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We have previously produced a series of antagalactose (anti-Gal) hybridomas and characterized their heavy chain gene usage. Here we have quantified the affinity of these Abs for the α-Gal epitope and characterized their in vitro effects on endothelial cell activation and apoptosis. We report that anti-Gal mAbs derived from Gal−/− mice show a range of affinity for the α-Gal epitope, and that affinity was generally increased as the VH gene usage transitioned from germline sequences to sequences exhibiting somatic maturation. Despite an 85-fold range in affinity, all the anti-Gal mAbs examined induced α-Gal-specific endothelial cell activation, and after prolonged exposure induced endothelial cell apoptosis in a complement-independent manner. Only murine anti-Gal mAbs of the IgM or IgG3 subclass, but not IgG1, were effective at initiating complement-dependent cell lysis. Using a novel rat to mouse xenograft model, we examined the in vivo ability of these mAbs to induce xenograft rejection and characterized the rejection using histology and immunohistochemistry. Infusion of complement-fixing IgG3 mAbs resulted in either hyperacute rejection or acute vascular rejection of the xenograft. Surprisingly, infusion of an equal amount of a high affinity anti-Gal IgG1 mAb, that fixed complement poorly also induced a rapid xenograft rejection, which we have labeled very acute rejection. These studies emphasize the importance of in vivo assays, in addition to in vitro assays, in understanding the role of anti-Gal IgG-mediated tissue injury and xenograft rejection. The Journal of Immunology, 2003, 170: 1531–1539.

Naturally occurring anti-Gal Abs in human and Old World monkeys bind to galactose α-(1,3)-galactose terminal carbohydrate epitopes (α-Gal) expressed on pig cells, where they initiate a rapid, complement-driven, hyperacute rejection of pig organs when transplanted into primates (1–4). This rejection process can be largely averted by depletion of anti-Gal Abs (5, 6) or systemic complement inhibition (7) or through the use of organs from transgenic pigs that provide intrinsic complement regulation by expressing human complement-regulating proteins (8–11). When hyperacute rejection (HAR) is prevented, the grafts are rejected within a few days to weeks by a vascular rejection process termed acute vascular rejection (AVR) (12) or delayed xenograft rejection (13). Studies involving serial biopsies reveal that AVR is characterized by a progressive deposition of IgG, IgM, and fibrin; diffuse thrombosis; edema; ischemic damage; necrosis; and a lack of cellular infiltrate (5, 14–16). Deposition of complement components is sometimes detected, but generally at levels far lower than observed during HAR. There is clear evidence from humans exposed to pig tissues (17–19) and from pig to primate xenografts (20–22) for a vigorous induction of anti-Gal Abs. The removal of anti-Gal Abs can at least delay the onset of AVR, and this has led to the hypothesis that these Abs may cause rejection by an as yet unidentified mechanism.

The Gal−/− mouse represents a model immunological system for studying anti-Gal responses to Gal−/+ xenografts (23–26). We have used this system to produce a series of anti-Gal hybridomas derived from naïve Gal−/− mice and from Gal−/− mice after rat to mouse heterotopic cardiac xenotransplantation (27). Previous characterization of these Abs demonstrated that naïve anti-Gal IgM and IgG mAbs derived early in the immune response used a variety of VH genes in a germline configuration. In contrast, anti-Gal IgG mAbs derived 21 days after transplantation were encoded by a single VH4606 14A gene family and showed evidence of somatic mutation. These observations were consistent with the recent reports by Cramer and his colleagues (28) that the VH genes that encode Abs with αGal reactivity are restricted to a small number of genes.

In this study we have measured the binding affinity of both germline-encoded and affinity-matured anti-Gal mAbs and characterized the effects of these anti-Gal mAbs on endothelial cells in vitro. We show that all the anti-Gal mAbs are capable of activating endothelial cells and that prolonged exposure to anti-Gal mAbs can lead to endothelial cell apoptosis. More importantly, we extend these in vitro studies to a preliminary analysis of the in vivo effects of these mAb on xenograft rejection using a unique rat to Gal−/− recombinase-activating gene (Rag)−/− transplant model. In this model we can reproduce both HAR and AVR using complement-fixing anti-Gal Abs. Surprisingly, we observed that a high affinity anti-Gal IgG1 mAb with poor complement-fixing activity was also very effective at inducing a rapid xenograft rejection (<2 h), which we have now labeled very acute rejection (VAR).
Materials and Methods

**Purification of anti-Gal Abs by ammonium sulfate precipitation**

Anti-Gal IgG-secreting hybridomas were cultured in protein-free hybridoma medium (Life Technologies, Grand Island, NY) for 3–5 days. Initial experiments were designed to purify anti-Gal mAbs using an affinity column containing a Gal-Tra2 trisaccharide. This method was unsuccessful since many of the anti-Gal mAb lost binding activity after elution by low pH buffer. As an alternative, ammonium sulfate precipitation was used to concentrate anti-Gal mAb from the supernatant. Following extensive dialysis, the purity of the precipitated Ab was confirmed by SDS-PAGE and Western blot analysis. No major contaminating protein bands were observed in the mAb concentrated in this manner. The Ab concentration was determined by a bicinchoninic acid protein assay and confirmed using an IgG ELISA based on the appropriate murine isotype standards. The specificity of each Ab was determined by an α-Gal ELISA using BSA or human serum albumin (HSA) glycoconjugates as a solid substrate Ag (29).

**Binding affinity of anti-Gal Abs**

The relative affinity of each anti-Gal IgG and the avidity of anti-Gal IgM Ab was determined using the methods described by Friguet et al. (29). This method relies on establishing an Ag-Ab equilibrium in solution and then determining the amount of Ab free at equilibrium using a solid phase ELISA. For the process to be accurate it was important to work with Ab concentrations that fall within the linear range of the solid phase ELISA. The α-Gal ELISA was therefore first calibrated for each hybridoma to determine an optimal Ab concentration for subsequent affinity measurements. Briefly, 30 μg/ml of an HSA-Gal glycoconjugate containing an average of 17 Gal-Tra2 trisaccharide moieties on each protein (HSA-Gal(17)) was coated in a 96-well plate overnight at 4°C. The plate was incubated with various concentrations of mAb (from 10 to 0.001 μg/ml) for 60 min at 4°C. After washing, the plate was blocked with PBS buffer containing 0.01% Tween 20 and 1% HSA. An alkaline phosphatase-conjugated goat anti-mouse IgG or IgM was placed into the plate for 60 min at 4°C to detect Ab binding. A standard hybridoma was repeatedly used to define the development time. The hybridoma concentration that produced an OD₄₀₅ reading between 0.6 and 0.8 under these conditions was chosen for subsequent affinity measurement.

To estimate Ab affinity a dilution series of the HSA-Gal(17) glycoconjugate was incubated at 4°C in solution with an optimal concentration (defined above) of each anti-Gal Ab. At equilibrium the concentration of free Ab in solution was determined by a solid phase ELISA used with the same HSA-Gal(17) glycoconjugate as a solid phase substrate. The following conditions applied for most Abs. The concentration of HSA-Gal(17) ranged from 10⁻¹⁰ to 10⁻⁸ M, and the optimal Ab concentration was ~10⁻⁸ M. The half amount of free Ab at equilibrium at 30 min incubation disturbed during the solid phase ELISA, a sample of the equilibrium solution after 30-min incubation in the solid phase ELISA was transferred to a second fresh ELISA well and incubated for an additional 30 min. The amount of free Ab estimated in the two wells and a fractional difference defined as f = (OD₄₀₅well2 - OD₄₀₅well1)/OD₄₀₅well1 was calculated. As shown in Figure 2 (29), low values of f (<0.1) indicate that the amount of Ab trapped in the solid phase ELISA was only a small fraction of the total free Ab at equilibrium, ensuring that the solid phase ELISA did not significantly displace the Ag/Ab equilibrium. Dissociation constants were determined by Klotz plots, since all mAb tested in this study were purified by ammonium sulfate precipitation, which is considered a semi-pure preparation. Procedures described by Stevens (30) were used to correct the error caused by the bivalent Ab structure of IgG. This analysis determined then the affinity of IgG and avidity of IgM anti-Gal mAbs.

A qualitative assessment of Ab affinity was also made in an ELISA format by assaying Ab binding to HSA glycoconjugates containing variable levels of α-Gal trisaccharide substitution. These HSA glycoconjugates were produced by coupling a galactose or 1,3-galactose α(1,4)-N-acetyl glucosamine trisaccharide containing a five-carbon linker and carboxylic acid group (TRFA) to HSA using N-hydroxysulfosuccinimide and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (ECD). The general reaction conditions consisted of mixing 16 ml of cold 200 mM Tris (pH 6.5) containing 1 g of HSA (Sigma-Aldrich, St. Louis, MO) with 4 ml of 1 M MES (pH 5.5) containing 80 mg/ml of hydroxysulfosuccinimide and 37.5 mg/ml of EDC. The mixture was reacted at 4°C for 4 h on a rotor shaker, and the reaction was stopped by the addition of 4 ml of 1 M Tris (pH 8.5). The conjugates were dialyzed through 10K membranes against distilled water and lyophilized. Increased TRFA densities were produced by increasing the amount of EDC in the reaction. The average TRFA density in each conjugate was determined by mass spectroscopy, and each conjugate was examined by silver-stained SDS-PAGE and anti-Gal Western blots to detect the presence of excess intermolecular cross-linking. Ab binding to HSA-Gal glycoconjugates with an average of 4, 8, 17, and 26 α-Gal trisaccharide epitopes was tested.

**Binding of anti-Gal hybridomas to Gal-expressing cells**

The ability of mAb to bind to pig RBC (pRBC) was determined by FACS. The pRBCs (5 × 10⁸) were incubated with purified anti-Gal mAb (1 μg) on a 1-h mixture, and the mixture was washed using 2% HSA-Gal(17) glycoconjugate. FITC-conjugated goat anti-mouse IgG was added to the pRBC, and Ab binding was detected by flow cytometry (FACSCalibur; BD Biosciences, San Jose, CA).

**Complement activation by anti-Gal Abs**

Anti-Gal hybridomas were assayed for the ability to activate complement with a hemoglobin release assay. Purified mAb at 5 μg/ml or 150 μg of hybridoma culture supernatant was incubated with 50 μl of pRBC in a 96-well, V-shaped microtiter plate (Costar, Corning, NY). After a 30-min incubation at room temperature, the cells were washed, and the anti-Gal-sensitized pRBCs were incubated at 37°C for 30 min in 2% guinea pig complement (Sigma-Aldrich). The plate was centrifuged, and the supernatants were monitored for released hemoglobin by spectrophotometric measurement at OD₅₄₀.

**Activation of pig microvesSEL endothelial cells (PMVEC) by anti-Gal Abs**

PMVEC were cultured to confluence in 96-well plates in endothelial culture medium (medium 199; Life Technologies) containing endothelial cell growth factor (30 μg/ml; Collaborative Biomedical Products, Bedford, MA) heparin (80 U/ml; Sigma-Aldrich), penicillin/streptomycin, and 10% FCS. After washing the cells, anti-Gal mAb or isotype controls (IgG3, IgG1, and IgM) at 1–10 μg/ml was added to the cells in the presence or the absence of 10 nM TRFA. A goat anti-mouse IgG Ab or a goat anti-human Ig was used to cross-link bound anti-Gal Abs. The plates were incubated for 4 h at 37°C in the endothelial cell culture medium containing 1% OVA. After the incubation, the cells were washed with PBS and fixed with 0.02% glutaraldehyde for 10 min. Subsequently, the plate was blocked with 1% OVA in PBS containing 0.1% Tween 20. The cells were then washed three times with PBS and treated with anti-C5b-9 ECD (ECL BioScience, San Diego, CA), anti-C5b-9 IgG, or anti-C5b-9 IgM for 1 h. After washing, the cells were stained with annexin V-Fluos and propidium iodide (PI) according to instructions from the manufacturer (Roche Diagnostics, Indianapolis, IN). Briefly, cells were washed with PBS and incubated with annexin V fluorescence in HEPES buffer containing PI, and then analyzed by flow cytometry (FACSCalibur; BD Biosciences).

**Ability of anti-Gal mAb to induce xenograft rejection**

Mice with engineered disruptions of the Rag (31) and GalT (26, 32) loci were bred and maintained at Rush Presbyterian-St. Luke’s Medical Center (Chicago, IL), an Association for the Assessment and Accreditation of Laboratory Animal Care International-accredited facility. The Rag⁻/⁻ Gal⁺⁺ mice were used as recipients for 10- to 16-day-old heterotopic Lewis rat (Harlan, Walkervville, MD) heart xenografts. Heterotopic rat hearts were transplanted into the abdomen of the recipient by anastomosing the donor aorta to recipient aorta and the donor pulmonary artery to recipient inferior vena cava as previously described (33). Anti-Gal mAbs were administered by i.v. injection on the day of transplantation or up to 7 days post-transplantation; this variable was monitored daily until rejection, and rejection was defined as the complete cessation of pulsation.

**Immunohistochemical staining**

Transplanted hearts were surgically removed upon complete cessation of palpable heart beat and snap-frozen in Tissue-Tek OCT (Sakura Finetek USA, Inc.)
USA, Torrance, CA). Sections (5 μm) were cut serially through the middle section of the heart, and one section was stained with H&E for histology. For immunohistochemistry, the sections were subjected to a modified avidin-biotin peroxidase method. Briefly, sections were fixed with cold acetone, and endogenous peroxidase was blocked with 0.015% H2O2. For identification of complement and fibrin deposition, sections were serially incubated with goat anti-C3/C5 (Quidel, San Diego, CA) and rabbit anti-thrombogen (DAKO, Carpinteria, CA) polyclonal Abs, biotinylated goat anti-goat IgG (Vector Laboratories, Burlingame, CA) or biotinylated goat anti-rabbit IgG (BD PharMingen, San Diego, CA), and HRP-streptavidin (Zymed). The TdT reaction was visualized with 3,3'-diaminobenzidine solution (Dako). Immunostaining was developed with 3,3'-diaminobenzidine solution and counterstained with Mayer’s hematoxylin.

**TUNEL staining**

A modified TUNEL assay was applied for detection of apoptosis in the transplanted xenografts (34). In brief, frozen sections of cardiac grafts were fixed with 10% buffered formalin and postfixed with alcohol and acetic acid mixture (2/1, v/v). Glucose plus glucose oxidase solution were applied for blocking endogenous peroxidase (35). The sections were rinsed briefly in TdT buffer (30 mM Trizma, 140 mM sodium cacodylate, and 1 mM cobalt chloride, pH 7.2), then incubated with TdT reaction solution (300 enzyme U/ml of TdT and 10 nmol/ml of biotinylated 16-dUTP (Roche) in TdT buffer (pH 7.2)) for 90 min at 37°C. The TdT reaction was terminated with 2× SSC buffer (300 mM sodium chloride and 30 mM sodium citrate, pH 7.2). The sections were then incubated with 2% BSA to block nonspecific binding, then incubated with HRP-conjugated streptavidin (1/200; Zymed). The TdT reaction was visualized with 3,3'-diaminobenzidine and counterstained with Mayer’s hematoxylin. The number of apoptotic cells was counted for a whole section of each cardiac xenograft sample.

**Results**

**Affinity of anti-Gal hybridoma Abs**

We determined the affinity of anti-Gal IgG and the avidity of anti-Gal IgM mAb using the method of Friguet et al. (29). The final IgG and IgM determinations for each Ab are listed in Table I, and a Klotz plot (Fig. 1) for anti-Gal IgM (GN-2-M2) IgG3 (GT21-2-G1 and GT21-1-G1.7) and IgG1 (GT21-2-G2) illustrates the range of results we obtained. The anti-Gal IgM mAbs were isolated from naive Gal−/− mice and used different germline VH chain genes. These mAbs exhibited the lowest binding avidity to HSA-Gal, ~5–6 × 10−7 M. Similar low affinity binding was observed for an IgG3-encoded Ab, GT21-2-G1. This hybridoma secretes an IgG3 Ab with the fewest replacement mutations in the VH sequence. In contrast, the other anti-Gal IgG3 mAb show 3- to 10-fold higher affinity than GT21-2-G1 and are encoded by VH genes with 7–11 replacement mutations. These other hybridomas probably represent products of a clonal expansion (27). The GT21-2-G2 hybridoma, an IgG1, had the highest level of replacement substitutions and showed the highest binding affinity among the mAb tested.

We further assessed the affinity of murine anti-Gal IgG and the avidity of anti-Gal IgM mAb using HSA-Gal glycoconjugates bearing different levels of α-Gal substitution. In human and primate sera, we typically observed anti-Gal IgM and IgG that bound effectively to an HSA-Gal substrate with an average of four α-Gal trisaccharide residues per monomer (36). In contrast, most of the murine anti-Gal hybridomas required a higher level of substitution to bind. The anti-Gal IgM hybridomas with the lowest binding avidity required an average of at least 19 trisaccharide residues per protein monomer to effectively bind in this assay. In contrast, the IgG anti-Gal mAb that exhibit 6- to 60-fold higher affinity than the IgM mAb bound effectively to glycoconjugates with an average of eight α-Gal trisaccharides. Only the anti-Gal IgG1Ab, GT21-2-G2, which had the most mutations in the gene sequence, bound to an HSA conjugate with an average of four trisaccharide epitopes per monomer (Table I). These results are consistent with our previous

![FIGURE 1. Klotz plot of anti-Gal Ab affinity for Gal-HSA. The graph is a reciprocal plot showing the relationship between the fraction of bound Ab (V0) and the total Ag concentration (A0). The slope of the curve is the estimate of binding affinity. The hybridomas GT21-2-G2 (●), GT21-2-G1 (▲), GT21-1-G1.7 (◆), and GN-2-M2 (○) illustrate the range of affinities between 5 × 10−7 and 7 × 10−9 M that we observed. Each sample was analyzed in duplicate.](http://www.jimmunol.org/)

Table I. Characteristics of mouse anti-Gal hybridomas

<table>
<thead>
<tr>
<th>Hybridomas</th>
<th>Isotypes</th>
<th>Mutations (R/S)</th>
<th>Affinity (M) (relative change)</th>
<th>Gal Substrate</th>
<th>Apoptosis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GN-1-M2</td>
<td>IgM</td>
<td>0</td>
<td>6 × 10−7 (1.0)</td>
<td>Gal (19)</td>
<td>ND</td>
</tr>
<tr>
<td>GN-2-M2</td>
<td>IgM</td>
<td>1/5</td>
<td>5 × 10−7 (1.2)</td>
<td>Gal (19)</td>
<td>ND</td>
</tr>
<tr>
<td>GT21-2-G1</td>
<td>IgG3</td>
<td>4/0</td>
<td>1 × 10−7 (6.0)</td>
<td>Gal (8)</td>
<td>68</td>
</tr>
<tr>
<td>GT21-1-G1.4</td>
<td>IgG3</td>
<td>7/1</td>
<td>6 × 10−8 (10.0)</td>
<td>Gal (8)</td>
<td>ND</td>
</tr>
<tr>
<td>GT21-1-G1.5</td>
<td>IgG3</td>
<td>8/1</td>
<td>6 × 10−8 (10.0)</td>
<td>Gal (8)</td>
<td>98</td>
</tr>
<tr>
<td>GT21-1-G1.1</td>
<td>IgG3</td>
<td>7/1</td>
<td>5 × 10−8 (12.0)</td>
<td>Gal (8)</td>
<td>67</td>
</tr>
<tr>
<td>GT21-1-G1.2</td>
<td>IgG3</td>
<td>8/1</td>
<td>3 × 10−8 (20.0)</td>
<td>Gal (8)</td>
<td>83</td>
</tr>
<tr>
<td>GT21-1-G1.6</td>
<td>IgG3</td>
<td>8/1</td>
<td>3 × 10−8 (20.0)</td>
<td>Gal (8)</td>
<td>95</td>
</tr>
<tr>
<td>GT21-1-G1.7</td>
<td>IgG3</td>
<td>11/1</td>
<td>3 × 10−8 (20.0)</td>
<td>Gal (8)</td>
<td>63</td>
</tr>
<tr>
<td>GT21-1-G1.8</td>
<td>IgG3</td>
<td>8/1</td>
<td>1 × 10−8 (60.0)</td>
<td>Gal (8)</td>
<td>ND</td>
</tr>
<tr>
<td>GT21-2-G2</td>
<td>IgG1</td>
<td>13/6</td>
<td>7 × 10−8 (85.0)</td>
<td>Gal (4)</td>
<td>54</td>
</tr>
</tbody>
</table>

a Affinity was measured by an ELISA as described in Materials and Methods. The R/S ratio represents the numbers of replacement/silent mutations as previously described (20). The relative differences in affinity between hybridomas (listed in parentheses) are represented by normalizing the lowest affinity Ab to a value of 1.0. The binding of hybridoma Abs to HSA-Gal glycoconjugates with an average of 4, 8, 17, 19, and 26 trisaccharide moieties was examined by ELISA. The glycoconjugate with the minimal level of trisaccharide substitution that gave OD405 > 0.200 in the binding ELISA is listed with the number in parentheses representing the level of α-Gal substitution. The ability of anti-Gal mAb to induce PMVEC apoptosis was determined by flow cytometric analysis as described in Materials and Methods. The percentage of apoptotic cells at 50 μg/ml of anti-Gal Ab was determined by positive annexin V and PI staining.
affinity measurements and suggest that upon affinity maturation some anti-Gal IgG from Gal$^{+/-}$ mice can attain affinity comparable to that of humans or primates. 

**In vitro characterization of anti-Gal Abs**

By FACS analysis, all the anti-Gal mAb bound effectively to pRBCs, and this binding appeared to be specific for the α-Gal epitope, since it was readily inhibited by competition with 2% HSA-Gal (Fig. 2A). Under conditions of Ab excess most of the mAb bound pRBC to a similar extent, exhibiting a mean channel fluorescence between ~100 and 200. The exception to this was hybridoma GT21-1-G1.1, which exhibited ~3- to 4-fold higher binding (mean channel fluorescence, 550). This difference does not appear to be associated with changes in Ab affinity per se, but may be due to differences in fine scale specificity of Abs for the α-Gal Ag. Similar observations of differential binding of human anti-Gal Abs and the lectin GS1B4 to different α-Gal-containing carbohydrates has been reported (37–39).

The binding of anti-Gal mAb to pRBC allowed us to determine the ability of these mAb to activate complement using a hemolytic assay. As expected, IgM and IgG3 anti-Gal mAb were effective at stimulating complement-dependent hemolysis (Fig. 2B). At a standard concentration (5 µg/ml) the IgG3 mAb exhibited 20–60% pRBC hemolysis with a background of ~10% hemolysis in the presence of 10 mM competing α-Gal trisaccharide. This background was similar to that observed for a mouse IgG3 isotype control. Note that the order of mAb in Fig. 2B reflects the order of affinity for the α-Gal epitope (Table I), suggesting that under these conditions of saturating levels of mAb, there was no obvious correlation between Ab affinity and the level of hemolysis. The highest affinity Ab, GT21-2-G2, failed to lyse pRBC (~10%). This result is consistent with the known ability of this isotype (IgG1) in the mouse to poorly fix complement (40).

**Activation and induction of apoptosis of endothelial cells by anti-Gal Abs**

It has been proposed that activation and apoptosis of endothelial cells by anti-Gal mAb play critical roles in AVR. We tested the ability of anti-Gal mAbs, affinity-purified human anti-Gal Ab, and human serum to activate and induce apoptosis of PMVEC in the absence of complement (Fig. 3A). In initial experiments incubation of endothelial cells with anti-Gal mAb alone did not result in endothelial cell activation, as determined by an induction of CD62E expression (data not shown). This was also true for naïve human serum and purified anti-Gal Abs derived from normal human serum (Fig. 3B). Under in vivo conditions, however, Abs bound to the cell surface may be cross-linked by a variety of mechanisms, including low levels of complement activation and interactions with cells bearing FeR. When endothelial cells were incubated with purified anti-Gal mAb in the presence of a cross-linking agent (goat anti-mouse IgG), the expression of CD62E was consistently induced (Fig. 3A). Similar induction of CD62E expression was evident using purified human anti-Gal Ab with the appropriate (goat anti-human IgG) cross-linking Ab (Fig. 3B). This induction was specific for the α-Gal epitope, since preincubating anti-Gal mAb with 10 mM free α-Gal trisaccharide prevented the increase in CD62E expression. The anti-Gal IgG1 mAb (GT21-2-G2) that was unable to effect complement-mediated lysis of pRBCs was equally effective at inducing CD62E expression in this assay. The effectiveness of the IgG1 anti-Gal mAb further suggests that cross-linked anti-Gal IgG bound to the endothelial cell surface can induce CD62E expression in the absence of complement activity.

![Figure 2](https://example.com/image.png)

**FIGURE 2.** A. Representative FACS analysis of anti-Gal hybridoma binding to pRBC. The pRBCs (5 × 10$^7$) were incubated with 1 µg of each anti-Gal Ab in the presence (●) or the absence (□) of 10 mM α-Gal. FITC-conjugated goat anti-mouse IgG was used for detecting the binding of anti-Gal to pRBC. The mean channel fluorescence for Ab binding in the absence of 10 mM α-Gal is given in each binding profile. B. Ability of anti-Gal Abs to induce complement-mediated pRBC lysis. The ability of each hybridoma Ab to fix complement was examined by a hemoglobin release assay as described in Materials and Methods. The pRBC were diluted to 5% and incubated with 10 µg/ml of anti-Gal mAb alone (●) or in the presence (□) of 10 mM α-Gal. Guinea pig serum (1/50) was used as a source of complement. Each sample was analyzed in duplicate and tested in at least two separate experiments. Water was used to obtain complete lysis of pRBC (100%), and isotype-unrelated, murine-specific Abs were used as negative controls.
anti-Gal Abs appeared to be dose-dependent, requiring a minimum of 10 mM competitive α-Gal trisaccharide (XNA) or in the presence of goat anti-human Ig to enhance cross-linking (XNA + anti-hulg). Endothelial cell activation was evident in the presence of the cross-linker and could be inhibited by blocking Ab binding with 10 μg/ml human xenoantibodies (XNA) purified from normal human serum (Fig. 4A). Under these conditions apoptotic en-

**FIGURE 3.** A. Endothelial cell activation induced by anti-Gal Abs. Anti-Gal Abs were incubated at the 10 μg/ml with PMVEC for 4 h at 37°C alone (■) or in the presence of 10 mM competitive α-Gal trisaccharide (□). Cells were fixed, and the level of E-selectin was quantified by ELISA as described in Materials and Methods. Each sample was analyzed in triplicate, and mouse IgG3 and IgG1 were included as negative controls. B. Endothelial cell activation using human anti-Gal IgG and IgM purified from an α-Gal affinity column. Human anti-Gal Ab was incubated alone (XNA) or in the presence of goat anti-human Ig to enhance cross-linking (XNA + anti-hulg). Endothelial cell activation was evident in the presence of the cross-linker and could be inhibited by blocking Ab binding with 10 mM α-Gal trisaccharide free acid (XNA + anti-hulg + TRFA).

Unlike the short term exposure of endothelial cells to cross-linked anti-Gal mAb, we found that prolonged incubation of endothelial cells and anti-Gal Ab (50 μg/ml) in the absence of a cross-linking agent was effective at inducing endothelial cell apoptosis (Table I and Fig. 4A). Under these conditions apoptotic endothelial cells stained positively for both annexin V and PI. This is a stage annexin V−PI− apoptosis (41). The apoptosis induced by anti-Gal Abs was dependent on the presence of α-Gal trisaccharide. Anti-Gal mAbs, GT21-1-G1.6, at 50, 20, 10, and 5 μg/ml (data not shown), was performed. Cells were incubated with Ab for 18 h at 37°C. Apoptotic cells were determined by annexin V and PI staining, and the frequency of apoptosis was determined by flow cytometry. B, Anti-Gal Abs induce apoptosis in a dose-dependent manner. Anti-Gal mAbs, GT21-1-G1.6, at 50, 20, 10, and 5 μg/ml were cultured with PMVEC for 18 h. Cells were also incubated with human xenoantibodies (XNA) purified by an α-Gal-conjugated affinity column, and mouse IgG3 isotype control (50 μg/ml; M-IgG3), human IgG (H-IgG), and human IgM (H-IgM) served as negative controls, respectively. The percentage of apoptotic cells was determined by positive annexin V and PI staining.

**In vivo activity of anti-Gal Abs**

Our in vitro analysis indicated that anti-Gal mAb of the appropriate isotype were effective at complement-mediated lysis, while all anti-Gal mAb in the absence of complement can induce endothelial cell activation and apoptosis. To determine what effect these mAb might have on xenograft survival, we examined the effects of infused purified Ab (50 μg/mouse) in rat to mouse heterotopic cardiac xenograft recipients. In this model system mice with targeted disruptions in the Rag and GALT loci are used as xenograft recipients. These recipient mice do not express the α-Gal Ag, and the presence of the RAGT mutation blocks the development of B and T cells. In the absence of any immunosuppressive agent, baby rat cardiac xenografts survive for >60 days in the double knockout recipients (Table II), while mice containing only the Gal−/− reject baby rat cardiac xenografts in 4–5 days. Thus, the double-knockout Rag−/−Gal−/− mice are ideal recipients for studying the effects of infused anti-Gal Ab on the survival of rat heart xenografts.

<table>
<thead>
<tr>
<th>Recipients</th>
<th>Sera/Ab</th>
<th>Xenograft Survival</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>GT-KO</td>
<td>None</td>
<td>44,5,5,5,5,5 d</td>
<td>4.7 ± 0.6 d</td>
</tr>
<tr>
<td>Rag−/−/Gal−/−</td>
<td>None</td>
<td>&gt;60 (×5) d</td>
<td>&gt;60 d</td>
</tr>
<tr>
<td>Rag−/−/Gal−/−</td>
<td>0.35 ml of serum</td>
<td>0.3, 0.3, 3 h</td>
<td>1.2 ± 0.9 h</td>
</tr>
<tr>
<td>Rag−/−/Gal−/−</td>
<td>0.5 mg of GT21-1-G1.1</td>
<td>0.1, 0.1, 0.25, 1, 1 h</td>
<td>0.5 ± 0.2 h</td>
</tr>
<tr>
<td>Rag−/−/Gal−/−</td>
<td>0.5 mg of GT21-2-G1</td>
<td>24 (×4) h</td>
<td>24 ± 0 h</td>
</tr>
<tr>
<td>Rag−/−/Gal−/−</td>
<td>0.5 mg of GT21-2-G2</td>
<td>0.6, 1.75, 2 (×5) h</td>
<td>1.8 ± 0.2 h</td>
</tr>
</tbody>
</table>

* Serum was harvested from Gal−/− mice immunized (i.p.) with 100 mg of homogenized pig kidney membranes at 2-wk intervals. This serum was harvested 2 wk after the third immunization, heat-inactivated at 56°C for 30 min, and stored at −70°C before use.
because they do not express the α-Gal epitope (so the infused Ab binds only to the transplanted organ), and there is no possibility of a pre-existing or induced humoral response to compete with the infused mAb.

We examined the effects of infusing two different complement-fixing anti-Gal IgG3 and one IgG1 Ab on xenograft survival (Table II). The IgG3 mAb exhibited different affinities to α-Gal, with the GT21-2-G1 showing the lowest affinity of all the IgG we studied and an ~2-fold lower affinity then the GT21-1-G1.1, which exhibited an average affinity among the IgG3 mAb we examined. The IgG1 mAb (GT21-2-G2) exhibited the highest affinity of all the anti-Gal mAb, but was ineffective at complement-mediated lysis of pRBCs. Each of these purified mAb was injected (500 μg/mouse) i.v. into Rag−/−Gal−/− recipients that had previously received heterotopic baby rat cardiac xenografts. As expected, the infusion of a complement-fixing IgG3 (GT21-1-G1.1) produced rapid organ failure. The speed of this rejection, the presence of widespread microcapillary platelet thrombosis, and the strong deposition of C3 and C5 were consistent with the general characterization of HAR (Fig. 5, A–D). Organ failure on a similar time scale and with the same pathology (data not shown) was also observed after the infusion of 0.35 ml of serum from Gal−/− mice immunized with pig kidney membranes. Surprisingly, the infusion of the lower affinity IgG3 Ab did not produce a classical HAR, but led to xenograft failure in 24 h. The immunohistopathology presented at rejection (24 h after mAb infusion) consisted of widespread Ab binding, myocyte necrosis, fibrin deposition, and low to undetectable levels C3 and C5 complement deposition (Fig. 5, E–H). Some of these features, such as pervasive IgG3 deposition in the heart tissues, may reflect the time of harvest of the grafts relative to mAb infusion; nonetheless, it is important to note that this pathology was similar to the AVR observed in pig to primate xenografts (14–16).

We expected that the high affinity IgG1 GT21-2-G2 mAb, which fixed complement poorly, would induce a protracted AVR; however, we observed a rapid organ failure. The time scale of this

FIGURE 5. Histology and immunohistochemistry of rejection by GT21-1-G1.1 A–E, Interstitial congestion, hemorrhage, and the lumen of arteries/microvessels were blocked by platelet thrombi (A; H&E staining). B, Thrombi deposits were predominantly composed of platelets (fibrinogen staining; magnification, ×400). There was extensive IgG3 (C) and C3 (D) and moderate C5 (E) deposition. F–J, Histology and immunohistochemistry of rejection by GT21-2-G1. F, Extensive intravascular coagulation, myocyte coagulative necrosis, and thrombosis of arteries accompanied by arterial fibrinoid necrosis (H&E staining). Fibrin thrombi in the artery (G) and microvessel (inset) were found (magnification, ×400). Extensive deposition of IgG3 (H), but low to undetectable C3 (I) and C5 (J) deposition, were present. Macrophage infiltration was focal and moderate (data not shown).

FIGURE 6. Histology of anti-Gal IgG1 (GT21-2-G2)-mediated VAR 2 h after mAb infusion (A–E). Interstitial congestion, hemorrhage, and the lumen of microvessels were blocked with platelet microthrombi (A; H&E staining). Rejection was associated with fibrin deposition (B) and extensive anti-Gal IgG1 deposition in the vessels of the xenograft (C). Minimal focal C3 deposition (D), but no C5 deposition (E), and minimal macrophage (Mac-1) infiltration (data not shown) characterized this IgG1-mediated VAR. F–J, Histology of anti-Gal IgG1 (GT21-2-G2)-mediated VAR 10–15 min after mAb infusion. F, Interstitial congestion, hemorrhage, and the lumen of microvessels were blocked with platelet microthrombi (H&E staining). Extensive fibrin deposition (G) and anti-Gal IgG1 deposition were observed in the vessels of the xenograft (H). Significant C3 deposition (I), but no C5 deposition (J), characterized the early stages of VAR. All slides were subject to avidin-biotin peroxidase staining and viewed at ×200 unless indicated otherwise.
organ failure was consistent with HAR, but immunopathology revealed that it was subtly different. Hearts harvested at the time of rejection, ~2 h after IgG1 infusion, were associated with strong fibrin deposition, minimal focal C3 deposition, and minimal macrophage infiltration (Fig. 6, A–D), but no C5 deposition (Fig. 6E). Examination of the xenografts at earlier time points (10–15 min after IgG1 infusion) revealed widespread microcapillary platelet thrombosis and a modest deposition of C3, but still no C5 deposition (Fig. 6F–J). Since HAR is completely dependent on activation of the terminal components of complement, further studies are underway to define the mechanism of IgG1-mediated xenograft rejection.

Observations of the in vitro ability of anti-Gal mAb to induce apoptosis in PMVEC prompted us to further examine for the presence of apoptotic cells in the xenografts following infusion of anti-Gal mAb (Fig. 7). In the grafts that underwent HAR 7–15 min after GT21-1-G1,1 infusion, the number of apoptotic cells was low, averaging 14 ± 3 for each heart section. The apoptotic cells in this and all of our analyses were located within the capillaries, consistent with endothelial cells. Hearts undergoing AVR examined 15 min after GT21-2-G1 mAb infusion revealed comparably low numbers of apoptotic cells, which were not significantly different from those in control hearts or HAR-rejected grafts (Fig. 7). Hearts undergoing VAR examined 10–15 min after the infusion of GT21-2-G2 had 31 ± 6 apoptotic cells/section, which was higher, but not statistically significant, compared with control hearts and HAR- or AVR-rejected grafts at an equivalent time (p > 0.05 compared with HAR and AVR groups, by ANOVA). At 120 min after infusion of GT21-2-G2, the number of apoptotic cells (46 ± 4) in VAR-rejected hearts increased. These observations are consistent with our in vitro data showing that apoptosis is a relatively slow process compared with complement-mediated lysis and suggest that at later time points apoptosis may contribute to the process of Ab-mediated xenograft rejection.

Discussion

In this study we have characterized a set of anti-Gal mAbs and, through a series of in vitro and in vivo tests, sought to determine what significance these mAb might have in xenograft rejection. A modest increase (85-fold) in anti-Gal affinities was observed in mAb isolated after an induced immune response. These observations confirm and extend reports by Cramer and colleagues (19, 28, 42–44) that a closely related group of Ig V genes is used to encode the Gal-Ab binding specificity, that these genes undergo an isotype switch to IgG, and that somatic mutation consistent with Ag-driven affinity maturation can be observed. Most of our anti-Gal mAb, however, appeared to have lower affinity for α-Gal than was typical of human and primate sera, since our anti-Gal mAb did not effectively bind to Ags that were easily detected by pre-existing anti-Gal IgM and IgG present in human serum. Apparent avidity measurements ranging from 10⁻⁸ to 10⁻¹⁰ M for human XNA or affinity-purified anti-Gal Ab from pre- and postimmune human serum have been reported for Ab binding to porcine aortic endothelium (45–47). Although the results from these studies may have been effected by the target Ag, the presence of a minority of higher affinity Abs, Abs with non-α-Gal specificities, or the affinity purification procedures, in general it appears that the mAb we studied exhibited lower relative avidity compared with human serum. In contrast, Wang and colleagues (48) isolated human anti-Gal mAb from EBV-transformed cells and reported an apparent affinity range of 10⁻⁷–10⁻⁸ M, similar to the range we detect. Together these results suggest that human serum contains a wide range of anti-Gal Ab concentrations and affinities and that a small proportion of high affinity Abs, possibly elicited from some previous exposure event, may dominate most α-Gal assays.

Exposure of human and primates to pig tissue leads to an elicited anti-Gal immune response dominated by the production of anti-Gal IgG. The elicited Ab includes both complement-fixing and noncomplement-fixing isotypes that appear to have increased affinity for α-Gal (46). Unlike mAb to defined peptide epitopes, anti-Gal mAb bind to a carbohydrate structure present on many glycoproteins expressed across the endothelial cell surface. This means that anti-Gal Abs, including mAbs, have the potential to cause the aggregation of many disparate cell proteins. Several groups have suggested that chronic exposure to this elicited IgG, with or without complement activity, may underlie the AVR process. We have investigated this question by determining the ability of our mAb to induce endothelial cell activation in vitro in the absence of serum complement and xenograft rejection in vivo. Our in vitro analysis indicated that all the anti-Gal Abs we studied were capable of inducing endothelial cell activation in the absence of serum complement, but only under conditions that favored cross-linking of the bound Ab. Thus, low Ab concentrations over short periods of time required specific cross-linking agents to induce CD62E expression, whereas prolonged exposure to high concentrations of Ab, which favors cross-linking of surface proteins, resulted in endothelial cell activation and apoptosis without a specific cross-linker. Palmevoofer et al. (49, 50) investigated the ability of GS1B4 lectin and sensitized serum (containing anti-Gal Abs) to induce a protein synthesis-independent (type I) and protein synthesis-dependent (type II) endothelial cell activation. Their studies suggested that the increased concentration and affinity of elicited anti-Gal Abs more efficiently cross-linked glycoproteins on the endothelial cell surface, producing endothelial activation that could then lead to AVR. Our in vitro results, which examined only type II activation, corroborate that the amount of anti-Gal Ab bound to the cell and the level of cross-linking are critical for endothelial cell activation; however, anti-Gal affinity, at least within the range we have examined, appears less important.

We also examined the role that anti-Gal Abs may play in xenograft rejection by infusing some of the mAb into Rag⁻/- Gal⁻/- mice that had previously received a rat heterotopic cardiac xenograft. These experiments were attempts to recreate HAR and AVR, to examine both the significance of anti-Gal isotype and affinity on xenograft rejection, and to test the suitability of this small animal
model. We expected that infusion of complement-fixing anti-Gal IgG3 would induce HAR, while infusion of an IgG1, which fixes complement poorly, would induce AVR. We observed, however, that with a constant amount of Ab, HAR was induced by only one of the two anti-Gal IgG3 mAbs tested. At this time it is difficult to determine whether the small difference in affinity or other factors affect the in vivo effects of anti-Gal IgG3. Differences in fine scale specificity of anti-Gal Abs and variations in the accessibility of α-Gal epitopes could affect the amount of Ab bound to the cell surface (37–39). Another factor that affects our interpretation is the density of α-Gal epitopes. The rat and mouse tissues used in our in vivo experiments express much lower levels of α-Gal Ag than commonly detected in the pig cells used in our in vitro experiments. Thus, the amount of anti-Gal mAb bound to rat xenografts in our in vivo experiments cannot be simply predicted based on Ab affinity or binding to porcine cells. With these caveats in mind, it nonetheless appears that mAbs GT21-1-G1.1 is likely to achieve a higher level of Ab binding to the cell surface compared with GT21-2-G1, and this difference may be sufficient to account for the different forms of rejection. If this is the case, HAR would require a threshold of Ab bound to the graft to elicit the classical complement-dependent pathological features. Lower levels of Ab bound to the graft may require more time to induce organ rejection. When the rejection process is slower, there may be a greater potential for endothelial cell activation, membrane remodeling, and physiological processes, such as thrombosis, which might affect the histology at rejection, producing an apparent reduction in complement deposition and a more AVR-like pathology.

Infusion of the anti-Gal IgG1 Ab elicits rapid organ rejection that can occur with minimal activation of the terminal components of complement. This was a surprising observation and suggests that rapid deposition of anti-Gal IgG1 in the xenograft may result in VAR. Unlike HAR, where strong deposition of C3 and C5 was observed, minimal deposits of C3 and no C5 were evident in VAR, consistent with the poor complement-fixing ability of IgG1 in the mouse. Increased numbers of apoptotic cells (by TUNEL staining) and up-regulated von Willebrand factor and P-selectin expression during VAR (data not shown) are consistent with rapid activation and injury to the graft endothelium during VAR. Experiments to further dissect the mechanism of VAR and the significance of complement activation, Ab affinity, and apoptosis are ongoing. Regardless of the mechanism, our observations suggest that rapid organ failure in pig to non-human primate xenotransplantation may occur in the absence of widespread complement activation. This could account for unexplained instances of apparent HAR if high levels of pre-existing noncomplement-fixing anti-Gal Abs were present. When the level of induced high affinity anti-Gal Ab increases more slowly compared with our infusion experiments, as is commonly the case in pig to non-human primate xenotransplantation, AVR may occur.

In summary, we have characterized the biological activities of a series of anti-Gal mAb isolated from Gal(−/−) mice. The in vitro behavior of these mAbs suggests that all anti-Gal Abs, independently of affinity or isotype, are capable of inducing endothelial cell activation and apoptosis, given a sufficient capacity to cross-link endothelial cell surface proteins. In contrast, the in vivo activity of these mAbs was not easily predicted from their in vitro behavior. Our in vivo data suggest that rapid xenograft rejection can be induced not only by complement-fixing anti-Gal Ab, but also by high affinity anti-Gal Ab with poor complement fixation. This rapid rejection process may be independent of terminal complement fixation and might explain the unexpected HAR of transgenic organs in the pig to primate models. Further study of this unusual rejection and more detailed analysis of the influence of Ab concentration, isotype, and affinity will be possible using the rat heart to Gal(−/−)−/−Rag(−/−) model. Finally, these studies emphasize the importance of in vivo assays, in addition to in vitro assays, in understanding the pathogenicity of anti-Gal IgG in xenograft rejection.

Acknowledgments

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References


