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Antibody-Mediated Immune Suppression of Erythrocyte Alloimmunization Can Occur Independently from Red Cell Clearance or Epitope Masking in a Murine Model

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Anti-D can prevent immunization to the RhD Ag on RBCs, a phenomenon commonly termed Ab-mediated immune suppression (AMIS). The most accepted theory to explain this effect has been the rapid clearance of RBCs. In mouse models using SRBC, these xenogeneic cells are always rapidly cleared even without Ab, and involvement of epitope masking of the SRBC Ags by the AMIS-inducing Ab (anti-SRBC) has been suggested. To address these hypotheses, we immunized mice with murine transgenic RBCs expressing the HOD Ag (hen egg lysozyme [HEL], in sequence with ovalbumin, and the human Duffy transmembrane protein) in the presence of polyclonal Abs or mAbs to the HOD molecule. The isotype, specificity, and ability to induce AMIS of these Abs were compared with accelerated clearance as well as steric hindrance of the HOD Ag. Mice made IgM and IgG reactive with the HEL portion of the molecule only. All six of the mAbs could inhibit the response. The HEL-specific Abs (4B7, IgG1; GD7, IgG2b; 2F4, IgG1) did not accelerate clearance of the HOD-RBCs and displayed partial epitope masking. The Duffy-specific Abs (MIMA 29, IgG2a; CBC-512, IgG1; K6, IgG1) all caused rapid clearance of HOD RBCs without steric hindrance. To our knowledge, this is the first demonstration of AMIS to erythrocytes in an all-murine model and shows that AMIS can occur in the absence of RBC clearance or epitope masking. The AMIS effect was also independent of IgG isotype and epitope specificity of the AMIS-inducing Ab. *The Journal of Immunology*, 2014, 193: 2902–2910.

The development of alloantibodies to RBCs can result in significant clinical sequelae complicating transfusions and pregnancy. The Ab response to RBCs can be downregulated by the simultaneous administration of an RBC-specific Ab concurrent with exposure to the foreign red cell (1–4). This effect has been termed Ab-mediated immune suppression (AMIS), and AMIS has been observed with a variety of different cell types, including platelets, leukocytes, microbes, and particulate Ags, such as vaccines in different mammals (5–8).

In the case of hemolytic disease of the fetus and newborn (HDFN), the prophylactic administration of anti-D has been highly

successful in preventing immunization of the mother to the D Ag on fetal red cells. Much of our information on how anti-D may prevent immunization to D⁺ RBCs has come from seminal studies dating back to the 1960s on human male D⁻ volunteers immunized with D⁺ RBCs. In many of these studies and others that have followed, it was noticed that the dose of anti-D that caused clearance of the D⁺ RBCs from the circulation was related to its ability to prevent the immune response to the Ag (3, 9–12). In addition, Abs reacting with the human Kell glycoprotein (a transmembrane protein distinct from RhD) were shown to inhibit the Ab response to the RhD Ag expressed on the same cell (9), cementing the concept that RBC clearance rather than epitope masking likely explains the AMIS effect with allogeneic erythrocytes. Observations in mice injected with SRBCs unfortunately cannot directly address the validity of the red cell clearance hypothesis, as these xenogeneic cells are cleared within minutes of their injection. Although the SRBC Ags are poorly defined, it has nevertheless been suggested that epitope masking by the anti-SRBC Ab likely plays a major role in reducing the immune response to these cells (13–15). Although not universally agreed upon, the most commonly accepted theories to explain the AMIS effect with erythrocytes are 1) that the AMIS Ab is able to rapidly clear Ag-positive target cells before they can be recognized by the immune system (10–12, 16, 17), theoretically based on FcγR-mediated phagocytosis of D⁺ RBCs preventing B cells from recognizing the D Ag (10, 11, 17), or 2) that the AMIS Ab sterically hinders the ability of the immune response to see or detect the Ag (4, 15, 16, 18). Other theories with direct experimental support have suggested that inhibition may occur through the inhibitory FcγRIIB (19), that inhibitory cytokines may be produced (20), that glycosylation of the AMIS Ab may play a role (21–23), and/or that Ab-mediated immune deviation (AMID) away from the Ag and directed toward the sensitizing Ab may be involved (5). In addition to these theories, a number of others have been proposed (Table I).

In the case of anti-D, this clinically effective biological is derived from pooled plasma, is available in limited supply, can contain

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Abbreviations used in this article: AMID, Ab-mediated immune deviation; AMIS, Ab-mediated immune suppression; HDFN, hemolytic disease of the fetus and newborn; HEL, hen egg lysozyme.

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amounts of other Abs, and will always carry a theoretical risk of infection (24, 25). As a result, a mAb capable of mimicking the effects of anti-D is highly desirable (12, 26, 27). There have been many attempts to make monoclonal anti-D Abs, and extreme efforts have gone into choosing those Abs that give the highest rates of RBC clearance for use in clinical studies (11, 28–30). In fact, regulators consider that for the prevention of HDFN a mAb specific for the RhD Ag should be demonstrated to cause RBC clearance before exposing pregnant women to the product. Along the lines of enhancing red cell clearance to derive an optimized mAb, additional attempts to biochemically increase the ability of some RhD-specific mAbs to cause increased red cell clearance characteristics by manipulating the carbohydrate structure on the Fc portion of the molecule have been performed (21, 22). Unfortunately, we do not as of yet have a monoclonal anti-D Ab that has proved successful for the prevention of HDFN, and some anti-D Abs with good red cell clearance abilities have actually led to an enhanced alloimmune response rather than inhibition (22, 23).

A good understanding of the mechanism of AMIS will be important in rationally designing mAbs for clinical use. In particular, the contribution of RBC clearance and epitope masking to an AMIS mechanism needs to be addressed. We demonstrate herein that neither RBC clearance nor epitope masking is required for the AMIS effect, in a full murine model.

Materials and Methods

Mice

C57BL/6 mice and B10.BR mice were obtained from Charles River Laboratories (Montreal, QC, Canada) and The Jackson Laboratory (Bar Harbor, ME). HOD mice on the FVB background were created as described (31). HOD mice on the FVB background were used because they are excellent breeders. All animal studies were approved by the appropriate animal care committees.

Abs

To make the polyclonal hen egg lysozyme (HEL)-specific Ab, C57BL/6 mice were immunized with 200 μ g HEL (Cat# L4919-1G; Sigma-Aldrich, St Louis, MO) in 100 μ l of an emulsion of CFA (Cat# 7001; Chondrex, Redmond, WA), followed by two boosts 4 wk apart with 200 μ g HEL in 100 μ l IFA (Cat# 7002, Chondrex). The sera from several mice were pooled and then IgG purified using a Protein G Spin Kit (Thermo Scientific, Rockford, IL) according to the manufacturer's directions. The mAbs to HEL (4B7 [IgG1], GD7 [IgG2b], and 2F4 [IgG1]) were as previously described (32, 33). These Abs were made and purified by cell culture services (BioExpress, West Lebanon, NH) using protein A chromatography. MIMA 29 was purified by protein G affinity chromatography. The mouse monoclonal IgG anti-Duffy, CBC-512 (anti-Fy3, IgG1) (34, 35), and K6 (NYBC-BG6, anti-Fy6, clone K6, IgG1) (36, 37) were used as a tissue culture supernatant fluid. MIMA 29 (mouse anti-Fy3, IgG2a) (35) and K6 were generously provided by Dr. Marion Reid and Gregory Halverson of the New York Blood Center. Control IgG was an affinity purified gel-filtered mouse IgG (Cat# 015-000-002; Jackson ImmunoResearch, West Grove, PA).

Immunizations

Blood collection and storage were performed as essentially described (38, 39). Briefly, HOD blood was collected into citrate-phosphate-dextrose-adenine solution (CPDA-1, Cat#C4431; Sigma-Aldrich) as an anticoagulant, at a ratio of 1 part anticoagulant to 9 parts whole blood, by retro-orbital bleeding or cardiac puncture; leukocytes were depleted; and the RBCs were washed with PBS (pH 7.22). Mice were transfused (i.v. injected into the tail vein) with 10^7 HOD-RBCs in 200 μ l PBS, and then 1 h later injected i.v. with 0.4 μ g (200 μ l) of control IgG or a HOD-specific polyclonal Ab or mAb, unless otherwise indicated. Mice were bled for serum on days 0, 6, 12, and 18.

Detection of the HOD-specific Ab response

To detect IgM and IgG subclasses specific for HEL or OVA, an ELISA-based assay was used as previously described (5). Briefly, ELISA plates were coated with 10 μ g/ml HEL or OVA in PBS. After overnight incu-

bation at 4°C, plates were washed and blocked with 1% BSA (Cat# A3059; Sigma-Aldrich). The plates were washed, and serum samples from mice were then serially diluted (1:50 for IgG and IgG isotypes and 1:200 for IgM) and added for 1.5 h at 22°C. The plates were next washed and incubated with alkaline phosphatase-labeled anti-mouse IgM (μ) (Cat# M31508; Invitrogen, Camarillo, CA), biotin-conjugated goat anti-mouse IgG, IgG1, IgG2b, IgG2a/2c, or IgG3 from Jackson ImmunoResearch. Next, the plate was developed using 1 mg/ml *p*-nitrophenyl phosphate in 9.7% diethanolamine or incubated with HRP-streptavidin and then developed by tetramethylbenzidine (Invitrogen).

RBC clearance

RBCs were labeled with the lipophilic carbocyanines DiOC₁₈ (DiO) or DiI₁₈ (DiI) (Invitrogen), and the posttransfusion survival rate was determined as described previously (32, 38). Briefly, FVB-RBCs were incubated with dye and DiO and HOD-RBCs were incubated with DiI, respectively, at 37°C for 30 min then washed to remove unincorporated dye. The labeled RBCs were then examined by a FACScan flow cytometer (BD Biosciences) to determine the extent of dye labeling for both dyes. Mice were challenged i.v. with 10^7 labeled HOD-RBCs and labeled FVB-RBCs in PBS and, 1 h later, transfused with PBS or with 0.4 μ g of one of the following Abs: control IgG, polyclonal (IgG) anti-HEL, 4B7, 2F4, GD7, CBC-512, MIMA 29, or K6. Recipients were bled 10 min after RBC transfusion, and at 2 h post Ab infusion, and the percentage of transfused HOD⁺ RBCs circulating in the recipient was assessed by flow cytometry. Survival of HOD-RBCs was calculated as a ratio between the percentage of surviving HOD-RBCs versus FVB-RBCs, and the ratio at 10 min post RBC transfusion was used as the baseline. Detection of Ab engagement on HOD-RBCs was accomplished by incubating cells with goat anti-mouse Ig followed by flow cytometric detection of bound Ab.

Epitope masking

B10.BR mice were challenged with 10^7 HOD-RBCs and bled for serum on day 6 or day 18 to obtain a high titer IgM (IgM anti-HOD) and IgG (IgG anti-HOD) Ab, respectively. Serum samples were pooled and stored at -20°C until used. HOD-RBCs were reacted with serial dilutions of Abs 4B7, GD7, 2F4, or MIMA 29 for 45 min at 22°C, washed, and then incubated with 20 μ l pooled IgM anti-HOD sera for 1 h at 22°C. Binding of the IgM anti-HOD to the RBCs was detected using PE-labeled goat anti-mouse IgM (μ) (Invitrogen), whereas binding of the IgG was detected with biotin-labeled anti-mouse IgG subclass followed by allophycocyanin-streptavidin (BD Biosciences) for 45 min at 22°C.

Statistical analysis

Data were expressed as mean \pm SEM and analyzed by Student *t* test or one-way ANOVA plus Bonferroni. The level of significance was set at a *p* value \leq 0.05.

Results

The IgM and IgG immune response to HOD-RBCs

Although previous studies have examined the immune response to HOD-RBCs at doses relevant to transfusion (100 μ l packed RBCs; $\sim 2 \times 10^9$ per transfusion) (39), transfusion of lower numbers of cells (relevant to AMIS induction) has not yet been evaluated. Recipient mice were transfused with nothing, or once with $10^4 \rightarrow 10^9$ HOD-RBCs, and IgM and IgG subclasses of responding Abs were evaluated. Although an Ab response to the HEL portion of the molecule was clearly detectable at different RBC doses (Fig. 1), consistent with a previous report, no immune response was detected to OVA or the Duffy portion of the Ag (40). The lowest dose of red cells giving rise to a significant response was 10^5 cells per mouse, which induced both IgM (Fig. 1A) and IgG (Fig. 1B–F) classes of Ab. The IgM response was characterized by a distinct peak responsiveness, which occurred at a dose of 10^7 RBCs (Fig. 1A), whereas the IgG classes of Abs generally displayed an increasing response as the cell number increased. The predominant IgG isotypes produced appeared to be IgG2b and IgG3, with a lower but significant amount of IgG1 being made (Fig. 1C–F). In comparison, although IgG1 isotype Abs appeared relatively minor in response to HOD-RBCs, this subclass was the dominant isotype in mice immunized with the HEL protein in

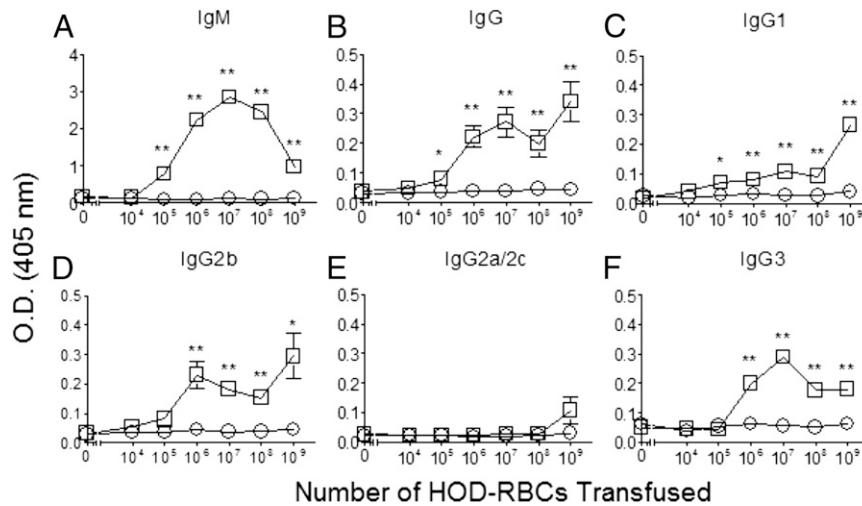


FIGURE 1. Mice challenged with HOD-RBCs make HEL-specific IgM and IgG. Mice were transfused on day 0 with nothing (prebled, O) or 10^4 – 10^9 HOD-RBCs (HOD-RBC, □) given as a single transfusion. Mice were bled for serum on day 0, on day 6 for IgM (A), and on day 18 for IgG and IgG isotypes. HEL-specific IgM (A), IgG (B), IgG1 (C), IgG2b (D), IgG2a/2c (E), and IgG3 (F) were assessed by ELISA. The concentration of IgM and IgG produced in response to immunization with 10^7 HOD-RBCs in (A) and (B) corresponds to roughly 1.5 and 1.0 $\mu\text{g/ml}$ Ab, as assessed using HEL-specific IgM and HEL-specific IgG as standards in the ELISA. The IgM standard was derived from MD4 mice, whereas the IgG standard was HEL-specific IgG, as described in *Materials and Methods*. Data are presented as mean \pm SEM. $n = 2$ mice per experiment from three separate experiments. * $p < 0.05$, ** $p < 0.01$ HOD-RBC versus prebled values at each respective point.

Freund's adjuvant (not shown and Ref. 33). Based upon the data in Fig. 1, a dose of 10^7 HOD-RBCs (roughly equivalent to a 2- to 5-ml bleed in a human on a blood volume basis) was selected for the evaluation of AMIS induction.

AMIS induction using HEL-specific polyclonal IgG

To evaluate AMIS, mice were transfused with 10^7 HOD-RBCs followed after 1 h by 0.4 μg of a mouse polyclonal IgG specific for HEL. Mice injected with HOD-RBCs alone or with HOD-RBCs plus a control IgG made a significant IgM and IgG response, as expected (Fig. 2A–E). In contrast, the IgM immune response in the AMIS

group was clearly inhibited (Fig. 2A). Because the AMIS-inducing Ab was a polyclonal mouse IgG that reacts in the ELISA (much like injected anti-D interferes in humans), we could at best evaluate only the rate of decay of IgG anti-HEL. In comparison with the amount of anti-HEL detected at 24 h (a time point well before IgG can be made in the mice), the relative amount of IgG detected from this point decayed over time, suggesting successful AMIS induction at the level of IgG (Fig. 2B). In comparison with the HEL-specific IgM and IgG response in HOD-RBC-treated mice, this response was significantly inhibited under AMIS conditions ($p < 0.01$), but not inhibited when an AMIS control IgG was used (Fig. 2A, 2B).

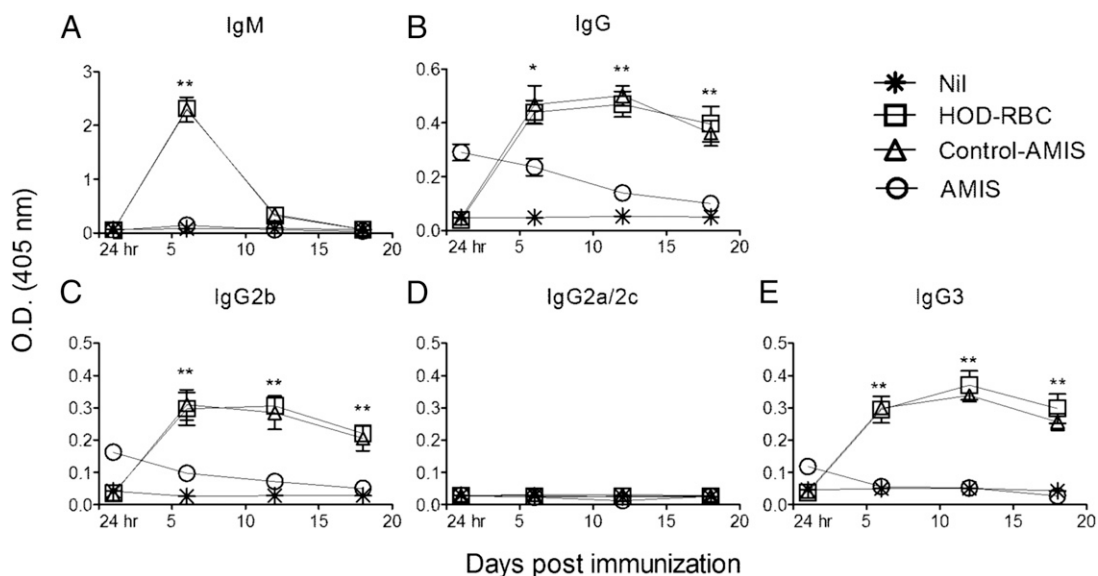


FIGURE 2. Induction of AMIS using polyclonal HEL-specific Ab. Mice were challenged with nothing (Nil, *), 10^7 HOD-RBCs (HOD-RBC, □), 10^7 HOD-RBCs followed after 1 h with 0.4 μg per mouse (i.v.) of control mouse IgG (Control-AMIS, Δ), or 10^7 HOD-RBCs followed after 1 h with 0.4 μg per mouse (i.v.) HEL-specific polyclonal Ab (AMIS, ○). HEL-specific IgM (A), IgG (B), IgG2b (C), IgG2a/2c (D), and IgG3 (E) were evaluated from the sera by ELISA. The level of HEL-specific IgG in the serum of all mice was first assessed 24 h after the start of the experiment. This time point is included to distinguish the anti-HEL in the mice caused by the injection of the AMIS Ab from that produced as the result of an immune response. An increased OD at this time point was observed only in the AMIS group; it decayed over time (B, C, and E) and is consistent with detection of the injected AMIS Ab. Data are the mean \pm SEM of three independent experiments performed in duplicate. * $p < 0.05$, ** $p < 0.01$ HOD-RBC versus AMIS values for the same time point.

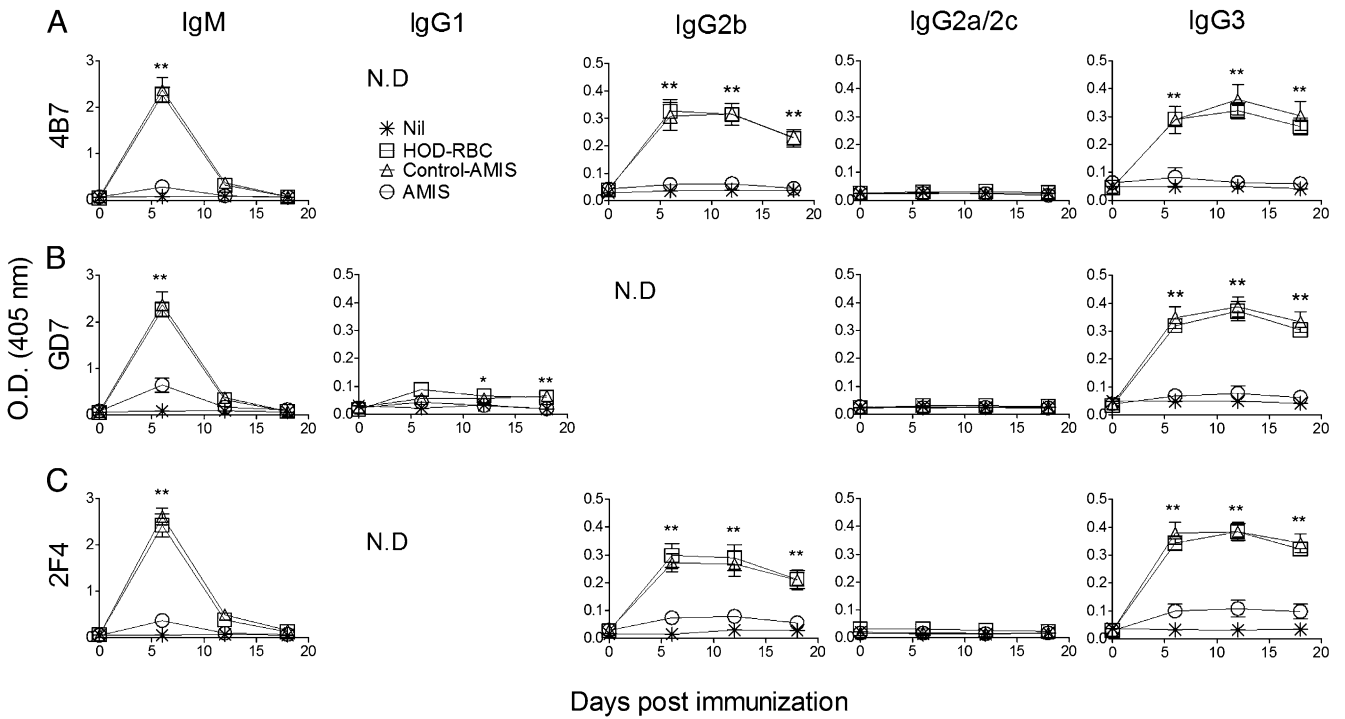


FIGURE 3. Induction of AMIS using monoclonal HEL-specific Abs. Mice were first challenged with nothing (Nil, *), 10^7 HOD-RBCs (HOD-RBC, □), 10^7 HOD-RBCs followed after 1 h with 0.4 μ g per mouse (i.v.) of control mouse IgG (control-AMIS, △), or AMIS induced using 10^7 HOD-RBCs followed after 1 h by i.v. injection of 0.4 μ g per mouse of the HEL-specific mAbs indicated on the Y-axis (AMIS, ○): 4B7 (IgG1 anti-HEL) (A), GD7 (IgG2b anti-HEL) (B), or 2F4 (IgG1 anti-HEL) (C). HEL-specific IgM, IgG, IgG1, IgG2b, IgG2a/2c, and IgG3 in serum from challenged mice were determined by ELISA. Data are the mean \pm SEM of three independent experiments performed in duplicate. N.D., “not done,” as this is the isotype of the AMIS-inducing IgG. * p < 0.05, ** p < 0.01 HOD-RBC versus AMIS values for the same time point.

The inhibition of specific subclasses of IgG was also evaluated in AMIS mice. Unfortunately, the polyclonal IgG anti-HEL used to induce AMIS had a predominance of IgG1 Abs (not shown); as a result, we were not able to evaluate inhibition of the IgG1 subclass in these experiments. An examination of the other IgG isotypes, however, showed that IgG2b and IgG3 were significantly inhibited

under AMIS conditions (Fig. 2C–E). There was no IgG2a/2c immune response detected in any of the mice, and therefore AMIS induction did not cause a shift in the immune response from the other subclasses to IgG2a/2c (Fig. 2D). Thus polyclonal IgG Ab specific for the HOD Ag on RBCs inhibited immunization at the level of IgG to transfused HOD-RBCs.

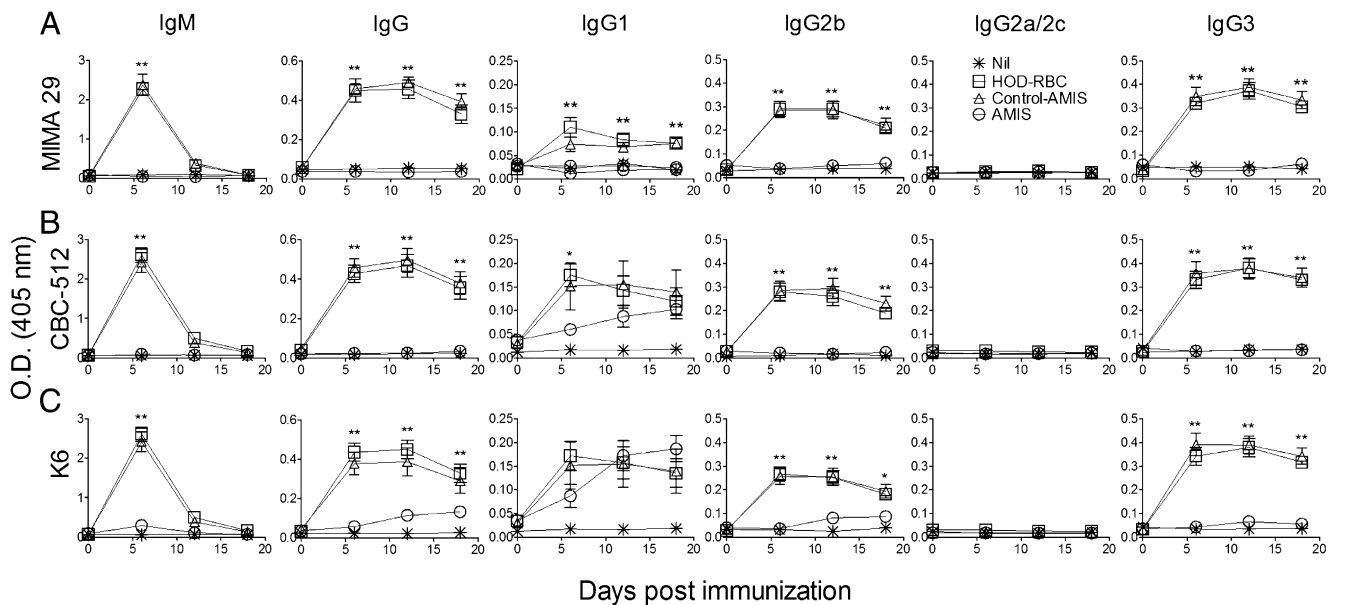


FIGURE 4. Duffy-specific monoclonal IgG prevents the HEL-specific Ab response. Mice were challenged as in Fig. 3 but AMIS was induced using anti-Duffy Abs: MIMA 29 (IgG2a anti-Fy3) (A), CBC-512 (IgG1 anti-Fy3) (B), or K6 (IgG1 anti-Fy6) (C). HEL-specific IgM and IgG, as well as IgG subclasses IgG1, IgG2b, IgG2a/2c, and IgG3, in serum from challenged mice were determined by ELISA. Data are the mean \pm SEM of three independent experiments performed in duplicate. * p < 0.05, ** p < 0.01 HOD-RBC versus AMIS values for the same time point.

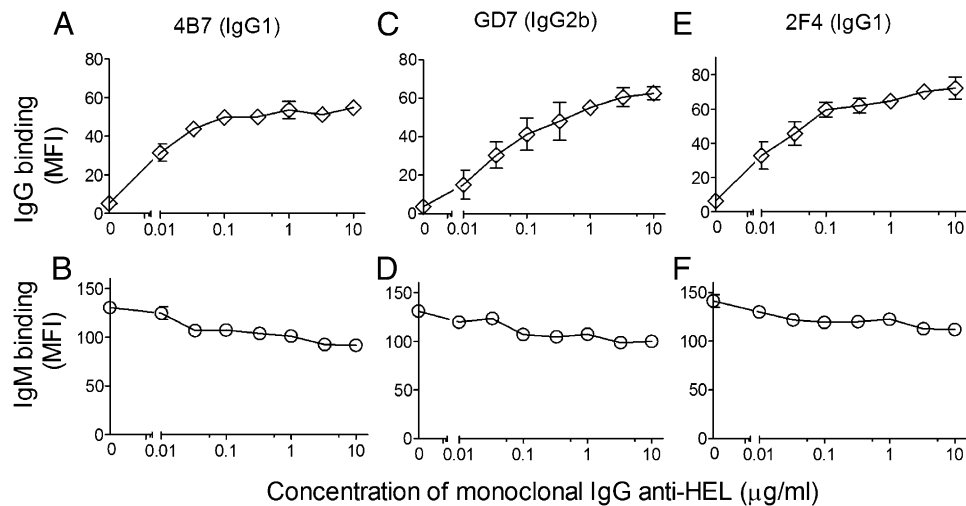


FIGURE 5. Epitope masking assay of the HEL-specific Abs. Mice were transfused with 10^7 HOD-RBCs, and serum was removed on day 6 to derive an HEL-specific IgM. Fresh naive HOD-RBCs were incubated with the indicated concentration of the mAbs: 4B7 (IgG1 anti-HEL), 2F4 (IgG1 anti-HEL), and GD7 (IgG2b anti-HEL) for 45 min, followed by the HEL-specific IgM (0.5 $\mu\text{g/ml}$) as described above, and the extent of binding of both Abs was assessed by flow cytometry. The binding of the mAbs 4B7 (A), GD7 (C), and 2F4 (E) with HOD-RBCs was assessed by fluorescence on FL4. Steric interference of HOD-specific IgM binding by 4B7 (B), GD7 (D), and 2F4 (F) was determined by fluorescence on FL2. Data are the mean \pm SEM of three independent experiments performed in duplicate. MFI, mean fluorescence intensity.

Induction of AMIS using mAbs

As AMIS induction worked well with polyclonal IgG against HEL (Fig. 2), we then attempted AMIS induction with mAbs against HEL. Mice were challenged with 10^7 HOD-RBCs followed by i.v. injection of the IgG1 subclass Ab 4B7 over a dose range of 10^{-4} – 10 μg per mouse. AMIS was successfully induced for the IgM immune response at doses of 4B7 ≥ 0.01 μg per mouse (Supplemental Fig. 1A). The IgG2b and IgG3 immune responses were optimally inhibited by Ab 4B7 at doses in the range of 0.01–0.1 μg per mouse (Supplemental Fig. 1B, 1D). IgG2a/2c Abs were not made in the mice (Supplemental Fig. 1C), and inhibition of IgG1 Abs could not be evaluated, as this was the subclass of the 4B7 AMIS-inducing Ab. Based upon this analysis, a dose of 0.4 μg per mouse was used with all of the subsequent Abs to induce AMIS.

Attempts to induce AMIS to RBCs in humans with mAbs have thus far been attempted only with human IgG1 and IgG3 subclass Abs, based in part upon the rationale that these subclasses are ones thought to be capable of causing red cell clearance. AMIS was induced with Abs 4B7 (IgG1) and GD7 (IgG2b), which are different isotypes but recognize an overlapping sequence on the HEL portion of the HOD Ag (Supplemental Fig. 2). Both isotypes were able to induce an AMIS effect, with Ab 4B7 giving slightly better AMIS induction for the production of IgM Abs (Fig. 3A, 3B). Another HEL-specific Ab 2F4 (IgG1) with a different specificity, compared with the other two Abs (not shown), also induced successful AMIS (Fig. 3C). The results showed that AMIS was induced by monoclonal IgG Abs of two different isotypes and different epitope specificities.

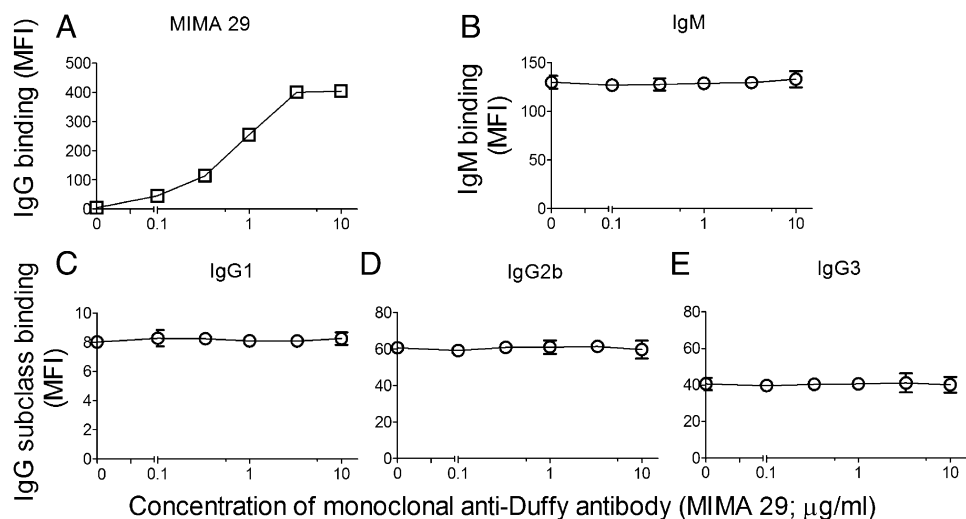


FIGURE 6. Duffy-specific Ab MIMA 29 does not block recognition of the HEL Ag on erythrocytes. Mice were transfused with 10^7 HOD-RBCs, and serum was removed on day 6 to derive HEL-specific IgM and on day 18 to derive HEL-specific IgG. Fresh naive HOD-RBCs were incubated with the indicated concentration of MIMA 29 (IgG2a anti-Fy3) followed by the HEL-specific IgM (0.5 $\mu\text{g/ml}$) or IgG (0.35 $\mu\text{g/ml}$) serum, and cells were examined by flow cytometry, as described in *Materials and Methods*. The binding of MIMA 29 (A) with HOD-RBCs was assessed by fluorescence on FL2. The ability of MIMA 29 to sterically prevent HOD-specific IgM (B), IgG1 (C), IgG2b (D), and IgG3 (E) from recognizing HOD-RBC was assessed on FL4. Data are the mean \pm SEM of three independent experiments performed in duplicate. MFI, mean fluorescence intensity.

AMIS can be induced by mAbs with epitope specificity distinct from the humoral immune target

The HOD Ag is composed of HEL in tandem sequence with known T cell determinants of OVA and the complete sequence of the human Duffy^b transmembrane protein. As B10.BR mice do not mount a humoral immune response to the Duffy portion of the molecule (not shown), this allowed us to ask if Abs directed to other portions of the molecule could induce an AMIS effect. Targeting the Duffy portion of the molecule also allowed us to examine inhibition of all anti-HEL IgG isotypes in the responding

mice. The MIMA 29 (IgG2a) Ab with specificity for the Fy3 loop of the Duffy protein was first evaluated. This Ab induced an AMIS effect at the level of IgM as well as all isotypes of IgG (Fig. 4A). Ab CBC-512 (IgG1), also with specificity for the Fy3 loop, induced a strong AMIS effect at the IgM and IgG levels (Fig. 4B, panels 1 and 2), but when specific IgG isotypes were evaluated, incomplete suppression of IgG1 was evident (Fig. 4B, panel 3). In fact, by day 18 of the experiment the IgG1 Ab increased within the normal range. This Ab was available only as a tissue culture supernatant, but when the dose of CBC-512 was increased, more complete suppression of the

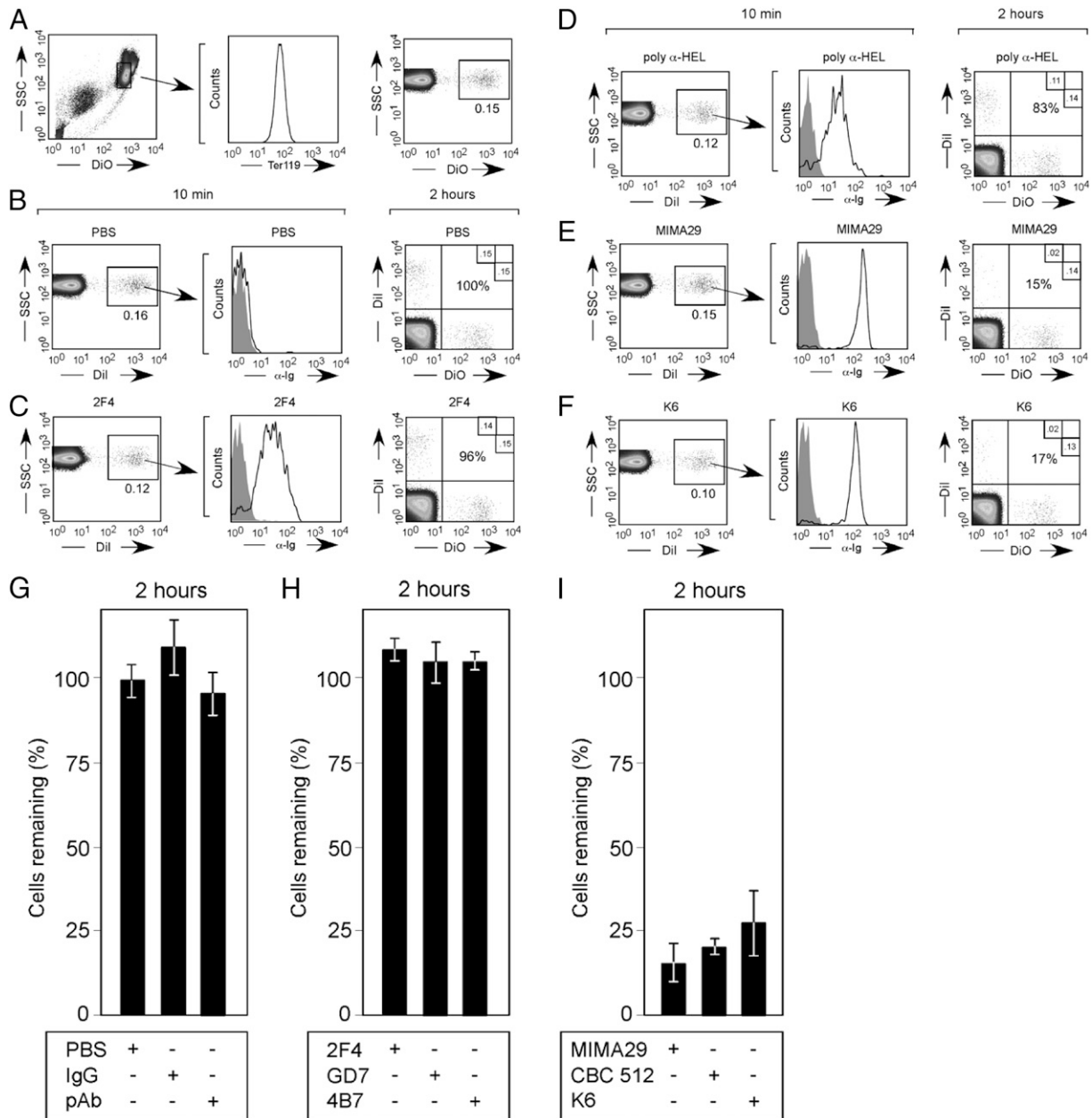


FIGURE 7. Clearance of HOD-RBCs under AMIS conditions. RBCs were isolated from FVB and FVB.HOD mice, incubated at 37°C with fluorescent dyes DiO and DiI for 30 min, respectively. Mice were challenged with both RBCs at 10⁷ per mouse, and HOD-RBC clearance was examined by flow cytometry. Gating strategy was used to interrogate FVB cells following transfusion in vivo (A). Analysis of cells for Ab engagement using anti-mouse Ig 10 min following injection or for the presence of the transfused cells 2 h following injection of PBS (B), 2F4 (IgG1 anti-HEL) (C), polyclonal anti-HEL (poly α-HEL) (D), MIMA 29 (IgG2a anti-Fy3) (E), and K6 (IgG1 anti-Fy6) (F). Solid gray, DiO-labeled HOD⁻ cells. Black line, DiI-labeled HOD⁺ cells. MIMA 29, CBC 512 (IgG1 anti-Fy3), and K6, but not 2F4, GD7 (IgG2b anti-HEL), 4B7 (IgG1 anti-HEL), or polyclonal anti-HEL, induce clearance of FVB.HOD RBCs (G–I). Quantitative analysis of FVB.HOD RBC clearance 2 h following injection of PBS, IgG control, or polyclonal anti-HEL (G), 2F4, GD7, or 4B7 (H), and MIMA 29, CBC 512 (CBC), or K6 (I) as indicated. Data are the mean ± SD of three independent experiments performed in duplicate. *p* < 0.01 for all Duffy-specific IgGs in (I) versus the IgG control in (G).

Table I. Selected potential mechanisms to explain AMIS effects with mAbs

Mechanism ^a
AMID ^b
Inhibition of synapse formation between the B cell and the Ag (RBC)
Disruption of B cell–T cell interaction
Induction of inhibitory cytokines
Fc γ RIIB-mediated inhibition
Impaired APC function
Immune suppression
Ag clearance
Epitope masking
IgG glycosylation
T cell inhibition

^aFor a discussion of these mechanisms see Ref. 27.

^bThis mechanism is presented in Ref. 5.

IgG1 response was observed (data not shown). Ab K6 (IgG1) with specificity for the Fy6 Ag on the Duffy molecule gave rise to an AMIS pattern similar to that with CBC-512. These results indicated that AMIS effects could be mediated by epitopes physically distant (along the polypeptide chain) from the antigenic portion targeted in the humoral immune response.

AMIS induction can occur independently from epitope masking

Although the Duffy portion of the HOD molecule is far removed in sequence from the HEL portion of the molecule, we do not know the tertiary structure of this experimental Ag. Thus it is possible that Abs directed to the Duffy portion of the molecule could interfere in the binding of immune Abs from HOD-RBC-challenged mice. To evaluate if any epitope masking was occurring with any of the Abs used in this study, HOD-RBCs were first treated with the three monoclonal anti-HEL Abs, the cells were washed, and then they were reacted with IgM anti-HEL. All three mAbs were serially diluted and assessed for their ability to bind to the HOD-RBCs (Fig. 5A, 5C, 5E) and simultaneously assessed for the ability of these mAbs to block or interfere in the binding of the IgM sera (Fig. 5B, 5D, 5F). As can be observed, all three Abs caused some diminution of binding of the HEL-specific IgM (Fig. 5B, 5D, 5F), suggesting partial but incomplete epitope masking. Even in the presence of maximal concentrations of these Abs, 100% of the HOD-RBCs stained positive using the IgM anti-HEL sera (not shown). Under these conditions, the IgM anti-HEL did not have any effect on the bound IgG anti-HEL (not shown). Of interest, when the monoclonal anti-HEL IgG Abs were added simultaneously with the IgM anti-HEL, the IgM interfered with the binding of the IgG (Supplemental Fig. 3A versus Supplemental Fig. 3B). In contrast, there was only minimal interference with the ability of the IgM to bind under these conditions (Supplemental Fig. 3C).

To evaluate if any epitope masking was occurring with a Duffy-specific Ab, MIMA 29 was evaluated. MIMA 29 bound well to HOD-RBCs (Fig. 6A), yet exhibited no steric inhibition of IgM anti-HEL binding to HOD-RBC, even at a saturating dose of MIMA 29 (Fig. 6B). This Ab also did not interfere in the binding of IgG anti-HEL of any subclass (Fig. 6C–E). When MIMA 29 was added simultaneously with the IgM, there was no evidence of epitope masking (Supplemental Fig. 4). Together with the above findings, these data indicate that AMIS mediated by the anti-Duffy Ab can occur in the absence of epitope masking and that blocking exposure to the Ag is thus not an absolute requirement for AMIS.

AMIS can occur independently of erythrocyte clearance

To address the requirement for erythrocyte clearance in AMIS effects, mice were injected with HOD-RBCs versus control RBCs

labeled with the Dil and DiO fluorescent dyes, respectively. Erythrocytes were identified using Ab Ter119 (Fig. 7A). After 1 h the mice were injected with PBS (Fig. 7B) or the HEL-specific and Duffy-specific Abs used in this study. The survival of HOD-RBCs in the presence versus absence of these Abs was assessed in relation to the survival of control labeled RBCs. Each of the Abs displayed specific recognition of the HOD-RBCs (e.g., Fig. 7C–F). Consistent with our previous studies, the polyclonal anti-HEL IgG did cause a minimal drop in red cell numbers (Fig. 7G, column 3), but none of the HEL-specific mAbs caused any observable specific clearance of HOD-RBCs (Fig. 7H) (32, 33). In contrast, all of the Duffy-specific Abs caused HOD-RBC clearance (Fig. 7I). To exclude the nonspecific effect of IgG on red cell clearance, we used mouse IgG, which does not specifically bind to HOD-RBCs. As expected, mouse IgG did not induce the clearance of HOD-RBCs (Fig. 7G, column 2). Together with the above immunization data, these findings indicate that RBC clearance is not a prerequisite for an AMIS effect with the anti-HEL Abs but do not rule out and perhaps may suggest that the Duffy-specific Abs could mediate AMIS effects through a red cell clearance mechanism or a different mechanism (Table I).

Discussion

Observations of the AMIS effect on erythrocyte Ags can be traced back to 1900 by von Dungern (41), two decades before the discovery that Abs were actually proteins. Since that landmark discovery, AMIS effects have been observed with a large number of cellular and other particulate Ags (4, 27). In terms of the immune response to D⁺ erythrocytes, the ability to prevent this response in humans has been achieved with both intentionally immunized males as well as during pregnancy (1, 10, 42). In the case of anti-D prophylaxis, considerable interest has been shown in replacing anti-D with a mAb. Although anti-D prophylaxis may today be considered generally safe, there will always be concerns with infection (24, 25), the presence of other Abs such as anti-A, batch-to-batch variation, and the need to intentionally alloimmunize males. The development of monoclonal anti-D to circumvent these issues is highly desirable.

To date, only monoclonal anti-D Abs of the IgG1 and IgG3 isotype have been attempted in human AMIS experiments, based upon their ability to mediate rapid red cell clearance (11, 12, 26, 30, 43, 44), as well as because of the predominance of these two isotypes after immunization with D⁺ RBCs (44, 45). Other isotypes of anti-D have also been detected (11, 44–48), and it is not yet clear which anti-D Abs actually mediate AMIS effects versus some that could be disadvantageous. In human clinical trials, some IgG1/3 mAbs were shown to mediate an enhanced immune response to the D Ag, whereas others mediated AMIS effects almost as well as polyclonal anti-D (12, 23, 30, 49, 50). If rapid red cell clearance is not an important or contributing attribute to an AMIS effect, then the selection of such isotypes of Abs could be detrimental. In fact, some indirect evidence suggests that red cell clearance is not strikingly related to a successful AMIS effect in some individuals (4, 12, 23, 50, 51), and work from our laboratory using SRBCs as the Ag in a murine model has shown that forcefully directing the sensitized erythrocytes to phagocytic macrophages gave rise to enhancement rather than suppression (52).

In the current study, we have used erythrocytes from HOD mice expressing a unique, well-characterized immunological Ag on their erythrocytes (31). AMIS induction was first achieved with a polyclonal anti-HEL IgG (somewhat analogous to the use of anti-D in humans), and this polyclonal IgG provided significant AMIS effects. One attribute of polyclonal anti-HEL is that it can

induce some degree of Ag loss or Ag modulation (32, 33). To circumvent this, we also used monoclonal HEL-specific Abs, which do not induce Ag loss. In our evaluation of mAbs with specificity for the HOD molecule, all mAbs were capable of inducing an AMIS effect. Although it would be interesting to perform a careful titration of the polyclonal sera against each of the mAbs in inducing an AMIS effect, and combining different mAbs to examine potential synergistic effects, a careful titration with the monoclonal 4B7 anti-HEL Ab showed a dose-dependent AMIS effect that decreased all of the IgG isotypes evaluated. AMIS effects on other potential classes of Abs (IgA, IgE, and IgD) were not evaluated in this article.

Studies performed in the 1960s using polyclonal anti-D showed that suboptimal doses can sometimes cause a small amount of immune enhancement (2, 3), and this has been seen with some monoclonal anti-D Abs (23, 50). In addition, some anti-D mAbs did not adequately prevent the immune response, although they induced rapid RBC clearance (12, 23, 50). More work needs to be performed to determine which attributes of a polyclonal Ab or mAb give rise to AMIS effects versus those that may lead to AMIS failure or, in a worst-case scenario, may actually cause immune enhancement.

To evaluate several mAbs for induction of AMIS, we evaluated Abs representing the mouse IgG1, IgG2a, and IgG2b subclasses. Of interest, although different IgG subclasses do have different attributes relating to Fc γ R binding and complement activation (53) all of these subclasses of IgG were capable of inducing AMIS. All of these subclasses of murine IgG are able to bind to Fc γ Rs; however, the IgG1 isotype is, in general, the poorest binder when taken as a whole (53).

One of the key findings of this study was the ability to induce AMIS using Abs that react with sequences of the molecule far removed from the sequences encoding the actual (humoral) antigenic site. This observation would indicate that AMIS, mediated by these Abs, does not occur through steric hindrance. In fact, studies done many years ago showed that AMIS could occur across the Rh Kell boundary (9). Although this was originally thought to be a good indication that red cell clearance was involved, in fact we know today that the RhD and Kell transmembrane proteins are part of the protein 4.1R complex and are therefore in very close proximity to each other (54).

Finally, we evaluated the ability of each of the HOD-specific Abs to mediate red cell clearance. Although all of the Duffy-specific Abs caused rapid red cell clearance, none of the HEL-specific mAbs caused any noticeable red cell clearance at this 2-h time point. Additional clearance studies performed 24 h after anti-HEL Ab injection gave similar results (not shown). In addition, HOD mice injected with all three HEL-specific Abs used in this study, as well as two additional HEL-specific Abs, did not cause any noticeable anemia in mice assessed for ≤ 6 d post Ab injection (not shown). Thus, Abs directed to the HEL portion of the molecule do not seem to cause red cell clearance, whereas Abs directed to the Duffy portions of molecule can.

Two potential interpretations of the data may be made on the basis of the work presented in this article. First, *neither* red cell clearance nor epitope masking is involved in this model, or second, *either* red cell clearance *or* epitope masking is involved. If the second interpretation is correct, then one would have to speculate that all of the HEL-specific Abs mediate AMIS effects by epitope masking, whereas all of the Duffy-specific Abs mediate AMIS effects by red cell clearance. Although a clear distinction at present cannot be definitively made, we favor the second interpretation. In particular, a variety of other hypotheses can explain AMIS induction (Table I). Although any of these hypotheses could

potentially be responsible for AMIS effects, our recent finding of AMID being related to AMIS induction is of interest (5). In the AMID work, mice were injected with SRBCs or the diphtheria/tetanus vaccine in the presence versus absence of Ag-specific IgG. We observed that under conditions in which the IgG successfully mediated an AMIS effect against the Ag, a dose-dependent humoral immune response against the AMIS-inducing IgG was observed (5). These Abs (anti-IgG) observed in our prior work somewhat resemble rheumatoid-like factors, which may themselves be immune regulatory, or their appearance could simply be an indication of immune deviation away from the original Ag. We have not looked for the appearance of rheumatoid-like factors in the current work because unlike our previous work, the AMIS-inducing Abs in this study are not antigenically distinct (by species or by allotype) from the responding Abs in the mice, making their detection problematic.

In summary, the AMIS experiments reported in this article are, to our knowledge, the first to evaluate AMIS in a fully allogeneic murine model. The results demonstrate that these mAbs can induce AMIS across different IgG isotypes, Ag binding sites, ability to induce steric hindrance, and ability to cause RBC clearance. Whether all Abs induce AMIS by the same fundamental mechanism or through different mechanisms remains to be resolved.

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Disclosures

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