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Xenoantibodies in the rat against guinea pig tissues

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Abstract. The role of naturally occurring antibodies in discordant xenograft rejection is poorly defined. This is partly attributable to a lack of information regarding their tissue specificity and titers. Sera from different rat strains were studied for naturally occurring antibodies against guinea pig tissues using immunofluorescence and immunoperoxydase staining techniques. All sera contained IgG and IgM antibodies in low titers against erythrocytes, lymphoid cells, and a variety of tissue structures. Intentional immunizations of adult rats with guinea pig cells resulted in the production of xenoantibody specificities that were not detectable in nonimmunized animals. Immunizations of Munich-Wistar rats with guinea pig skin grafts occasionally resulted in the formation of antibodies that reacted with allogeneic and syngeneic cells of the liver, lung, and lymphoid organs. We conclude that rats have low titers of naturally occurring xenoantibodies against various tissue structures of the guinea pig, but their importance in xenograft rejection remains to be established.

Key words: Discordant xenografts – Naturally occurring antibodies – Guinea pig tissue

Vascularized discordant xenografts are rejected rapidly and vigorously, very much like kidney allografts transplanted into presensitized recipients with high titers of alloantibodies against donor transplantation antigens [3, 16, 23]. In the latter case, the rejection is mediated by complement-fixing antibodies that bind to alloantigens that are present in high density on the graft vascular endothelium [3]. Based on histopathological similarities in the pattern of graft destruction, as well as on the presence of naturally occurring xenoantibodies in many combinations [12], it has been widely assumed that these antibodies mediate discordant xenograft rejection. Attempts to produce prolongation in discordant xenograft survival by antibody depletion have, however, resulted in limited success, and it is uncertain to what extent the success of such procedures is due to depletion of mediators of tissue injury other than antibodies [17, 20].

The guinea pig to rat model is a widely used model for discordant xenograft rejection [1, 14, 19, 26, 28]. It was recently reported that the rat has no naturally occurring antibodies against guinea pig blood cells [19], something which raises doubts as to the importance of naturally occurring antibodies in the rejection process in this particular combination. In this paper, we describe the antibody reactivity pattern of normal rat sera with guinea pig tissues and compare it with the pattern of rat antisera obtained after intentional immunizations with guinea pig tissues.

Materials and methods

Animals

Inbred adult Lewis (LEW), Brown Norway (BN), ACI, Wistar Kyoto (WKY), and (LEW × BN)F_1 rats were obtained from Harlan Sprague Dawley (Indianapolis, Md.); inbred Munich-Wistar (MW) rats were purchased from Simonsen Laboratories (Gilroy, Calif.). MW newborn and infant rats were obtained by mating litter mates of adult MW rats under conventional conditions in our own facilities at the University of Calgary Animal Resources Centre. Randomly bred Sprague Dawley (SD) rats were obtained from Charles River (St. Constant, Quebec, Canada). Randomly bred guinea pigs (GP) were obtained from High Oak Ranch (Goodwood, Ontario, Canada).

Normal rat sera

Normal adult rat sera were obtained by cardiac puncture or sacrifice of the animal under ether anesthesia. Experiments with normal sera were done with pooled sera from four to six individual animals from

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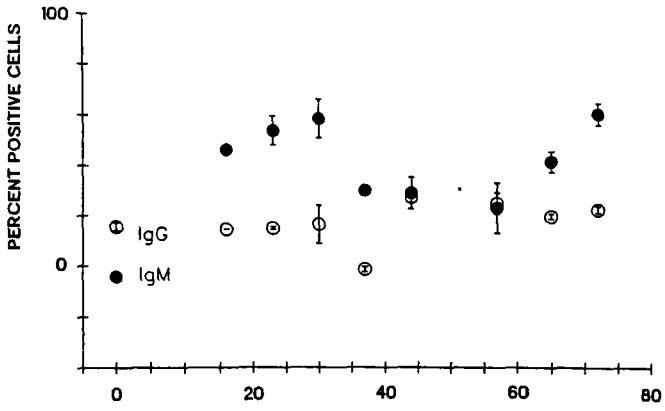

Fig. 1. Percentage of guinea pig lymph node cells stained after incubation with rat sera (1: 2 dilution in phosphate-buffered saline) and antisera against rat IgG (○) or IgM (●); sera were drawn on different days after birth

each strain, unless specified otherwise. Serum was further drawn from four MW animals on the day of birth, as well from seven MW rats that underwent weekly bleedings, starting after the 1st postnatal week for up to 10 weeks.

Rat anti-guinea pig antisera

Eight MW and four LEW rats were immunized with guinea pig tissues using either two consecutive split-thickness ear skin grafts 2 weeks apart, followed 2 weeks later by an injection of 10^7 lymphoid cells divided s.c. and i.p. [5], or three biweekly injections of 10^6–10^7 guinea pig spleen cells i.p. and s.c.

Target tissues

Red blood cells were prepared from heparinized normal blood while lymphocyte suspension were prepared from spleens and lymph nodes, as described elsewhere [5]. Tissues for immunohistochemical studies were harvested after partial exsanguination of the animal, followed by perfusion with heparinized phosphate-buffered saline (PBS) through a cannula in the aorta. A uniform blanching of the organs was taken to indicate effective perfusion. Tissues were snap-frozen in liquid nitrogen and stored at −70°C. Rat liver tissue with reduced numbers of class II-positive bone marrow-derived cells were obtained 9–11 days after treatment of the animals with 1000 rad CS irradiation administered for 3 min (gamma cell 1000; Atomic Energy Canada, Ottawa, Canada), as described by others [13]. Reconstitution was done with 5×10^8 syngeneic nucleated bone marrow cells. Tissues obtained from 11 animals, including 2 that were reconstituted with syngeneic bone marrow, were investigated.

Analysis of the rat sera

Serial dilutions of rat sera in PBS were analyzed for antibody reactivity against guinea pig cell suspension using an indirect immunofluorescence technique. The cells were washed three times in PBS with 0.1% azide; 5×10^5 cells were incubated with 50 µl of rat sera in different dilutions for 30 min at 4°C. After another three washings, the cells were incubated with 50 µl (FITC) fluorescein isothiocyanate conjugated goat anti-rat IgG or IgM for 30 min at 4°C, followed by washing and fixation in 1% phosphate-buffered formalin. Incubation conditions were based on pilot experiments with a selective serum from a female LEW rat; this serum produced plateau staining with guinea pig erythrocyte concentrations below 10^6 cells/50µl and saturating amounts of FITC-labeled antisera. The cells were examined with a modified system 50H cytofluorograph, and data were recorded and analyzed using a 2150 computer system (Ortho Instruments, Westwood, Mass.). Enumeration of positive cells was determined by channel-by-channel subtraction of the positive histogram from that of the control suspensions that had been incubated with either normal guinea pig serum or normal saline and stained as above [31]. Control preparations usually stained less than 2% of the cells; experiments with more than 4% control staining have been excluded from analysis.

The titer of hemagglutinating antibodies was determined using a standard hemagglutination assay with serial dilutions of the test sera and 0.5%–2% solutions of target cells [27].

The antibody reactivity of sera against tissue structures was studied with the use of indirect immunofluorescence, as described elsewhere [22]. Indirect immunoperoxydase staining was done for more precise tissue localization of antibody binding. The sections were fixed in cold acetone for 30 s, incubated with normal goat serum for 30 min, with rat serum in serial dilutions for 90 min, and with peroxydase-conjugated goat anti-rat Ig for 30 min, followed by diaminobenzidine for 4 min. Control sections were incubated with serial dilutions of normal guinea pig serum, followed by staining procedures as described above.

Antisera

The monoclonal antibody ER13 (mouse anti-rat Ia) was obtained from Dr. J. Rozing, REP Institutes TNO, Rijswijk, The Netherlands. FITC-conjugated goat anti-rat IgG, IgM, and total immunoglobulins, as well as goat anti-mouse IgG, were purchased from Organon Technika (Scarborough, Ontario, Canada). Since the FITC-conjugated antisera against rat and mouse IgG cross-reacted to some extent in immunofluorescence assays with mouse and rat IgG, respectively, these antisera were, in some experiments, diluted in normal mouse or rat sera, as described elsewhere [22].

Absorptions

Absorptions with red blood cells or spleen cells were done at 4°C and room temperature. One volume of serum was absorbed four to six times with two volumes of packed red blood cells or one volume of packed spleen cells until no remaining antibody reactivity was detected against the absorbing cells.

Results

Reactivity of normal rat sera with guinea pig blood cells

Normal adult rat sera showed no hemagglutinating activity with guinea pig erythrocytes, as reported elsewhere [19], while all LEW and WM sera obtained after immunization with guinea pig tissues and cells showed high titers of hemagglutinating antibodies (titers > 1: 128, in most instances > 1: 1024). Sera from seven different inbred rat strains were investigated for antibody reactivity against guinea pig cells using cytofluorographic analysis. Normal rat sera diluted 1: 2 in PBS reacted with 2%–26% of guinea pig erythrocytes using FITC-labeled anti-rat IgG for staining and with 10%–33% of cells using FITC-labeled anti-rat IgM; the highest percentage of staining was observed with sera from WM rats. Antibody titers were, however, low and plateau staining was usually not observed. On the other hand, rat sera obtained after intentional immunizations with guinea pig tissues contained

high titers of both IgG and IgM antibodies against 100% of red blood cells.

Normal rat sera were investigated for antibody reactivity with guinea pig spleen and lymph node cell preparations. Such sera contained IgG antibodies that reacted with 29%–44% of guinea pig spleen cells and with 39%–57% of the lymph node cells; the same sera contained IgM antibodies that reacted with 23%–37% of guinea pig spleen cells and 35%–55% of lymph node cells. Because the highest antibody activity was observed in sera from MW rats, sera from six MW animals were tested individually. In a typical experiment, approximately 40%–50% of spleen and lymph node cells were stained with antibody titers up to 1: 16 or 1: 32.

The time of appearance of naturally occurring antibodies was investigated in sera obtained from newborn MW rats (Fig. 1). Low titers of naturally occurring IgG antibodies were present at birth, while IgM antibodies became detectable in the 1st postnatal week. After the 1st week, the IgM antibodies reacted with a larger fraction of the cells than the IgG antibodies.

Reactivity with tissue sections

Pooled sera from MW, LEW, BN, WKY, ACI, SD, and (LEW × BN)F_1 rats were tested against a variety of guinea pig tissues using indirect immunofluorescence and peroxydase staining techniques. Incubation of the guinea pig tissue sections with PBS and FITC-labeled anti-rat immunoglobulins showed weak autofluorescence of some vessel walls. While most normal rat sera showed variable binding to tissue structures, the strongest and most consistent reactions were observed with sera from MW rats. No specific staining was observed with normal guinea pig sera in dilutions higher than 1: 2.

Incubation of guinea pig liver sections with normal rat sera, followed by FITC-labeled anti-rat IgG, showed staining of parenchymal cells, the arterial vessel wall, the arterial and venous endothelium, as well as the smaller structures between the parenchymal cells (Fig. 2). In higher serum dilutions, the parenchymal staining was no longer observed. Immunoperoxydase staining with the sera in 1: 10 dilutions showed definite staining of the liver sinusoids and vascular endothelial cells. In the kidney, the sera gave diffuse staining of the cortical and medullary tubular cells, of the vessel walls, and of the glomerular mesangium, while some sera contained additional IgG antibodies that reacted weakly with the luminal aspect of the tubules. With heart tissue, diffuse staining of myocardial cells and weak endothelial cell staining were seen. The antibody titers against guinea tissues were generally low and varied between 1: 4 and 1: 32.

Absorptions

Two normal MW sera that contained naturally occurring anti-guinea pig IgG antibodies in titers between 1: 16 and 1: 32 against liver tissue were absorbed with guinea pig erythrocytes until no anti-erythrocyte activity was left. Prior to absorption, these sera reacted with ± 45% of guinea pig lymph node cells, while after absorption they reacted with ± 30% of the cells in dilutions from 1: 2 to 1: 8. When these sera were tested by indirect immunofluorescence using sections of guinea pig liver and heart, it was found that the overall staining intensity had decreased but the pattern was not substantially influenced. Absorptions of normal WM sera with guinea pig lymphoid cells resulted in complete removal of all antibody activity against guinea pig tissue structures.

Tissue reactivity of rat antisera raised against guinea pig

Sera from MW and LEW rats that had been immunized with either guinea pig skin grafts and lymphoid cells or lymphoid cells only contained predominantly IgG antibodies against guinea pig liver parenchymal cells, vessels, and interstitial cells; serum dilutions of 1: 64 to 1: 256 showed IgG binding to the vessels and the sinusoid cells (Fig. 3). The same sera contained IgG antibodies against renal tubular cells, interstitial cells, possibly extracellular matrix material, mesangium, and blood vessels, including the endothelium. Serum dilutions in excess of 1: 128 showed IgG binding to interstitial cells, glomerular mesangial cells, and vascular endothelium (Fig. 4). Finally, with the use of heart tissue sections, IgG antibodies were found that bound diffusely to cardiac muscle fibers, cells between the fibers, the vessels, and the endocardium, while virtually all cell types except the bronchial epithelium were stained in sections of lung tissue.

When the xenoantisera were tested against rat tissues, almost all sera contained low titers of IgG that bound to the arterial vessel wall, just as many normal rat sera do [22]. Two anti-guinea pig sera raised in MW rats by skin grafting and injections with lympoid cells contained IgG antibodies that reacted extensively with allogeneic and syngeneic tissues. These sera reacted in dilutions up to 1: 128 with venous endothelium as well as with a population of dispersed cells that resembled class II-positive Kupffer or dendritic cells of normal rat livers [6, 7, 13] (Fig. 5a). Class II positivity of the dispersed cell population was confirmed by double staining techniques using the mouse monoclonal antibody ER13 and sections from normal livers as well as livers from irradiated and bone marrow-reconstituted animals. In these experiments, the FITC-labeled anti-rat IgG was diluted in normal mouse serum while the rhodamine-labeled anti-mouse IgG was diluted in normal rat serum prior to use. Most interstitial cells that reacted with ER13 reacted with MW anti-guinea pig antisera and vice versa, although there was an inverse relationship between the staining intensity with either reagent (Fig. 5a, b). The irradiation produced variable depletion of the number of cells that reacted with ER13 as well as with the MW anti-guinea pig antisera. The overall concordance rate of cells that stained with both reagents was 80%–90%. The MW anti-guinea pig antisera bound, in addition, to a variety of other rat tissues, such as the large vessel endothelium in the lung, the central arteriole in the spleen (Fig. 6), and lymphatic vessel endothelium in lymph nodes. No specific staining

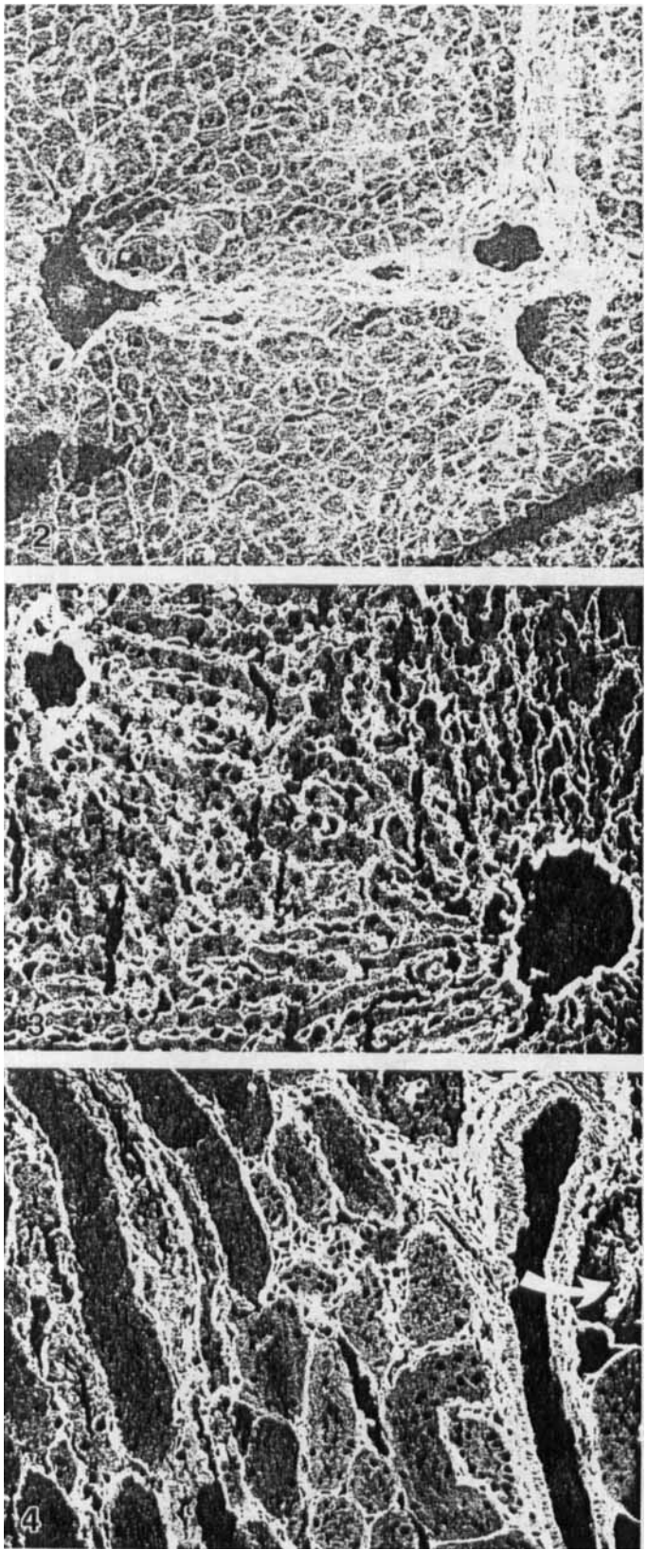

Fig. 2. Indirect immunofluorescence photograph of guinea pig liver section incubated with normal MW serum and stained with FITC-labeled anti-rat IgG; specific staining of vessel wall structures and the sinusoidal lining cells (× 100)

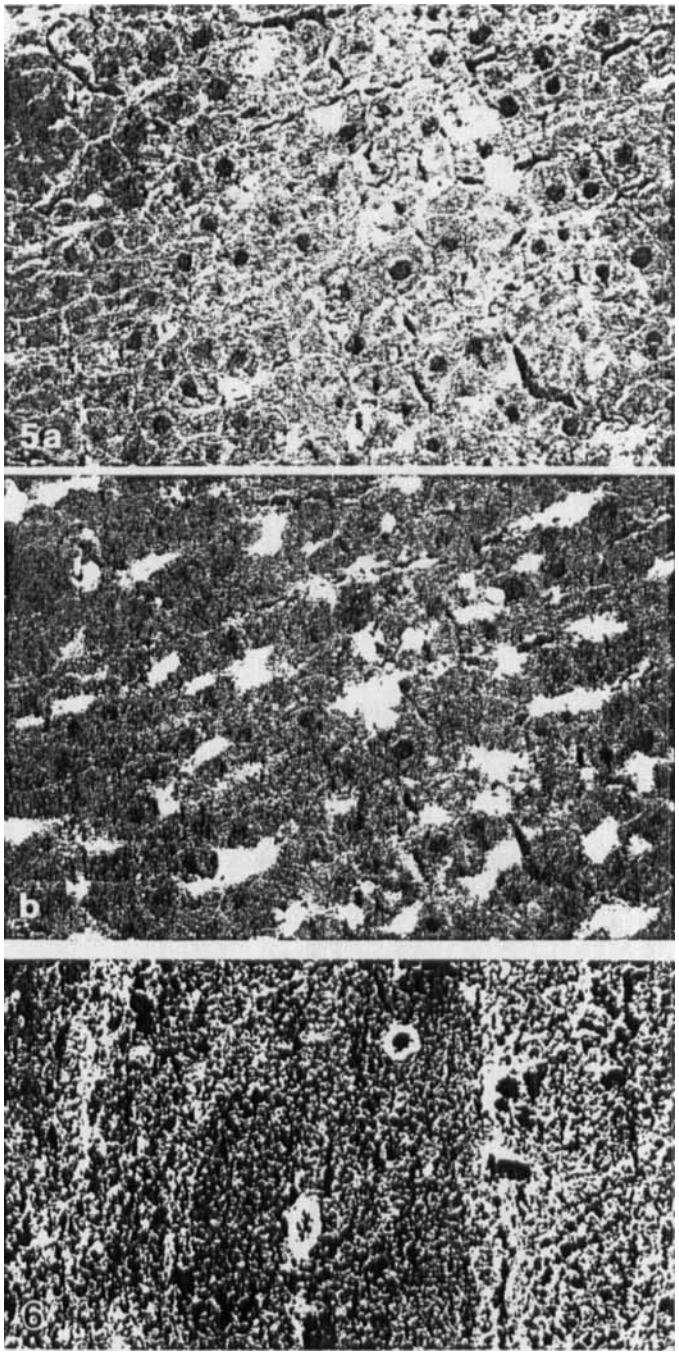

Fig. 3. Guinea pig liver section incubated with MW anti-guinea pig serum; specific staining of the vessel wall and the cells that line the sinusoids (× 100)

Fig. 4. Guinea pig kidney section incubated with MW anti-guinea pig serum and stained with FITC-labeled anti-rat IgG; specific staining of the vessel wall, interstitial cells and mesangium (*arrow*; × 100)

Fig. 5 a, b. Liver section from a SD rat incubated with: **a** MW anti-guinea pig serum and FITC-labeled anti-rat IgG or **b** ER13 monoclonal antibody (mouse anti-rat class II) and rhodamine-labeled anti-mouse IgG. The cells that are stained by MW anti-guinea pig serum are also stained by the monoclonal antibody ER13

Fig. 6. Rat spleen section incubated with MW anti-guinea pig serum and FITC-labeled anti-rat IgG; specific staining of the splenic central arteriole (× 100)

was observed with isolated rat lymphoid cells, red blood cells, or sections of rat kidney and heart. Finally, the MW anti-guinea pig sera did not exhibit any cytotoxicity in complement-dependent cytotoxicity assays with isolated rat lymphoid cells (data not shown). Since the preimmunization sera dit not contain any allogeneic or syngeneic antibodies, the antibodies described resulted from the xenogeneic immunizations.

Discussion

Guinea pig heart or liver tissues transplanted into untreated rats are rejected within minutes or hours with the histopathological picture of interstitial hemorrhages, edema, and vascular thrombi [1, 14, 26]. Such an xenogeneic rejection pattern has been classified as discordant and, based on the speed and histological similarity with hyperacute rejection in presensitized recipients with high titers of donor-directed antibodies, it has been assumed that this type of rejection is mediated by naturally occurring, complement-fixing xenoantibodies [11, 12, 23]. The evidence that antibodies participate in such kinds of rejections includes an arteriovenous decline in antibody titers across the graft, immunoglobulin deposition within the graft, and delayed xenograft rejection in neonatal animals that have no circulating antibodies or in animals that have undergone various procedures aimed at removal of antibodies [15, 20, 23, 25]. Furthermore, several histological and physiological aspects of discordant graft rejection have been reproduced by ex vivo perfusion of organs with xenogeneic serum [18, 25], as well as by transfer of antibodies to immunosuppressed recipients that carry vascularized xenogeneic skin grafts [30].

Recent studies have challenged the concept that complement-fixing antibodies mediate discordant xenograft rejection in the guinea pig to rat heart graft model [19, 20]. Miyagara et al. found in normal rat sera no naturally occurring hemagglutinating or complement-fixing lymphocyte antibodies against guinea pig cells [19]. We also found no naturally occurring hemagglutinating antibodies, although low titers of anti-erythrocyte and anti-lymphocyte antibodies were found in the sera of virtually all rat strains investigated using the more sensitive cytofluorographic analysis. More relevant for transplantation experiments are the findings that normal rat sera contained IgG antibodies against a variety of guinea pig tissue structures, such as liver vascular endothelial cells, liver sinusoid cells, renal tubular cells, glomerular mesangium, and cardiac muscle cells. The precise serological specificity of these reaction patterns is, however, unknown and is difficult to address because of the low antibody titers. Based on absorption experiments with two selected sera, it would appear that all xenoantigens that are recognized by naturally occurring antibodies on liver and heart parenchymal tissue are also present on lymphoid cells since absorptions with lymphoid cell suspension removed all antibody reactivity with tissue structures. On the other hand, absorptions of the sera with erythrocytes removed reactivity with a fraction of the lymphoid cells, whereas residual reactivity with lymphoid cells and tissues remained, indicating the presence of at least two naturally occurring antibody specificities. It is unknown whether the latter antibodies react with parenchymal tissue structures; absorption-elution studies would be required to address this question. A precise definition of antibody specificities with crossabsorptions and elutions is hampered by the low antibody titers, while monoclonal antibodies against guinea pig surface markers are presently not available.

Following intentional immunizations of rats with guinea pig tissues, antisera with high titers of IgG antibodies were obtained with a tissue reactivity pattern that was more extensive than that of normal rat sera. The induced antisera showed stronger and more extensive staining of the vascular endothelium, undefined interstitial cells, and possibly extracellular matrix components of guinea pig organs. We cannot definitely answer the question of whether this is due to additional antibody specificities or higher titers of pre-existing, naturally occurring antibodies. The reactivity pattern with heart and kidney tissues suggests, however, that additional antibody specificities emerged following immunizations. In view of the immunodominance of the major histocompatibility complex (MHC) in both allogeneic and xenogeneic immunization, it is tempting to speculate that intentional immunization resulted in MHC-directed xenoantibodies, as described in other systems [29].

The serological specificities of the naturally occurring antibodies remain unknown at present. The fact that they are present at birth without any obvious prior direct exposure of the animals to xenoantigens, together with their low titers, may suggest that the naturally occurring antibodies are antibodies directed against a variety of antigens that exhibit low-affinity cross-reactivity with xenogeneic cell surface [2, 8]. Alternatives for the proposed conventional antibody-antigen interactions include immunoglobulin binding through interaction of its Fc region with Fc receptors on xenogeneic cells [11] or nonspecific "sticking" of rat immunoglobulins to xenogeneic cell surfaces. The latter possibility seems unlikely, however, in view of the distinct and reproducible staining pattern of the rat sera with a fraction of blood cells rather than with all cells, as well as the staining of distinct anatomic structures in tissue sections. Absorption experiments of rat sera with guinea pig erythrocytes removed only red cell reactive antibodies, rather than all anti-guinea pig activity, lending further support to the possibility that naturally occurring antibodies react with xenogeneic cell surfaces through antigen-specific interactions.

To assess the role of naturally occurring antibodies in xenograft rejection more precisely, we attempted unsuccessfully to suppress their formation by neonatal injections of rats with guinea pig cells (data not shown). Although neonatal tolerance for alloantigens can be readily induced in rodents, this has not yet been achieved for complex xenogeneic antigens [9, 21].

The low titers of naturally occurring xenoantibodies raise the question as to how effective they are in mediating "hyperacute" or discordant xenograft rejection. Observations from allogeneic transplantation suggest that comple-

ment-fixing antibodies are mostly effective in mediating tissue damage if the recipient has high titers of circulating antibodies against donor antigens expressed in high density on the graft endothelium [3]. Under these conditions, complement can be activated along the classical route, resulting in the generation of mediators of tissue injury. On the other hand, small amounts of noncomplement-fixing antibodies can cause hyperacute rejection of xenogeneic grafts through the antibody-dependent cellular cytotoxicity mechanism [4]. Whether such a mechanism participates in the rapid destruction of xenografts in donor-recipient combinations with low titers of naturally occurring xenoantibodies remains to be established. The rejection tempo in many discordant graft rejection processes casts doubts on the potential importance of this mechanism in these conditions.

The question has been raised of whether xenogeneic immunization influences the course of a subsequent allograft. Etheredge et al. reported that rabbits that have been exposed to xenogeneic skin grafts exhibit accelerated rejection of a subsequent skin allograft [10], suggesting crossimmunity between xenogeneic and allogeneic transplantation antigens. We observed in the postimmunization sera of two MW rats that had been exposed to guinea pig tissues IgG antibodies that reacted with allogeneic and syngeneic vessel wall cells in the liver, lung, spleen, and lymph nodes, as well as with a population of class II-positive Kupffer cells in the liver. Since the sera did not react with class II-positive cells in other tissues, it is unlikely that the antibodies are directed against class II molecules themselves. Although the frequency and extent of such reactions have not been studied extensively, it is conceivable that they may influence the course of a subsequent allograft.

We conclude from the present study that rats have naturally occurring antibodies in low titers against a variety of guinea pig tissue structures. These antibodies are very likely low-affinity antibodies that crossreact with xenogeneic cell surfaces. It is at present, unclear whether such antibodies play a pathogenetic role in this model of discordant xenograft rejection.

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