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Understanding the Induced Antibody Response

Uri Galili, Zhao-chun Chen, Masahiro Tanemura, Tatiana Seregina and Charles J. Link

The unique evolutionary event of inactivation of the α1,3galactosyltransferase gene and the resulting elimination of α-gal epitopes (Galα1-3Galβ-4GlcNAc-R), which occurred approximately 20 million years ago in ancestral Old World primates,[1] erected an immunological barrier that currently prevents transplantation of pig organs into humans. This barrier is comprised of the natural anti-Gal antibody produced in humans, apes and Old World monkeys as 1% of immunoglobulins, interacting with α-gal epitopes abundantly expressed on pig cells. This antigen/antibody interaction results in the destruction of xenograft cells, either by complement-induced lysis (mediated by anti-Gal IgM molecules), or by antibody-dependent cell-mediated cytotoxicity (ADCC, mediated by anti-Gal IgG molecules).[2] The prolongation of xenograft survival time in monkeys depleted of anti-Gal[3] is a direct indication that anti-Gal is the major natural antibody that induces xenograft rejection. However, xenograft rejection is exacerbated by the extensive anti-xenograft immune response which is characterized by the production of large amounts of high affinity anti-Gal IgG antibodies, as well as antibodies to a wide variety of xenoproteins. We designate these latter antibodies as "anti-non gal" antibodies. Here, we summarize the characteristics of the induced anti-Gal and anti-non gal antibodies and speculate on their possible effects in clinical xenotransplantation trials.

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Induced Anti-Gal Antibodies

We estimate that ~1% of B cells in humans can produce anti-Gal. This is based on the finding that as many as 1% of EBV-transformed human B cells secrete this antibody.[4] Most of these B cells (designated "anti-Gal B cells") are in a quiescent state within the lymph nodes and spleen. Natural anti-Gal is produced primarily by anti-Gal B cells along the gastrointestinal tract, because of continuous stimulation by bacteria of the natural flora.[5]

Xenografts transplanted into humans release into the circulation α-gal epitopes on xenoglycoproteins that reach the quiescent anti-Gal B cells, activate them and induce extensive production of high affinity anti-Gal IgG molecules. The efficacy of this induced anti-Gal response was first observed in diabetic patients transplanted by Groth and colleagues[6] with an allogeneic kidney and with xenogeneic fetal pig islet cell clusters. Despite immunosuppression regimens which successfully prevented rejection of the kidney allograft, anti-Gal IgG titers increased by 50- to 100-fold.[7] The induced anti-Gal displayed increased affinity in comparison to the natural anti-Gal, probably as a result of the affinity maturation process. Induced anti-Gal can bind effectively to α-gal epitopes on xenograft cells and exacerbate xenograft destruction, primarily by ADCC.

We could recently monitor this induced anti-Gal IgG response in an ovarian carcinoma patient with recurrent metastatic tumor. This patient can be regarded as a discordant xenograft recipient because she was treated at the Human Gene Therapy Research Institute in Des Moines, Iowa, with repeated intraperitoneal infusions (three times in seven week intervals) of 6 x 10^9 mouse fibroblasts that released a replication-defective retrovirus containing the thymidine kinase gene. Tumor cells infected in situ by the virus were killed by subsequently administered ganciclovir.[8] The amount of cells in each infusion corresponded to ~50 gm of

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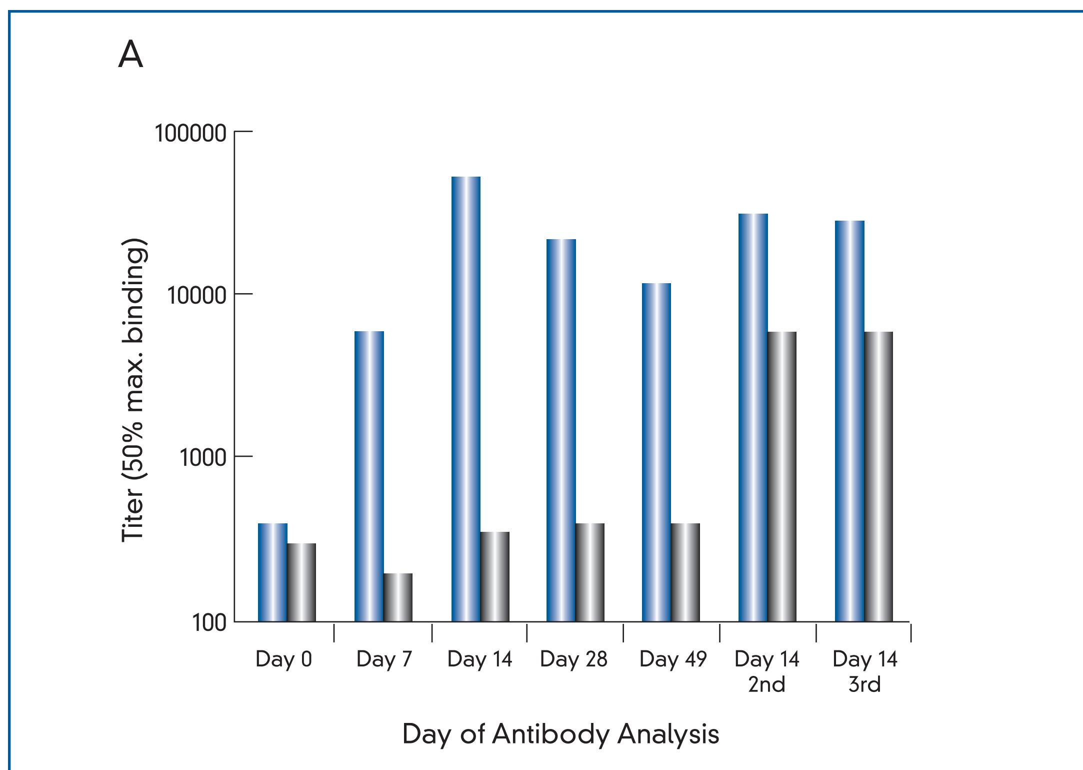

Figure 1A. Kinetics of induced anti-Gal and anti-non gal in a human xenograft recipient. Production of anti-Gal (blue) and anti-non gal (gray columns) IgG antibodies in an ovarian carcinoma patient transplanted three time i.p. with 6 x 10^9 mouse fibroblasts that produce retrovirus containing the thymidine kinase gene. Anti-Gal activity was measured by ELISA with α-gal BSA as solid-phase antigen,[10] and anti-non gal activity (in anti-Gal depleted sera) by ELISA with the mouse fibroblast cell line used for the i.p. transplantation treatment.

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mouse fibroblasts, which express α-gal epitopes, like other mouse cells.[9] Therefore, this treatment represents a repeated mouse-to-human transplantation of single cell xenograft suspensions.

Monitoring of antibody production in the treated patient indicated that anti-Gal IgG titer increased by ten-fold within the first week post transplantation (Figure 1A). A further ten-fold increase was observed on day 14. The overall ~100-fold increase in anti-Gal titer observed by day 14 was comprised of a ten-fold increase in the concentration of the antibody, detected already by day 7, and a further ten-fold increase in the affinity of anti-Gal which occurred within the second week post transplantation. Approximately, 90% of the induced anti-Gal was of the IgG2 subclass and the rest was IgG3. No significant changes were observed in anti-Gal IgG1, IgG4, IgM or IgA antibodies. We observed predominance of anti-Gal IgG2 also in sera of the previously reported diabetic patients transplanted with pig fetal islet cell clusters (not shown).[6,7]

The data in Figure 1A suggest that within two weeks after transplantation of a xenograft, there is an expansion of anti-Gal B cell clones, most of which undergo isotype switch and affinity maturation, to produce large amounts of high affinity anti-Gal IgG. The life span of an anti-Gal producing plasma cell is relatively short (i.e., few days), because no further increase in anti-Gal titer was observed on days 28 or 49. In recent studies in knock-out mice for the α1,3galactosyltransferase gene, we found that this induced anti-Gal IgG response is dependent on helper T cells, activated by xenopeptides that are processed and presented by anti-Gal B cell.[10] This induced anti-Gal response can be temporarily prevented by anti-CD40L antibodies[10] but the response resumes if treatment with anti-CD40L is discontinued.

Interestingly, anti-Gal IgG titer did not increase after second and third infusions, above the titer observed post-first infusion of mouse fibroblasts. It is probable that high affinity anti-Gal IgG molecules bind strongly to α-gal epitopes on xenoglycoproteins and prevent them from interacting with B cell receptors on many of the anti-Gal B cells. Overall, this results in maintaining induced anti-Gal production at a constant level which corresponds to ~100-fold higher titer than the original titer, despite the repeated transplantation of xenograft cells. We have observed a similar phenomenon of

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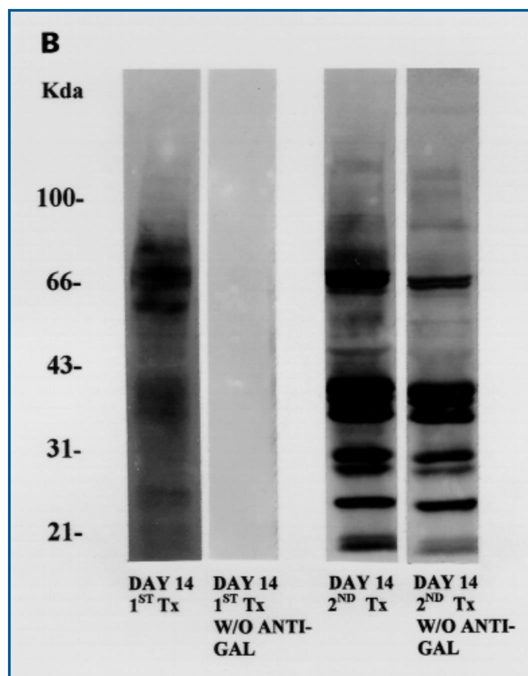

Figure 1B. Western blots representing binding of anti-Gal and anti-non gal IgG antibodies to mouse fibroblast proteins after first and second transplantation of these cells. The sera were diluted 1:200 and anti non-gal antibodies were studied in sera depleted of anti-Gal (lanes w/o anti-Gal).

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stable induced anti-Gal IgG titers in cynomolgus monkeys transplanted for prolonged periods (>2 months) with pig cartilage.[11]

Induced Anti-Non Gal Antibodies

Humans and nonprimate mammals, such as pigs or mice, belong to separate evolutionary lineages that evolved independently for ~75 million years. Thus, each species has accumulated species-specific mutations that result in 10-25% differences in amino acid sequences of many homologous proteins. Therefore, mice and pigs have hundreds and possibly thousands of peptides that are immunogenic in humans. In xenograft recipients, these immunogenic xenopeptides can induce production of anti-non-gal antibodies with a very large number of specificities. The production of these antibodies is shown in Figure 1A, where this activity was measured by ELISA with the mouse fibroblasts as solid phase antigen. The serum samples assayed were depleted of anti-Gal by adsorption on rabbit red cells. Anti-non-gal antibodies were produced only after the second infusion of mouse fibroblasts, implying that the initial number of anti-non gal B cells is very low. Therefore, a boost with xenoproteins was required for this T cell-dependent B cell-response to reach a level that enables detection of anti-non-gal antibodies in the serum.

In Figure 1B we immunostained mouse fibroblast proteins in western blots with sera obtained after the first or second transplantation with these fibroblasts. The only anti-xenograft antibody in serum from day 14 post-first transplantation was anti-Gal that bound to α-gal epitopes on a large variety of mouse glycoproteins. This was indicated by the complete removal of antibodies binding to mouse proteins when the serum was adsorbed on rabbit red cells. However, the serum from day 14 post-second infusion contained antibodies that bound to the many separate fibroblast proteins, also after removal of anti-Gal. The intensity of various bands is likely to reflect the abundance of different immunogenic proteins in the fibroblasts. The subclass distribution of anti-non gal antibodies was IgG1>IgG2>IgG3>IgG4.

Concluding Remarks

1. Xenotransplantation induces in humans an extensive T cell-dependent anti-Gal IgG response (mostly IgG2), which is detrimental to xenografts. Immunosuppressive regimens effective in preventing allograft rejection do not prevent the induced anti-Gal response in xenograft recipients. Prevention of this response may be achieved by either elimination of α-gal epitopes on pig cells, or specific elimination of anti-Gal B cells and induction of tolerance toward the α-gal epitope.
2. Induction of anti-non gal antibodies against multiple immunogenic proteins in the xenograft is a much slower process than induction of anti-Gal. Because anti-non gal antibodies are T cell-dependent and produced by many different B cell clones of initial limited size, it may be possible that immunosuppression targeted towards helper T cells and B cells will effectively prevent production of these antibodies..

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