Immunology 2000 **101** 467–473

Distribution of, and immune response to, chicken anti-αGal immunoglobulin Y antibodies in wild-type and αGal knockout mice

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SUMMARY

Chicken antibodies (immunoglobulin Y; IgY) to the αGal epitope (galactose α-1,3-galactose) bind to αGal antigens of mouse and porcine tissues and endothelial cells *in vitro* and block human anti-αGal antibody binding, complement activation and antibody-dependent cell-mediated lysis mechanisms. The activities and toxicity of anti-αGal IgY have not been tested *in vivo*. In this study, we tested the effects of multiple injections of affinity-purified anti-αGal IgY (AP-IgY) in both wild-type (WT) and α-1,3-galactosyltransferase knockout (Gal KO) mice. WT and Gal KO mice were injected once, twice, three, or four times intravenously (i.v.) with AP-IgY and killed at 1 hr or 24 hr. Mice displayed no toxicity to four injections of AP-IgY. Heart, lung, liver, kidney, spleen and pancreatic tissue were evaluated using immunohistochemical techniques for the presence of the αGal epitope using the GSI-B4 lectin, and for bound IgY, as well as mouse IgM and IgG. The binding of AP-IgY antibodies to the endothelium of WT mouse tissues was essentially identical to the pattern of binding of the GSI-B4 lectin after injection of WT mice and death at 1 hr. WT mice killed 24 hr after i.v. injection of AP-IgY showed little remaining bound IgY in their endothelia, indicating that IgY is cleared over that time period. We also evaluated the blood drawn at the time of death for the presence of anti-αGal IgY, anti-IgY IgM and anti-IgY IgG by enzyme-linked immunosorbent assay. Anti-αGal IgY was almost undetectable in WT mouse sera at all injection and killing times. In contrast, Gal KO mouse sera showed increasing anti-αGal IgY levels until 24 hr after the fourth injection, when anti-αGal IgY levels were almost undetectable. Anti-IgY IgM and IgG levels in WT and Gal KO mouse sera showed a typical increase in anti-IgY IgM 24 hr after the second injection (3 days after the first injection) and an increase in anti-IgY IgG 24 hr after the third injection (5 days after the first injection). These results show that IgY binds to αGal epitopes in the WT mice and is cleared sometime over a 24-hr time period and that IgY is an expected immunogen in mice eliciting a rather typical anti-IgY IgM and IgG response.

INTRODUCTION

Due to the inadequate supply of human organs, xenografts are being considered as a potential source of organs.[1,2] The pig is considered to be the optimal animal for organ donation to human recipients. However, exposure of unmodified pig organs to human blood results in hyperacute rejection (HAR). HAR is initiated by human preformed xenoreactive natural antibodies (XNAs) against galactose α-1,3-galactose (αGal) epitopes found on the cell surfaces of all mammals except man, apes and Old World monkeys.[3,4] Although temporary depletion of these antibodies by plasmapheresis or adsorbent columns appears promising in the prevention of HAR, there are currently no effective strategies to prevent antibody deposition for longer periods. αGal XNAs are thought to play an important role in acute vascular xenograft rejection (AVXR), which is the major immunological obstacle beyond HAR. Furthermore, the long-term effects of chronic exposure to αGal antibodies are unknown.[5,6] Therefore, strategies to remove and/or block αGal antigens will be of critical importance to the success of clinical xenotransplantation.

Received 9 June 2000; revised 7 August 2000; accepted 21 August 2000.

Abbreviations: αGal, galactose α-1,3-galactose; αGal-BSA, Galα1,3Galβ1,4GlcNAc-bovine serum albumin; AP-IgY, affinity-purified anti-αGal immunoglobulin Y; AVXR, acute vascular xenograft rejection; BSA, bovine serum albumin; FT-IgY, IgY flow-through; Gal KO, galactosyltransferase knockout mouse; HAR, hyperacute rejection; IgY, immunoglobulin Y (chicken antibodies); PBS, phosphate-buffered saline; TBS, Tris-buffered saline; WT, wild-type; XNA, xenoreactive natural antibodies.

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Like humans, birds and some reptiles do not express the αGal epitope and they have XNAs to αGal.[7] Avian antibodies isolated from chicken egg yolk [and thus termed immunoglobulin Y (IgY)] have potential for therapeutic uses in humans because IgY does not activate mammalian complement nor bind to mammalian Fc receptors.[8,9] Chickens have been immunized with αGal antigens to boost titres of anti-αGal IgY antibodies and methods to isolate anti-αGal IgY have been described.[10,11] In studies from this laboratory, we have shown that IgY can block human antibody binding to porcine endothelial cells, with subsequent blocking of human blood complement- and antibody-dependent cell-mediated lysis of the target cells.[12] Preliminary experiments in our laboratory have shown that IgY binds to mouse endothelial cells within 30 min of intravenous (i.v.) injection. The potential toxic effects of administering IgY have not been previously evaluated *in vivo* nor have the kinetics of the antibody response to IgY.

In this paper we examined the distribution of IgY in both the α-1,3-galactosyltransferase knockout mouse (Gal KO) and the wild-type (WT) mouse after sequential injections of IgY. Gal KO mice are the only small animals that lack the αGal epitope on cell surfaces and which have very small amounts of anti-αGal antibodies in their blood.[13–17] The mouse IgM and IgG antibody responses to administered IgY were also characterized.

MATERIALS AND METHODS

Reagents

All reagents and chemicals were purchased from Sigma Chemical Company, St Louis, MO, unless otherwise noted. Phosphate-buffered saline (PBS) was made from premeasured tablets and contained 10 mM sodium phosphate, 2·7 mM KCl, 137 mM NaCl, pH 7·4. PBSat is PBS containing 0·08% sodium azide and 0·08% Tween-20. PBSA is PBS containing 1% bovine serum albumin (BSA). Tris-buffered saline (TBS) was made up of 100 mM Trizma base, 150 mM NaCl, 0·1 mM $MgCl_2$ and 0·08% sodium azide and was titrated to pH 7·4. High pH TBS was adjusted to pH 9·5.

Preparation of anti-αGal IgY

Anti-αGal IgY was prepared by immunizing chickens with αGal epitope conjugated to ovalbumin or keyhole limpet haemocyanin, isolating the IgY fraction from egg yolks, and affinity purifying the anti-αGal fraction using Synsorb-90 beads as previously described.[12] The portion of IgY which did not bind to the αGal epitope on the Synsorb-90 matrix was termed the IgY flow-through (FT-IgY). The portion of IgY eluted from the column using high pH conditions was termed affinity-purified anti-αGal IgY (AP-IgY).

Injections of mice

These experiments were performed in accordance with the guidelines set by the Animal Care and Use Committee of North-western University. WT B6D2F1 mice were obtained from Jackson Laboratories (Bar Harbor, ME). Gal KO mice of the genetic background C57BL/6 × DBA/2J × 129 sv (H-2K^b and H-2K^d)[18] were kindly provided by Dr J. Lowe at the University of Michigan. Progeny were tested to confirm homozygous knockout of the αGal epitope. Both WT and Gal KO mice were used as recipients of AP-IgY and FT-IgY.

WT and Gal KO mice were injected with 200 μl of 1 mg/ml of either AP-IgY or FT-IgY every 48 hr (time 0 = first injection). With the mice anaesthetized, injections were made in the dorsal vein of the penis using a 30½-gauge needle. Mice were killed following one, two, three, or four injections. Mice were killed 24 hr after the last injection (for example, a mouse with three injections received injections at 0, 48 and 96 hr, and was killed at 120 hr), except for the eight (four WT and four Gal KO) mice which received four injections and were killed 1 hr after injection. Three WT and three Gal KO mice were injected with AP-IgY and one WT and one Gal KO mouse with FT-IgY for each group based on the number of injections.

Mice were killed, under anaesthesia, by drawing blood from the abdominal vena cava using a 25-gauge needle, which yielded between 0·7 and 1·0 ml of blood per mouse. The blood was placed in an Eppendorf microcentrifuge tube, and after clotting, was centrifuged at 2000 *g* for 20 min. The serum fraction was removed and stored at −70°.

Organs were harvested immediately from exsanguinated mice. Heart, lung, liver, kidney, spleen and pancreatic tissues were placed in a cartridge filled with optimal cutting temperature (OCT) compound (VWR Scientific, S. Plainfield, NJ), the cartridge was placed in a beaker filled with 2-methylbutane and the beaker was dipped into liquid nitrogen until the OCT froze completely. The frozen blocks were then stored at −70°.

Immunohistochemical techniques

The immunohistochemical procedures were similar to those previously described.[12] Briefly, heart, lung, liver, kidney, spleen and pancreas tissues were sectioned at a thickness of 8 μm, placed on slides and incubated with biotinylated-GSI-B4 lectin followed by fluorescinated–avidin reagent to detect αGal epitopes, and incubated with fluorescinated anti-IgY, anti-mouse IgG or anti-mouse IgM reagents to detect the respective immunoglobulins. Slides were counterstained with 0·01% methylgreen, coverslips were placed on them and the fluorescent intensities, graded from 0 to 4+, were investigated using a Zeis Axioskop with wide-band fluorescein isothiocyanate (FITC) filter.

Enzyme-linked immunosorbent assay for the detection of anti-αGal IgY, and mouse anti-IgY IgG and IgM antibodies in mouse serum

Enzyme-linked immunosorbent assay (ELISA) was performed to determine the IgY anti-αGal levels in mouse sera. Galα1,3Galβ1,4GlcNAc-BSA (αGal-BSA) (V Labs, Covington, LA), 1 μg/ml in 0·1 M sodium bicarbonate, pH 9·0, was used to coat the wells of Titertek 96-well ELISA plates (Titertek, ICN Biomedicals, Costa Mesa, CA) at room temperature for 1 hr (or overnight at 4°) and washed with PBSat, then back-coated with 300 μl of PBSA per well at room temperature for 1 hr (or overnight at 4°). The plate was washed with PBS, and 100 μl per well of mouse serum diluted 1 : 20 in PBSA was incubated for 1 hr at room temperature. Alkaline phosphatase-labelled rabbit anti-chicken secondary antibody diluted 1 : 3000 in PBSA, 100 μl, was added to each well, and the plate was incubated for 1 hr at room temperature and the wells were washed with TBS, pH 7·4. Alkaline phosphatase substrate (*p*-nitrophenylphosphate), 100 μl of 1 mg/ml, in high pH TBS, was added per well. The optical densities (ODs) at

405 nm were obtained at 10-min intervals up to 30 min incubation using a V_{max} Kinetic Microplate Reader (Molecular Devices Corp., Sunnyvale, CA). OD values were considered valid if product formation (OD at 405 nm) was linear with time. All OD values were plotted as 30 min times of product formation. In a few instances activities were high and the 10 min OD values were used and thus multiplied by three to normalize and for plotting purposes (Fig. 3).

ELISAs were also performed to determine mouse anti-IgY IgG and IgM levels in serum samples. FT-IgY (devoid of anti-αGal IgY) was used to coat the microtitre plate wells, the wells back-coated with PBSA and mouse serum samples (diluted 1 : 100 in PBSA) were added and incubated for 1 hr. Bound mouse IgG and IgM antibodies were detected using the appropriate alkaline phosphatase-labelled anti-mouse IgG or IgM (heavy-chain-specific) reagents, ODs were obtained and data were analysed as described above.

RESULTS

αGal epitope expression in WT and Gal KO mice

The αGal epitope expression in WT and Gal KO tissues was examined using biotinylated GSI-B4 lectin followed by fluorescinated-avidin applied to cryostat-cut tissue sections as described in the Materials and methods. The most intense fluorescence (4+) was observed on the endothelium of the WT mice heart and lung. The vessels of the kidney glomeruli were also strongly positive (3–4+), as were the tubules. The liver vessel endothelia were weakly positive (1–2+) as well as the lining cells of the sinusoids. The endothelial layer of vessels in the pancreas was moderately positive (2+) as were the lining cells of the exocrine ducts. The αGal epitope distribution was essentially identical to that reported by Tearle *et al.*[19] In contrast, there was no detectable αGal epitope in the Gal KO mouse tissues, confirming that the α-1,3-galactosyltransferase was not expressed in those mice.

IgY distribution in Gal KO and WT tissues

AP-IgY was injected into WT mice, which were then killed after 30 min, and the tissue distribution of IgY was determined using fluorescinated rabbit anti-IgY reagent. IgY was detected in heart, lung, kidney and liver tissues with the same location and relative intensities as observed for the GSI-B4 lectin binding.

AP-IgY was then injected into mice one to four times at 2-day intervals, tissues were harvested 24 hr after each injection (or 1 hr after the fourth injection) and the IgY was measured. For the WT mice, IgY was not detectable in any tissues after one, two, three, or four injections harvested 24 hr after the last injection, except slight positivity (1+) in the spleen associated with cellular elements. After four injections with AP-IgY and death 1 hr after the final injection, IgY could be detected in small amounts (1+) in the vessel endothelia of the heart, lung and liver (Table 1). Stronger staining (2+) was seen in the glomerulus of the kidney. Strong staining was also seen in the spleen (3+). WT mice given injections of FT-IgY (IgY lacking anti-αGal antibodies) and killed at 24 hr or 1 hr showed only minor staining (1+) in the spleen and 1+ staining of the kidney glomeruli of the mice injected four times and killed at 24 hr. Gal KO mice injected four times with AP- or FT-IgY showed only small amounts of IgY staining in the spleen or kidney (glomerulus) of animals killed 1 or 24 hr after the fourth injection. Gal KO mice injected two or three times and killed 24 hr later, exhibited only minor amounts of IgY staining (1+) associated with cellular elements of the spleen tissues.

IgY levels in WT and Gal KO mouse sera after injections of AP-IgY or FT-IgY

Mice were injected once, twice, three, or four times with AP-IgY and killed 24 hr after the last injection, or 1 or 24 hr after the fourth injection. Additional mice were injected with FT-IgY at the same injection intervals and killing schedules. At the time of death, sera were obtained and the levels of IgY were determined using the ELISA technique described in the Materials and methods. It was first determined whether the mouse sera would interfere with the quantitative assay of IgY. AP-IgY was added at different concentrations to either WT or Gal KO mouse sera and IgY anti-αGal reactivity was determined, showing there was no effect of either WT or Gal KO sera on the binding of IgY anti-αGal to the αGal epitope.

IgY anti-αGal reactivity was almost undetectable in WT mouse sera injected with AP-IgY at all injection and killing times (Fig. 1). WT mice injected with FT-IgY, as expected, showed no IgY reactivity with the αGal epitope in the ELISA. The same results were obtained for Gal KO mice injected with FT-IgY. However, IgY anti-αGal reactivity was present (OD 0·263) in sera from Gal KO mice injected with AP-IgY once and killed 24 hr after the injection (Fig. 1). Sera from Gal KO

Table 1. IgY distribution in various tissues in WT and Gal KO mice after four injections of AP-IgY or FT-IgY

	WT* AP-IgY†	WT AP-IgY	WT FT-IgY	WT FT-IgY	Gal KO AP-IgY	Gal KO AP-IgY	Gal KO FT-IgY	Gal KO FT-IgY
Number of injections	4	4	4	4	4	4	4	4
Time of harvest	1 hr	24 hr	1 hr	24 hr	1 hr	24 hr	1 hr	24 hr
Heart	+	0	0	0	0	0	0	0
Lung	+	0	0	0	0	0	0	0
Kidney	++gl	0	0	+gl	0	+gl	+gl	+gl
Liver	+	0	0	0	+	0	0	0
Spleen	+++	+	+	+	+	++	++	+
Pancreas	0	0	0	0	0	0	0	0

*WT and Gal KO, strains of mouse used; †AP-IgY or FT-IgY are substance injected. Organs were harvested 1 hr or 24 hr after the fourth injection. 0, no reactivity; +, weak reactivity; ++, moderate reactivity; +++, reactivity; ++++, strong reactivity; gl, glomerulus.

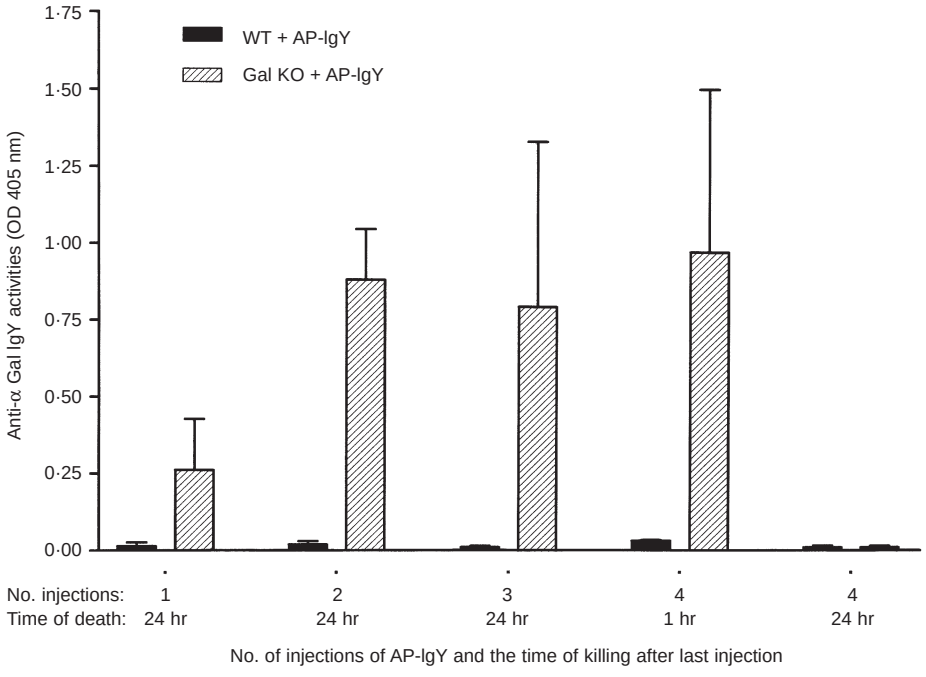

Figure 1. Anti-αGal IgY activities in WT and Gal KO mouse sera after i.v. injection with AP-IgY. WT or Gal KO mice were injected with AP-IgY or FT-IgY once, twice, three, or four times with AP-IgY and killed 1 hr or 24 hr after the last injection. Anti-αGal IgY levels were determined using ELISA and quantified in terms of the OD values. Anti-αGal IgY levels in Gal KO mouse sera were detectable after the first injection (blood drawn at 24 hr) and were higher 24 hr after the second and third injections, and 1 hr after the fourth injection, but not detectable 24 hr after the fourth. Anti-αGal levels in WT mice were almost undetectable. Results are the average OD values for three mice in each experimental group ± SD.

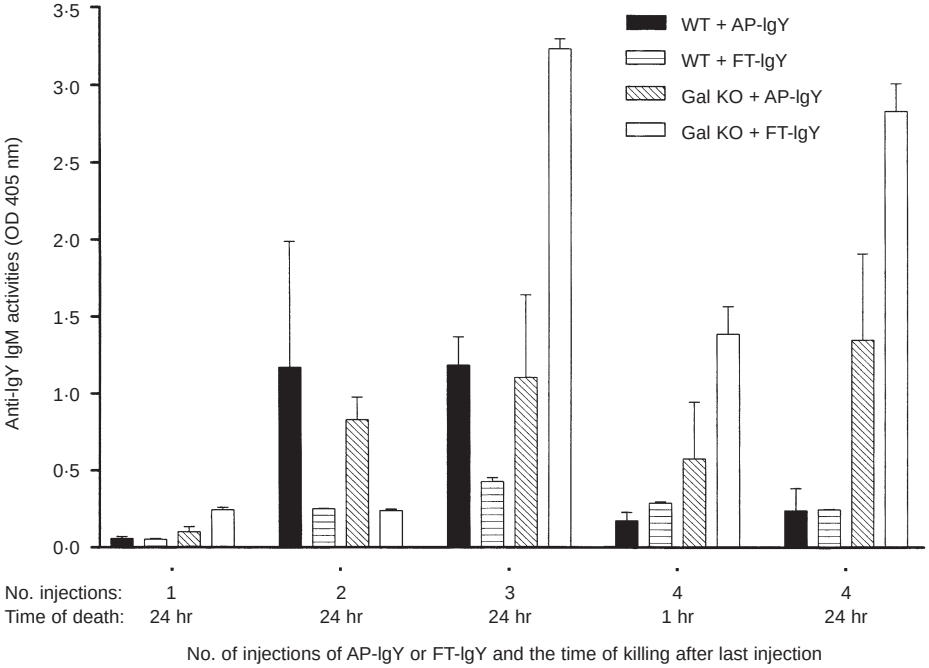

Figure 2. Anti-IgY IgM activities in WT and Gal KO mouse sera after i.v. injection with AP-IgY or FT-IgY. WT or Gal KO mice were injected with AP-IgY or FT-IgY once, twice, three, or four times and killed 1 hr or 24 hr after the last injection. Anti-IgY IgM levels were determined using ELISA and quantified by OD values. Anti-IgY IgM levels in WT mouse sera were detectable 24 hr after the first injection, rose greatly after the second and third injections and declined both 1 hr and 24 hr after the fourth injection. Anti-IgY IgM levels in Gal KO mouse sera rose similarly after the first, second and third injections, and also declined 1 hr after the fourth injection. In contrast to the WT mice, anti-IgY IgM levels in sera from Gal KO mice rose 24 hr after the fourth injection of AP-IgY. The anti-IgY IgM serum levels were lower for mice injected twice FT-IgY compared to AP-IgY injected mice, and the levels differed either higher (Gal KO mice) or lower (WT mice) after the third and fourth injections of FT-IgY. Results are the average OD values for three mice in each experimental group ± SD.

mice injected twice or three times with AP-IgY and killed at 24 hr contained greater IgY anti-αGal reactivities (OD 0·884 and 0·800, respectively). Similar high levels of IgY anti-αGal were found in Gal KO sera of mice injected four times and killed at 1 hr (OD 0·980). However, for Gal KO mice injected four times whose sera were drawn 24 hr later showed little detectable IgY anti-αGal activity.

Anti-IgY IgM levels in WT and Gal KO mouse sera after injection of AP- or FT-IgY

In WT mice injected with AP-IgY, the serum levels of anti-IgY IgM increased from small amounts (average OD 0·059) after one injection, to a large amount of anti-IgY IgM (ODs 1·172 and 1·185) after two and three injections (killed at 24 hr) (Fig. 2). The levels of anti-IgY IgM declined after four injections at both 1 hr and 24 hr after injection (OD 0·175 and 0·239, respectively). The sera from WT mice injected with FT-IgY contained less anti-IgY IgM compared to mice injected with AP-IgY. Anti-IgY IgM levels were low after one injection (OD 0·054), the levels rose after two injections (OD 0·251), peaked after three injections (OD 0·428) and declined after four injections [killed at 1 hr (OD 0·289) or 24 hr (OD 0·247)].

Anti-IgY IgM levels in sera from Gal KO mice yielded different results from the WT mice. The anti-IgY IgM levels were comparably low for sera from Gal KO mice injected with AP-IgY (OD 0·103) or FT-IgY (OD 0·244) after one injection (killed at 24 hr) (Fig. 2). The IgM levels were greater in sera from Gal KO mice injected twice with AP-IgY (OD 0·830) than sera from Gal KO mice injected with FT-IgY (OD 0·240). After three injections and harvesting at 24 hr, the anti-IgY IgM levels in sera from Gal KO mice injected with FT-IgY (OD 3·237) greatly increased in comparison to the increase observed in sera from Gal KO mice injected with AP-IgY (OD 1·106). Levels from Gal KO mice injected with AP- or FT-IgY decreased after four injections in those killed after 1 hr (OD 0·578 and 1·389, respectively) and were increased after four injections but with killing after 24 hr (OD 1·351 and 2·837, respectively). In the Gal KO mice injected four times, anti-IgY IgM levels remained higher in sera from mice injected with FT-IgY compared to mice injected with AP-IgY.

Anti-IgY IgG levels in WT and Gal KO mouse sera after injection of AP- or FT-IgY

Anti-IgY IgG levels in WT mice injected with AP-IgY were almost undetectable after the first (OD 0·019) and second injections (OD 0·037) (Fig. 3). The levels rose greatly after the third injection of AP-IgY (OD 5·970). After the fourth injection, anti-IgY IgG were low (OD 0·197) when the mice were killed 1 hr after the fourth injection, but the levels were

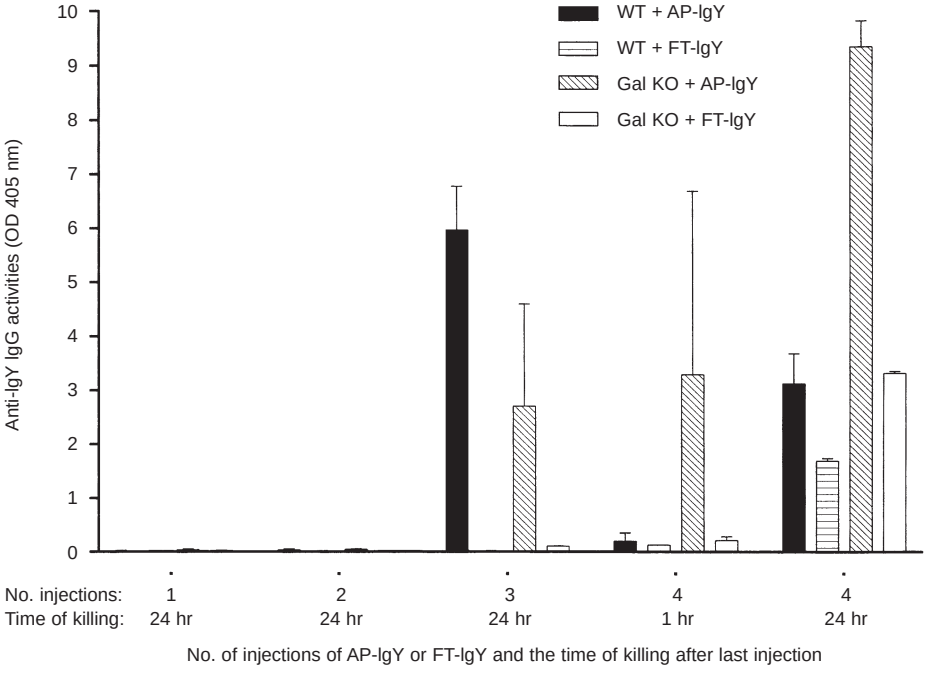

Figure 3. Anti-IgY IgG activities in WT and Gal KO mouse sera after i.v. injection with AP-IgY. WT or Gal KO mice were injected with AP-IgY once, twice three, or four times with AP-IgY and killed 1 hr or 24 hr after the last injection. Anti-IgY IgG levels were determined using ELISA and quantified by OD values. Anti-IgY IgG levels were undetectable in both WT and Gal KO mouse sera after the first and second injections. In WT mouse sera, the anti-IgY IgG levels rose greatly after the third injection, declined 1 hr after the fourth injection and rose again 24 hr after the fourth injection. Similarly, anti-IgY IgG levels in Gal KO mice were first detectable after the third injection. In contrast to the WT mice, the anti-IgY IgG levels continued to rise 1 hr after the fourth injection and peaked 24 hr after the fourth injection. In contrast to mice injected with AP-IgY, FT-IgY injected mice did not exhibit detectable levels of anti-IgY IgG until after the fourth injection. Results are the average OD values for three mice in each experimental group ± SD. The OD values for this figure were from 10-min times of product formation in ELISA. The OD values were therefore multiplied by three to normalize the data in comparison to Figs 1 and 2.

much higher (OD 3·127) in the mice killed 24 hr after the fourth injection. The WT mice injected with FT-IgY had very low levels after the first (OD 0·020), second (OD 0·015) and third (OD 0·020) injections (killed after 24 hr). Levels of the anti-IgY IgG were slightly higher after the fourth injection in mice killed after 1 hr (OD 0·127) and higher still in those killed 24 hr after the fourth injection (OD 1·690).

Anti-IgY IgG activities of Gal KO mice were barely detectable 24 hr after the first and second injections of either AP-IgY or FT-IgY (Fig. 3). The anti-IgY IgG levels in Gal KO mice injected with AP-IgY were detectable and about the same after the third injection of AP-IgY (OD 2·706) and 1 hr after the fourth injection (OD 3·291). Twenty-four hours after the fourth injection with AP-IgY, the anti-IgY IgG activity increased about 2·5-fold compared to after the third injection (OD 9·370). Gal KO mice injected with FT-IgY showed only small apparent activities of IgG anti-IgY levels after three injections and four injections of sera measured 1 hr after the fourth injection (OD 0·215). The anti-IgY IgG was much higher after the fourth injection in those sera collected 24 hr after the last injection (OD 3·323).

DISCUSSION

The αGal carbohydrate determinant of porcine and other mammalian organs is considered to be the major epitope responsible for rejection of xenotransplants to primates lacking that epitope.[20] The primate XNAs bind to the αGal epitope of transplanted organ endothelium eliciting HAR and AVXR mechanisms. Many attempts have been made to inhibit or block XNA binding to the αGal epitopes.[6] One possible way is to block the epitope with anti-αGal IgY, made in the chicken, because IgY does not activate mammalian complement or bind to mammalian Fc receptors. Previously we have shown that IgY anti-αGal pretreatment of porcine endothelial cells blocks human XNA binding, complement activation and antibody-dependent cell-mediated cellular cytotoxicity (ADCC)-type effector functions.[12]

One limitation of using IgY as a possible therapeutic agent is that mammals will make antibodies against the foreign protein IgY. Furthermore, we need to know how long IgY will remain in the mammalian system. In order to determine these parameters, we conducted the study described here using the mouse as a model system and where the WT and the Gal KO mouse could be compared. The WT mouse organs express αGal and are similar in that respect to a porcine xenograft tissue and the Gal KO mouse is similar to a primate lacking αGal epitopes as a recipient of xenografts.

IgY could be injected into mice on multiple occasions with little toxicity. Both WT and αGal KO mice survived four i.v. injections of 200 μg of AP-IgY or FT at intervals of 2 days. A fifth i.v. injection resulted in death, presumably due to the anti-IgY levels in the mouse and the formation of immune complexes with activation of complement. Both types of mouse survived 10 intraperitoneal injections of 200 μg AP-IgY at 2-day intervals. Presumably, the IgY flows from the peritoneum into the vascular compartment slowly so as to not elicit a life-threatening shock to the animal.

When WT mice were injected i.v. with a single dose of 200 μg of AP-IgY and killed 30–60 min later, the IgY was localized to the endothelial cell lining of vessels in all organs examined and was comparable to the distribution of αGal epitopes detected with the GSI-B4 lectin. The injected IgY is apparently cleared from the tissues and circulation rather rapidly; by 24 hr after injection of AP-IgY into the mouse, very little AP-IgY was detected on the mouse tissues. Part of the AP-IgY are possibly metabolized in the liver and spleen after forming antigen–antibody complexes with αGal epitopes on mouse blood glycoproteins. Also a portion of the AP-IgY may bind to αGal epitopes of the mouse peripheral blood leucocytes which have been shown to be αGal positive.[19]

When AP-IgY was injected into WT mice, IgY anti-αGal was barely detectable in the mouse sera either 24 hr after each injection, or 1 hr after the fourth injection. In the αGal KO mice, the IgY anti-αGal was readily detectable at 24 hr after the first, second and third injections, and 1 hr after the fourth injection. We estimated from the OD values (Fig. 1) and the IgY anti-αGal curves measured in the presence of mouse serum that the 1 : 20 diluted mouse sera contained 4 μg/ml of IgY anti-αGal. This translates into 80 μg/ml of whole mouse serum, and assuming a 2-ml blood volume for the mouse, 160 μg of IgY anti-αGal. Thus, most of the 200 μg of AP-IgY injected into the mice remained in the Gal KO mouse serum 24 hr after the second and third injections, and 1 hr after the fourth injection even though the anti-IgY IgM serum levels were high after two injections and anti-IgY IgG serum levels were detectable after three injections. We do not know the weighted amounts of the anti-IgY IgM and IgG levels and so we presume that the AP-IgY was in considerable excess over the anti-IgY levels such that the IgY could be assayed for anti-αGal activities. Alternatively, complexes of either mouse IgM or IgG with IgY (which would occur in sera after the second and third IgY injections) do not block the IgY anti-αGal binding to αGal antigen in the ELISA system. Twenty-four hours after the last injection, the anti-IgY IgM and IgG levels were apparently sufficiently high to have cleared the AP-IgY injected which was no longer detectable (Fig. 1).

The anti-IgY responses (Figs 2 and 3) in WT and Gal KO mice showed a typical initial IgM response (high levels 24 hr after the second injection) followed by the IgG response (high levels 24 hr after the third or fourth injections). The anti-IgY IgM responses were higher in the WT and Gal KO mice injected with AP-IgY than in those injected with FT-IgY 24 hr after the second injections; whereas, the Gal KO mice exhibited higher anti-IgY responses than WT mice, particularly 24 hr after the fourth injection. Also, the WT mice injected with FT-IgY had consistently lower anti-IgY IgM levels than those seen in the other groups; whereas, the Gal KO mice injected with FT-IgY showed the highest IgM responses 24 hr after the third and fourth injections.

We speculate that the stronger IgM response in WT mice injected with AP-IgY compared to WT mice injected with FT-IgY could be due to the formation and processing of the IgY–αGal antigen complexes that would form with αGal on cell surfaces and on plasma glycoproteins. However, the AP-IgY also elicited a comparable IgM response in the Gal KO mice 24 hr after the second, third and fourth injections, which could suggest that AP-IgY is a stronger immunogen preparation than the FT-IgY. This observation could be due to the conditions of preparation of AP- versus FT-IgY, or perhaps to the lesser heterogeneity of AP-IgY (IgY of anti-αGal-binding activity). The stronger immunogenicity of the AP-IgY is

supported by the findings (Fig. 3) that anti-IgY IgG was high in AP-IgY, three time-injected WT and Gal KO mice; whereas, only small IgG responses were detected in mice injected three times with FT-IgY.

The greater IgM response to the FT-IgY in the Gal KO mice after three and four injections could be due to IgY reactivities and binding of IgY to determinants expressed in greater amounts in the Gal KO mice resulting from lack of the αGal determinant. Such determinants could be directly related to lack of the αGal residues, for example, the βGal-1,4GlcNAc precursor sequence, the acceptor sequence for α1,3-galactosyltransferase enzyme. Alternatively, other carbohydrate sequence determinants, for example, sialylated or fucosylated, incorporating the same precursor sequence, could be increased in Gal KO mice.

Although *in vitro* studies have shown that IgY can block human antibody binding to porcine endothelial cells, thereby blocking human blood complement- and antibody-dependent cell-mediated lysis of the target cells,[12] AP-IgY alone probably will not be as successful *in vivo* in preventing HAR and AVXR except perhaps in a short time-frame of a few days. The results presented here show that AP-IgY is cleared from the mouse system within 24 hr after the first to fourth i.v. injections (WT mice injected with AP-IgY and tissues examined 24 hr after each injection; Table 1). AP-IgY was readily detectable on WT mice tissues when the animals were killed shortly after i.v. injection; whereas, after the fourth injection and death 1 hr later the IgY binding to tissues was apparent but much less. This we ascribe to the reactivity of IgM and/or IgG anti-IgY in mouse sera by the fourth injection (6 days after the first injection).

This study shows that for AP-IgY to be an effective *in vivo* therapeutic agent in blocking complement- and antibody-dependent cell-mediated lysis of xenografts containing αGal epitopes, AP-IgY can only be used for a short time, perhaps up to three injections and 4 days at which time the AP-IgY is still active in the serum fraction despite the presence of IgM and IgG anti-IgY. Alternatively, we can explore ways of protecting IgY from the recipient's immune system, for example, by making dextran or polyethylene glycol conjugates of AP-IgY; such conjugates exhibit reduced immunogenicity.[21]

ACKNOWLEDGMENTS

This research was supported in part by an Alpha Omega Alpha Student Research Fellowship, and the Kovler/Falk Fund.

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