were compared, there was 80 percent concordance (101/126, $p < 0.001$, chi-square) in the serum samples that were positive for the presence of human IgG anti-HuHb between the two assays.

Human RBC lysis study

To examine the HuHb cross-reactive IgG antibody's potential for RBC lysis, human RBCs from a group O subject were incubated with the three groups of serum samples in the presence of a complement source. None of the serum samples caused IgG antibodies to either detectably bind to the surface of the RBCs as assessed by hemagglutination with a standard antiglobulin test method or induced measurable RBC lysis in the presence of complement. After centrifugation, no free Hb was visually detected. Moreover, after testing the plasma specimens spectrophotometrically, the samples contained no Hb. The positive control antiserum sample induced visually detectable Hb in the supernatant of centrifuged RBCs, thus confirming the validity of the assay.

DISCUSSION

HbOCs such as HbOC-201 (glutamer-250, Hemopure, Biopure Corp.) have been shown to be effective alternatives to RBC transfusion in promoting tissue perfusion and minimizing hypoxic cellular damage during various surgical procedures in humans and animals.[1-4,14-16] Often,

multiple doses of HbOC are desired such as in the case of the patient with myeloid leukemia who needed long-term transfusions.[17] Repetitive dosing has raised potential safety concerns, especially as it relates to humoral immune responses directed at the HbOC that tend to increase with the dose administered.

In 2001,[6] we investigated the immunologic, histologic, and physiologic consequences of multiple doses of HbOC-301, an analogous preparation to HbOC-201 that is FDA-approved for use in canines. HbOC-301 was administered intravenously to eight canines at 1.3 g per kg, nine times over 50 weeks. HbOC-301-specific IgG antibody was detected in seven of eight dogs by Week 6, with levels that peaked after the third dose (Week 10). Immunofluorescence studies indicated no selective antibody-mediated tissue deposition from multiple HbOC-301 infusions. The pattern of IgG, IgA, IgM, and C3 deposition in the kidney and liver was comparable between control dogs receiving no HbOC-301 and the eight test dogs. Importantly, microgram per milliliter quantities of IgG anti-HbOC-301 in serum were unable to inhibit oxygen binding to HbOC-301 in vitro. This indicated that circulating antibody does not diminish the oxygen-binding capacity of HbOC-301 in circulation. All these data supported the safety of multidose administration of HbOC-301 in canines.

A variety of immune mechanisms exist that can lead to drug-induced immune hemolysis. For this reason, we studied whether antibodies reactive with HbOC-201 promote in vitro hemolysis or can sensitize RBCs. Our RBC serologic studies demonstrated no evidence for a direct lytic effect. This is consistent with the serum samples appearing clear and having no detectable Hb on spectrophotometric analysis. Furthermore, we were not able to detect RBC sensitization with human IgG anti HbOC-201, which might also potentially occur. The polyspecific anti-human immunoglobulin that we used as a positive control was from a single supplier, and although it is possible that the use of other antiserum samples might be used to confirm this finding, we did not pursue this. These data support the conclusion that IgG anti-HbOC-201 in the serum of individuals receiving HbOC-201 has no observable adverse effect on the RBCs that might lead to hemolysis.

In contrast to IgG antibody responses, IgE anti-HbOC-201 have not been detected in the serum samples of individuals receiving HbOC-201 as part of FDA-approved clinical trials.[18] The absence of detectable IgE antibody responses support the conclusion that the risk for Type 1 (IgE antibody-mediated) hypersensitivity reactions after repetitive administrations of HbOC-201 appear negligible. Overall, HbOC-201 when used repetitively as an alternative to RBC transfusions in the management of acute blood loss appears to be safe and its efficacy is not compromised by the presence of specific IgG antibodies.

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