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Cutting Edge: NK Cells Mediate IgG1-Dependent Hyperacute Rejection of Xenografts

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Classic hyperacute rejection is dependent on the activation of the terminal components of complement. Recently, xenoantibodies with limited abilities to activate the classical pathway of complement in vitro have been implicated in the acute vascular rejection of xenografts. It is unclear how these Abs affect their pathogenic activities in vivo. In this study, we demonstrate the ability of an anti-Gal-α1,3Gal (Gal) IgG1, with modest complement-activating abilities in vitro, to induce xenograft rejection. This rejection was dependent on the activation of complement, on FcγR-mediated interactions, and on the presence of NK cells. Inhibition of any one of these factors resulted in the abrogation of IgG1-mediated rejection. In contrast, an anti-Gal IgG3 mAb induced classic, hyperacute rejection that was solely dependent on complement activation. Our observations implicate two types of IgG-mediated rejection; one that is dependent on complement activation, and a second that is uniquely dependent on complement, FcγR, and NK cells. The Journal of Immunology, 2004, 172: 7235–7238.

The xenoantibody response in primates and humans following exposure to porcine cells or organs is predominantly directed against the Gal epitope (1–3). Anti-Gal IgM titers increase by 4- to 20-fold, while anti-Gal IgG titers increase by 20- to 100-fold after the transplantation of a xenograft (4–6). Anti-Gal IgM, IgG2, IgG3 and IgG4 are present in the preimmune serum, while IgM and all IgG subclasses, including IgG1, are present in the postimmune serum (6, 7). The ability of complement-fixing IgM to induce hyperacute rejection is well-characterized, but it is unclear what the pathological effects of weakly or non-complement-fixing IgGs are. To address this question, we generated anti-Gal IgG-producing hybridomas from galactosyltransferase-deficient (Gal−/−) mice sensitized with Lewis rat (Gal+/-) hearts (8, 9). We confirmed that the ability to mediate complement-dependent lysis in vitro was restricted to the anti-Gal IgG3 mAbs, while anti-Gal IgG1 was not able to trigger lysis; however, both anti-Gal IgG3 and IgG1 mAbs were able to elicit rapid xenograft rejection in vivo (9). In this report, we demonstrate that the mechanism of anti-Gal IgG1-mediated xenograft rejection differs from that mediated by anti-Gal IgG3 mAb in its unique dependence on FcγR and NK cells.

Materials and Methods

Transplantation

Gal−/− mice with a C57BL/6 background, and Rag−/− Gal−/−-deficient mice with the 129 background, were bred and maintained at the University of Chicago, an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited facility. Hearts from 10- to 16-day-old Lewis rats (Harlan Laboratories, Walkersville, MD) were transplanted heterotopically into the abdomens of the recipient mice. Anti-Gal Abs were injected (i.v.) on the day of transplant or up to 7 days posttransplant. Cobra venom factor (CVF; Quidel, San Diego, CA) was administered at 3.5 U/mouse on day −1 and 1.0 U/mouse on days 0, 1, and 2 posttransplantation. Anti-asialo GM1 (Waco Chemicals, Richmond, VA) was administered at 50 U/mouse on days −2, 0, and +2 posttransplantation. Anti-NK1.1 (PK136; American Type Culture Collection, Manassas, VA) was administered at 1 mg/mouse on days −2 and −1 and 2 h before administration of anti-Gal mAbs. Anti-C5 (BB5.1) mAbs were a gift from Dr. Y. Wang (Alexion Pharmaceuticals, Cheshire, CT), and were administered at 0.5–0.75 mg/mouse, 2 h before administration of anti-Gal mAbs. Anti-FcγRII/III (2.4G2; American Type Culture Collection) was administered at 0.125 mg/mouse on day −1 and 2 h before anti-Gal mAbs. All heart grafts were monitored half-hourly for the first 4 h, then daily. Rejection is defined as the complete cessation of heart pulsation.

Purification of mAbs

Anti-Gal IgG, anti-NK1.1, and anti-FcγRII/III hybridomas were produced in protein-free hybridoma medium (Life Technologies, Gaithersburg, MD) and purified by ammonium sulfate precipitation. Following extensive dialysis, the purity of the precipitated Ab was confirmed by SDS-PAGE, and the Ab concentration was determined by UV spectrometry (280 nm).

Binding and complement activation by anti-Gal mAbs

Pig RBCs (pRBCs; 5 × 107) were incubated with dilutions of anti-Gal mAbs on ice for 30 min. FITC-conjugated goat-anti-mouse IgG was added to the pRBCs and Ab binding was detected by flow cytometry (FACScan; BD Biosciences, Mountain View, CA). For complement activation, the cells were washed and then incubated with a 1:10 dilution of mouse serum and FITC-conjugated anti-mouse C3b (Cappel-Organon Teknika, Durham, NC) or goat anti-sera to human-C5 (Quidel), biotin-conjugated rabbit anti-goat IgG (Vector Laboratories, Burlingame, CA), and streptavidin-FITC (BD Pharmingen, San Diego, CA). Anti-Gal mAbs were also assayed for the ability to cause complement-mediated lysis with a hemoglobin release assay as previously reported (10).

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3 Abbreviations used in this paper: CVF, cobra venom factor; pRBC, pig RBC; vWF, von Willebrand factor.
In vitro characterization of anti-Gal IgG1 (GT6-27) and IgG3 (GT4-31) mAbs. The fine specificity of the mAbs was determined by ELISA with Gal-α,1,3Gal-disaccharides (Disac), trisaccharides (Trisac), or pentasaccharides (Pentasac) as substrates (a). The ability of anti-Gal IgG1 (□) and IgG3 (■) to bind pRBC (b); to induce mouse C3 (c; triangles), C5 deposition (c; circles), or to induce the lysis of pRBC (d) was determined as described in Materials and Methods. MCF, Mean channel fluorescence.

Results and Discussion

In vitro analysis of anti-Gal mAbs

We had previously determined the affinity of the anti-Gal IgG3 (GT4-31) and IgG1 (GT6-27) to be $5 \times 10^{-8}$ to $7 \times 10^{-9}$ M, respectively, for the Gal-α,1,3Gal disaccharide substrate (9). In this study, we analyzed the fine specificity of these mAbs by comparing their relative affinities for the Gal-α,1,3Gal di-, tri-, and pentasaccharides (Fig. 1a). The average numbers of Gal-α,1,3Gal epitopes per BSA molecule were 20, 12, and 8.8 for the di-, tri-, and pentasaccharides, respectively. Both anti-Gal mAbs, GT4-31 and GT6-27, demonstrated comparable fine specificities, with affinity predominantly for the Gal-α,1,3Gal trisaccharide.

We next tested the ability of anti-Gal IgG3 and IgG1 to bind to pRBC, and to induce the deposition of C3 and C5 and complement-mediated lysis. Anti-Gal IgG3 bound to, and induced the deposition of C3b and C5b at a 1:10 μg/ml (Fig. 1, b–d). Anti-Gal IgG1 was also able to bind to and induce the deposition of C3b and C5b (Fig. 1, b–d). Anti-Gal IgG1 was ~50-fold less effective than the IgG3 at binding to pRBC (Fig. 1b). It is unclear whether this difference reflects differences in affinity of the FITC-conjugated anti-mouse IgG for IgG1 and IgG3, or in the ability of IgG3 to self-associate through noncovalent interactions resulting in enhanced binding to Ag (11), or both. The ability of anti-Gal IgG1 to induce C3b and C5 deposition was 10 to 50-fold less effective, compared with anti-Gal IgG3 (Fig. 1c).

Despite the detectable ability to induce C3 and C5 deposition, anti-Gal IgG1 was unable to induce complement-mediated lysis even at the highest concentrations of 400 μg/ml (Fig. 1d). Collectively, these observations indicate real qualitative differences in the in vitro ability of anti-Gal IgG1 and IgG3 to activate complement. Both anti-Gal IgG1 and IgG3 induced the activation of early components of complement, but IgG1 mAbs could not activate the terminal components of complement necessary for the assembly of the membrane attack complex and the lysis of pRBCs.

Immunohistochemistry

Immunohistochemistry was based on a modified avidin-biotin peroxidase method as previously published (10). For identification of complement and von Willebrand factor (vWF) deposition, sections were serially incubated with goat anti-C3 or rabbit anti-vWF (DAKO, Carpinteria, CA) polyclonal Abs, biotinylated rabbit anti-goat IgG (Vector Laboratories) or biotinylated goat anti-rabbit IgG (BD PharMingen), and HRP-streptavidin (Zymed Laboratories, South San Francisco, CA).

Functional analysis of anti-Gal IgG mAbs

We next compared the activities of anti-Gal IgG1 and IgG3 in a murine xenograft rejection model. The anti-Gal IgG3 was more efficacious at inducing xenograft rejection. High doses of 0.5–0.25 mg/mouse, injected i.v. into Rag $^{-/-}$ Gal $^{-/-}$ mice, resulted in the rejection of Gal $^{+/-}$ rat heart grafts within 60 min, while lower doses of 0.05 mg/mouse resulted in rejection within 1–2 days (Table I). High doses of anti-Gal IgG1 (0.25–0.5 mg/mouse) were also able to induce xenograft rejection within 120 min (Table I). There was one notable difference in the dose response between the IgG3 and IgG1 mAbs. IgG3 mAbs induced rejection over a wide dose range and the time for rejection gradually increased with decreasing dose; in contrast, IgG1 mAbs induced over a narrower dose range, with an abrupt loss in the ability to induce rejection at doses below 0.25 mg/mouse. This difference could reflect a difference in the mechanism by which the IgG1 and IgG3 mediated xenograft rejection. Thus, the next series of experiments was performed to test this possibility.

Role of complement in anti-Gal IgG1- and IgG3-mediated rejection

Abs mediate their effects in vivo via the activation of complement, or through FcγR-mediated interactions (12, 13). Because of the defined role of complement in hyperacute xenograft rejection (14),
FIGURE 2. Specific inhibition of anti-Gal IgG1-mediated graft rejection by anti-FcγRII/III and anti-NK cell Abs. Rag^(-/−) Gal^(-/−) (a−d) or Gal^(-/−) (e−f) mice were pretreated with CVF, anti-C5, anti-FcγRII/III (FcγRII/III), or anti-NK1.1 mAbs, or anti-asialo GM1 (aGM1) polyclonal Abs as described in Materials and Methods. An additional experiment with anti-asialo GM1 and rabbit sera as a supplementary source of complement (C′) was performed for mice receiving IgG1.

The ability of treated mice to reject Lewis rat heart grafts were monitored for 24 h following administration of anti-Gal or IgG1 (a, c, and e), IgG3 (b, d, and f), 0.5 mg/mouse). All treatment groups had three to eight recipients per group.

we focused initially on testing whether complement activation is necessary for anti-Gal IgG1- or IgG3-mediated rejection. We pretreated Rag^(-/−) Gal^(-/−) recipients with CVF or anti-C5 Abs to deplete C3 or block C5, respectively (15, 16). Rejections mediated by anti-Gal IgG1 and IgG3 were equally inhibited by CVF or anti-C5 (Fig. 2, a and b), suggesting a critical role for activation of complement in the induction of xenograft rejection mediated by anti-Gal IgG1 or IgG3.

Role of FcγR in anti-Gal IgG1- and IgG3-mediated rejection

It has been reported that mouse IgG3 binds poorly to FcγR, while IgG1 binds to FcγR with intermediate affinity (13). We next tested whether FcγR-mediated interactions were critical for the induction of anti-Gal IgG1-mediated rejection. Blocking of FcγRII/III-mediated interactions with the 2.4G2 mAb resulted in a selective loss in the ability of anti-Gal IgG1 to mediate xenograft rejection (Fig. 2c). In contrast, rejection mediated by anti-Gal IgG3 was not affected (Fig. 2d). These observations demonstrate a critical difference in IgG1- vs IgG3-mediated xenograft rejection.

The classic pathological features of hyperacute rejection are platelet thrombosis and fibrin, C3 and C5 deposition in the microvessels and arteries (9). We previously reported that these features were evident in xenografts harvested 7–20 min after infusion of anti-Gal IgG3 mAbs (9). Similar features of hyperacute rejection were observed in xenografts after anti-Gal IgG1 infusion, with the exception that the levels of C3 and C5 deposition were significantly lower than that induced by anti-Gal IgG3 (9). We observed a substantial reduction of platelet thrombosis, C3 and vWF deposition in the xenografts of recipients pretreated with anti-FcγRII/III mAb (2.4G2; Fig. 3, a−d). These observations suggest that FcγRII/III-mediated interactions contribute to the process of platelet aggregation and thrombosis, which is critical for the induction of rapid vascular rejection of xenografts.

The contribution of other FcγRs and other FcγR-expressing cells to IgG1-mediated rejection was not exhaustively examined in this study due to the limited availability of blocking mAbs for specific FcγRs. The activating FcγRI, expressed primarily on macrophages, may have a role in mediating IgG1-mediated rejection. Our observations that the partial depletion of macrophages, with clodronate-encapsulated liposomes, did not result in the inhibition of either IgG3- or IgG1-mediated rejection (data not shown), do not support a role for FcγRI in IgG1-mediated xenograft rejection. FcγRI is an inhibitory receptor that expressed on a variety of cell types, including monocytes, B cells, T cells and granulocytes, but

FIGURE 3. Specific inhibition of anti-Gal IgG1-mediated graft rejection by anti-FcγRII/III and anti-NK cell Abs. Rag^(-/−) Gal^(-/−) mice were pretreated with saline (a and b), anti-FcγRII/III mAbs (c and d), or anti-asialo GM1 (aGM1) polyclonal Abs (e and f) as described in Materials and Methods. The Lewis rat heart grafts were harvested 18 min after the infusion of anti-Gal IgG1, and were stained with anti-C3 (a, c, and e) or vWF (b, d, and f).
not NK cells, while FcγRIII is more widely expressed on macrophages, neutrophils, mast cells, and NK cells (13). Because FcγRIII, but not FcγRII, is expressed on NK cells, we initiated the next series of experiments to gain insights into the role of FcγRIII and NK cells in IgG1-mediated xenograft rejection.

Role of NK cells in anti-Gal IgG1- and IgG3-mediated rejection

We tested whether depletion of NK cells with anti-asialo GM1 Abs would abrogate the ability of anti-Gal IgG1 to mediate xenograft rejection. We observed that mice pretreated with anti-asialo GM1 were unable to mediate anti-Gal IgG1-mediated xenograft rejection, but retained the ability to induce anti-Gal IgG3 rejection (Fig. 2, c and d). Histological examination confirmed a substantial reduction of platelet thrombosis, C3, and vWF deposition in the xenografts of recipients depleted of NK cells (Fig. 3). These observations suggest a unique mechanism of xenograft rejection that is dependent on FcγRIII on NK cells; the role for other cell types or FcγR remains possible and are currently under investigation.

To address the possibility that serum complement had been depleted in anti-asialo GM1-treated recipients, we measured the levels of serum C3 by flow cytometry. The levels of C3 in anti-asialo GM1-treated mice were comparable to untreated controls (data not shown). Additionally, we supplemented serum complement by infusing 0.2 ml of rabbit serum into anti-asialo GM1-treated recipients, 10 min before injection of anti-Gal IgG1 Abs. We have previously reported that this volume of rabbit serum restored the ability of anti-Gal Abs, in heat-inactivated human serum, to induce hyperacute rejection of Lewis rat hearts in Gal−/− mice (17). Injection of rabbit serum into recipients pretreated with anti-asialo GM1 did not restore IgG1-mediated hyperacute rejection, confirming that the effects of anti-asialo GM1 are independent of complement depletion (Fig. 2c).

It has been reported that anti-asialo GM1 eliminate NK cells and a subset of T cells. We believe that, in our system, the depletion of NK cells by anti-asialo GM1 is responsible for the loss in ability of anti-Gal IgG1 to elicit hyperacute rejection as T cells are not present in Rag−/− Gal−/− recipients. We also tested whether anti-NK1.1 mAbs, which preferentially deplete NK cells, prevented IgG1-mediated rejection (Fig. 2e). This experiment was performed in immunocompetent Gal−/− C57BL/6 mice, which express NK1.1 (Rag−/− Gal−/− mice with the 129 background do not express NK1.1). Anti-NK1.1 inhibited IgG1-mediated rejection, but had minimal effects on IgG3-mediated rejection (Fig. 2, e and f). These observations confirm a critical and unique role for NK cells in IgG1-mediated rejection in both immunocompetent and immunodeficient mice.

The in vitro ability of NK cells to lyse endothelial cells, either through natural recognition or through Ab-mediated interactions, has been extensively reported (18–20). However, in vivo evidence for a definitive role of NK cells in mediating acute vascular rejection of xenografts has been lacking. We here demonstrate a role for NK cells in the rapid rejection of xenografts by anti-Gal IgG1 mAbs. At present, it is unclear whether FcγRIII molecules on NK cells serve to cross-link anti-Gal mAbs bound to graft endothelial cells to induce their activation, or whether the cytolytic effects of NK cells triggered by FcγRIII-cross-linking, are critical for IgG1-mediated rejection.

In conclusion, we report that anti-Gal IgG1 mAb mediates a novel form of vascular rejection that is dependent on complement activation, FcγR-mediated interactions, and NK cells. We hypothesize that IgG1 inefficiently induces complement activation to a level that is insufficient to induce hyperacute rejection. In contrast, the combined effects of complement and FcγRIII-mediated activation, and injury, of endothelial cell activation is sufficient to trigger platelet aggregation and coagulation, and hyperacute rejection. Blockade of either complement activation or FcγRIII-mediated events results in suboptimal endothelial cell activation or injury that is insufficient to induce IgG1-mediated rejection. This model of hyperacute rejection involving FcγRs and NK cells may explain the ability of Abs with weak complement-activating properties to elicit xenograft rejection.

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