

2029

CHIMERISM AND XENOTRANSPLANTATION

New Concepts

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Only two sweeping improvements have occurred in clinical organ transplantation since 1962, each associated with the advent of a more effective baseline immunosuppressant.³⁹ The first was replacement of azathioprine with cyclosporine as the baseline immunosuppressant (1979–1980); the second was substitution of tacrolimus (1989–1990); however, what was actually being accomplished with these drugs remained enigmatic until only 5.5 years ago and is still incompletely worked out. Nevertheless, it now can be suggested that the riddle of allograft acceptance has been solved in principle, if not in detail.³⁵ With this fresh insight, it may be possible to map effective strategies that will make xenotransplantation feasible.

INITIAL INSIGHT

Enduring interest in the clinical applications of transplantation usually is dated to the first demonstration of “acquired tolerance” to skin grafts in mice that had been infused with allogeneic splenocytes or bone marrow in utero or just after birth.^{2,3} This so-called “neonatal tolerance” was strongly associated with donor hematopoietic chimerism and was

Supported in part by research grants from the Veterans Administration and Project Grant No. DK-29961 from the National Institutes of Health, Bethesda, Maryland.

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generally construed as replacement of the native immune system before it was sufficiently mature to reject the hematopoietic grafts obtained from adult donors.

Bone marrow transplantation after cytoablation of the adult recipient with total body irradiation or myelotoxic drugs was, in essence, a simulation of the neonatal model.²¹ Ironically, bone marrow transplantation was not accomplished clinically until 1968, and then only with the use of human leukocyte antigen-matched donors⁴⁹ 9 years after the first successful organ transplantation in humans²⁴; however, because of its strong association with tolerance, attempts to produce donor leukocyte chimerism in preparation for, or at the same time as, tissue and organ transplantation dominated surgical research until the early 1960s.²⁵

These efforts to combine adjunct donor bone marrow infusion with organ transplantation were largely abandoned by the end of 1963. The loss of interest stemmed from evidence suggesting that kidney allografts were inherently tolerogenic, seemingly eliminating the need for the dangerous cytoablation that was assumed to be a prerequisite for donor leukocyte engraftment. The concept of organ tolerogenicity was encapsulated in the title of the article "The reversal of rejection in human renal homografts with subsequent development of homograft tolerance,"⁴¹ in which the combined use of azathioprine and dose-manueverable prednisone was described for the first time.

The use of "tolerance" to characterize the progressively diminishing need for maintenance immunosuppression after the early postoperative period of these patients⁴¹ was harshly criticized at the time, and subsequently; however, the term was apposite. Although none of the pioneer patients were weaned from immunosuppression until much later,³⁷ long-term drug-free allograft acceptance already had been recorded by 1964 in a few mongrel canine kidney recipients treated for a year post-transplantation with 6-mercaptopurine or its analogue, azathioprine. This was accomplished with even greater regularity after canine liver transplantation following only 4 months of similar immunosuppression.³³

Soon thereafter, permanent liver graft acceptance without any treatment at all was described in outbred pigs, many of which passed through spontaneously resolving rejection crises. Thus, it was obvious 30 years ago that chemotherapeutic immunosuppression was permitting, rather than causing, a fundamental change in the host, the graft, or probably both.^{33, 34, 37}

Finally, it was demonstrated, first in pigs⁵ and then in rodents,⁵⁴ that the tolerance self-induced by a liver allograft also extended to other organs transplanted simultaneously or later from the same donor (donor-specific nonreactivity; tolerance). Although these observations were most readily exemplified with liver transplant models, "hepatic tolerogenicity" was only an extreme manifestation of a property that is variably shared by all tissues and organs.^{25, 36-38} Eventually, it was shown that the unusual tolerogenicity of the liver was caused by its large content compared with other organ allografts of bone marrow-derived donor

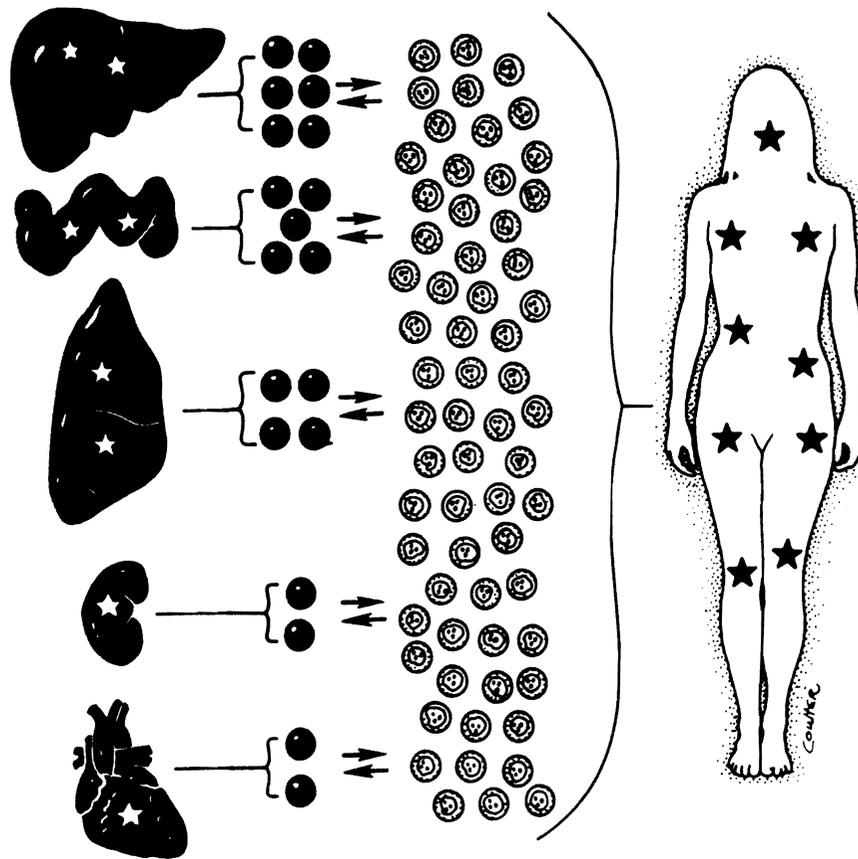


Figure 1. The explanation for the variable ability of different organ allografts to induce acceptance and ultimately tolerance. The authors postulate that the dendritic leukocyte is the single most important (see text), although not the only, tolerogenic cell by virtue of its role in antigen presentation and clonal activation, which leads to exhaustion/deletion. Although the intestine is leukocyte-rich, the total number of donor leukocytes in this hollow organ is less than in the liver; in addition, the high percentage of mature T cells makes the bowel allograft more GVHD prone than other organs. Stars represent chimeric donor cells in the recipient. (From Starzl TE, Demetris AJ, Trucco M, et al: Cell migration and chimerism after whole-organ transplantation: The basis of graft acceptance. *Hepatology* 17:1127–1152, 1993; with permission.)

leukocytes (Fig. 1).^{25, 36–38} These “passenger leukocytes” make up most of the nonparenchymal cells (NPCs) of all tissues and organs.

THE ENEMY: PASSENGER LEUKOCYTES

Because of their strong immunogenicity, passenger leukocytes (particularly mature dendritic cells⁴⁴) have long been known to provide the principal stimulus leading to organ rejection.^{20, 45} In addition to the

recipient immune reaction induced within the allografts by the donor hematopoietic cells,^{10, 11} it has been recognized for more than 15 years that these donor leukocytes induce widespread immune activation after their hematogenous migration from the transplanted organ to host lymphoid organs.^{10, 18, 27}

It also was demonstrated as early as 1969¹⁶ that the migratory cells following successful transplantation of hepatic allografts were replaced in the organ by an influx of recipient cells of the same lineages, a phenomenon that was wrongly thought for nearly 2 decades to be unique to the liver. In addition, destruction by the recipient immune system of the highly immunogenic donor leukocytes, either within the transplanted organ or after their peripheral migration, was incorrectly assumed to be a prerequisite for successful organ transplantation. This latter assumption, which already had become dogma by 1963, was not challenged for nearly 3 decades.

THE DISCOVERY OF DONOR LEUKOCYTE CHIMERISM

As a result of these conceptual errors, numerous chimerism-exclusionary theories were elaborated between 1962 and 1992 to explain organ allograft "acceptance" by different mechanisms than those responsible for neonatal tolerance or for the tolerance seen after clinical bone marrow transplantation.³⁷ When peripheralized donor leukocytes were discovered in 1992 to be present in small numbers (microchimerism) in host lymphoid and nonlymphoid areas as many as 30 years after organ transplantation,^{36, 38} the authors suggested that the different forms of acquired tolerance, including organ allograft acceptance, were all variations on the same theme (Fig. 2). As a practical matter, the implication was that "clinical success (after transplantation)—tolerance or graft acceptance—means that a characteristic lymphoid and dendritic cell [DC] chimerism has been introduced, which may be stable either without further treatment or only when further immunosuppression is provided; an unstable graft and its migrated cells may either be rejected or cause graft-versus-host disease.³⁶

MECHANISMS ASSOCIATED WITH CHIMERISM

Clonal Exhaustion or Depletion

In the first few days after organ transplantation, multilineage bone marrow-derived donor ("passenger") leukocytes, which include pluripotent stem cells^{25, 26} and dendritic cells,^{8, 9, 25, 30, 36, 38} constitute 1% to 20% of the host circulating mononuclear cells, depending on the kind of allograft (i.e., highest with liver and intestine, lowest with heart or kidney).^{25, 38} Although their primary migration is to lymphoid organs,^{8,}

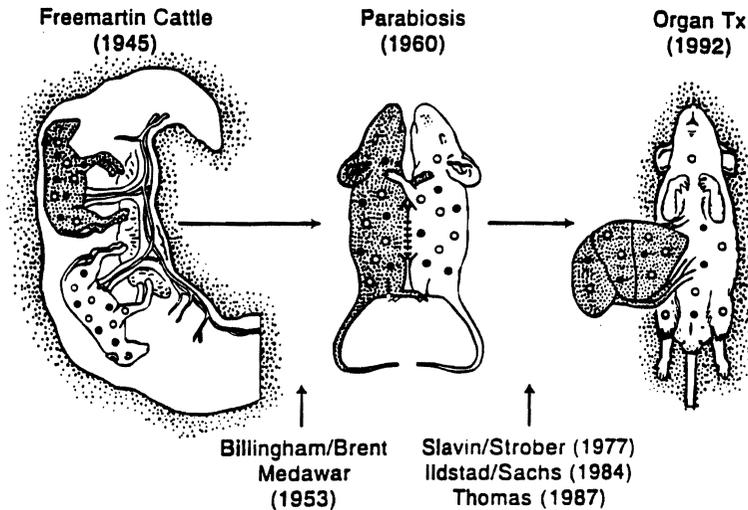


Figure 2. The continuum from observations of natural chimerism in Freemartin cattle to the present time. Chimerism was incorrectly discounted as a factor in organ allograft acceptance from about 1960 until the discovery in 1992 of microchimerism in organ recipients. (From Starzl TE, Demetris AJ, Murase N, et al: The lost chord: Microchimerism. *Immunol Today* 17:577-584, 1996; with permission.)

^{9, 30} beginning in approximately 2 weeks, small numbers of donor leukocytes can be found increasingly in other tissues; by 3 months, they are mostly in nonlymphoid sites (e.g., skin and native heart).^{35, 36, 38, 48}

Even with the limited information available in 1992, it was possible to suggest that organ allograft acceptance involved "[acute] responses of co-existing donor and recipient immune cells, each to the other, causing reciprocal clonal expansion, followed by peripheral clonal deletion."³⁶ The characteristic cycle of immunologic crisis and resolution, first observed in drug-immunosuppressed kidney recipients and most practically monitored by serial changes in allograft function,⁴¹ was not simply a host-versus-graft reaction (rejection). It was the product of the dual immune reaction: host-versus-graft and graft-versus-host (Fig. 3).^{25, 35-38}

The mutually canceling effect of the donor and recipient cell populations as both underwent clonal exhaustion and depletion explained the rarity of graft-versus-host disease following the engraftment in noncytoablated recipients of immunologically active organs, such as the intestine and liver.^{36, 38} Disruption of the leukocyte interaction with the host cytoablation used to prepare bone marrow recipients (Fig. 4) but not the recipients of whole organs (Fig. 4) obviously was responsible for the differences between bone marrow and organ transplantation (Table 1), including absolute dependence on human leukocyte antigen matching to avoid graft-versus-host disease in the first instance but not the second.

In the framework of the double immune reaction, treatment failure for immunologic reasons after essentially all transplantation procedures

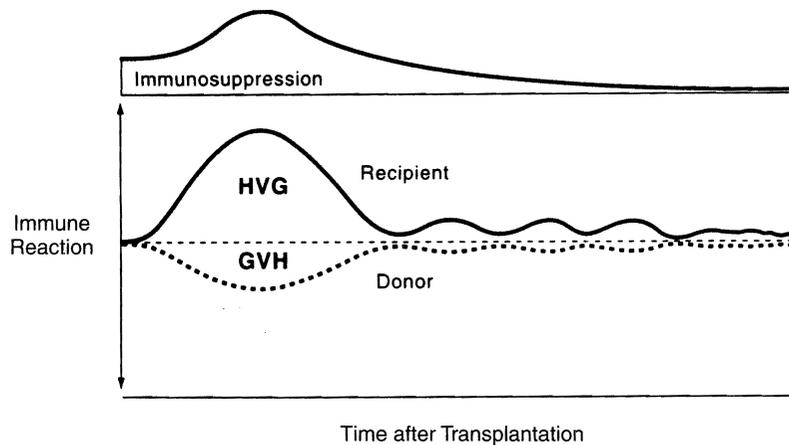


Figure 3. Contemporaneous host-versus-graft (HVG) and graft-versus-host (GVH) reactions in the two-way paradigm as applied to organ transplantation. Following the initial interaction, the evolution of nonreactivity of each leukocyte population to the other is seen as a predominantly low-grade stimulatory state that may wax and wane, rather than a state of absolute or irreversible clonal deletion. After bone marrow transplantation to a recipient who has undergone cytoablation, the conditions are a mirror image.

can be defined as uncontrollable ascendancy of one of the interactive arms (Fig. 3) or sometimes both together. The strongest reaction in organ recipients usually is host-versus-graft (rejection) (Fig. 3). In bone marrow recipients, it usually is graft-versus-host.

Immune Indifference

In addition to clonal exhaustion and depletion, it was proposed in 1992 that "acceptance" of transplanted organs was facilitated by the in situ depletion of leukocytes post-transplantation and a consequent loss of allograft immunogenicity.^{36, 38} This effect of leukocyte depletion has been demonstrated in many different experimental models.^{17, 20, 45, 46} Thus, with successful organ engraftment, three progressive changes presumably take place at about the same time: (1) diminished immunogenicity of the allograft, allowing the host immune system to view it with progressive indifference; (2) clone-specific host exhaustion and depletion of host-versus-graft reactivity; and (3) reciprocal passenger leukocyte exhaustion or depletion of graft-versus-host specificity. A stable allograft emerging from this triple process eventually may come to resemble an immunologically neglected infection.³⁵

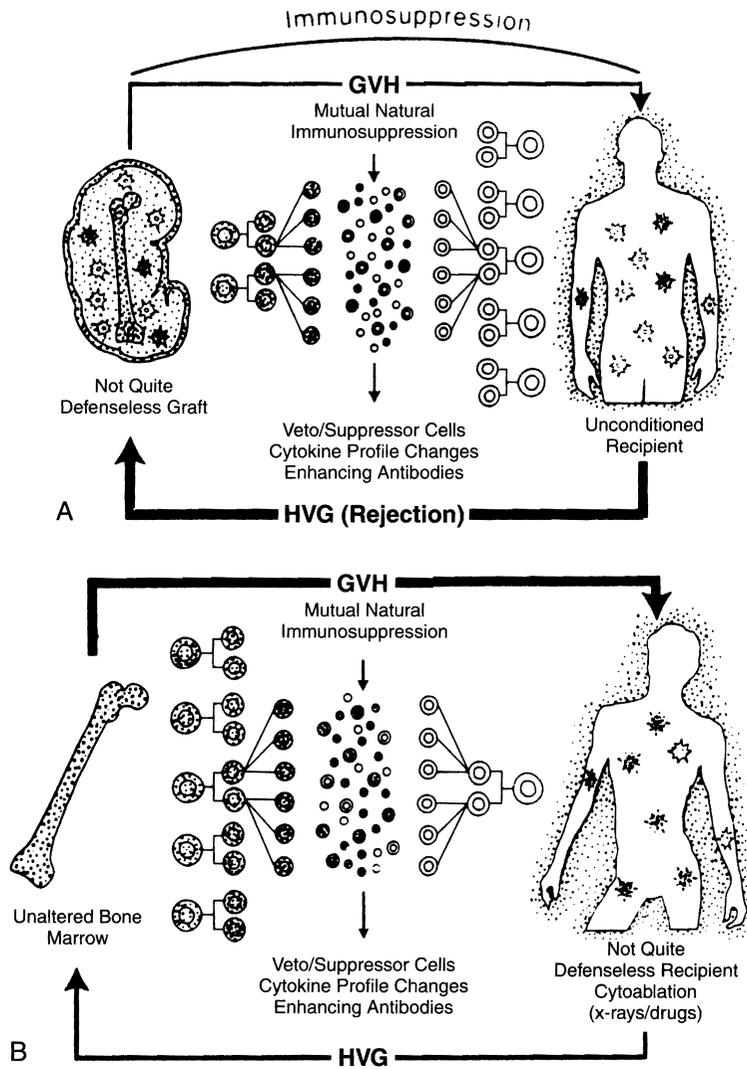


Figure 4. Two-way paradigm in which transplantation is seen as bidirectional and mutually canceling immune reactions that are (A) predominantly HVG with whole organ grafts and (B) frequently GVH with bone marrow grafts.

THE MICROCHIMERISM CONTROVERSY

Skepticism about the significance of microchimerism^{4, 53} has been based on (1) the inconsistency with which donor leukocytes can be found in blood or tissue samples from recipients of long-surviving organ allografts, (2) the development of acute or chronic rejection despite

Table 1. DIFFERENCES BETWEEN CONVENTIONAL BONE MARROW AND ORGAN TRANSPLANTATION

Bone Marrow		Organ
Yes	← Recipient cytoablation*	→ No
Critical	← MHC compatibility	→ Not critical
GVHD	← Principal complication	→ Rejection
Common	← Drug-free state	→ Rare
Tolerance	← Term for success	→ "Acceptance"†

*All differences derive from this therapeutic step, which, in effect, establishes an unopposed graft-versus-host reaction in the bone marrow recipient whose countervailing immune reaction is eliminated.

†Or "operational tolerance."

MHC, major histocompatibility complex; GVHD, graft-versus-host disease.

demonstrable chimerism, and (3) the inability to use microchimerism to guide post-transplant weaning of immunosuppression. All of these observations can be readily fitted into the concept of the various chimeric states, providing the chimerism is coupled with the degree of altered immunogenicity of the allograft and that the extent to which reciprocal clonal exhaustion or depletion has occurred is factored in.⁴² These parameters vary with time and are subject to a common governance.

GOVERNANCE OF MECHANISMS

One of the authors (TES) and Zinkernagel of Zurich³⁵ have proposed that the immunologic response or nonresponse against infections or tumors, and under the conditions of clinical transplantation, are governed primarily by the migration and localization of antigen. In this view, immune reactivity depends on migration of antigen to organized lymphoid tissue and can be viewed as "a balance between potentially reactive lymphocytes versus the qualities, quantity, kinetics, and distribution of the antigen (foreign or self) within the host."³⁵ In this context, nonreactivity can be the consequence of immune activation, followed by clonal deletion. Nonreactivity also may occur by default if the antigen never reaches lymphoid organs or is secondarily sequestered in non-lymphoid sites (immune indifference).³⁵

Thus, donor leukocyte chimerism is a prerequisite for, but not synonymous with nor a consequence of, the evolution of allograft tolerance.^{35-38, 42} Although the association of chimerism with organ allograft acceptance was discounted for 30 years,³⁷ the principle of chimerism-linked organ allograft acceptance is no different than in the rodent neonatal,^{2, 3} cytoablation-dependent,²¹ parabiosis-induced,²² and more complicated "mixed chimerism" tolerance models^{15, 32, 50} (Fig. 2). The theme came full-circle back to the observations by Owen²⁸ more than 50 years ago of natural reciprocal tolerance to allogeneic blood cells and skin grafts in nonidentical (Freemartin) twin calves whose circulations were joined in fetal life as the result of placental fusion (Fig. 2).

INFECTIOUS AND TRANSPLANT ANALOGIES

The barrier to successful transplantation of allografts and xenografts is the result of a survival-driven coevolution of the host immune system with environmental microorganisms.^{55, 57} The immune response is similar against lethal cytopathic pathogens and the less dangerous noncytopathic varieties, but with distinct and variable consequences.

Noncytopathic Pathogens and Allografts

In a prophetic early review, Lawrence¹⁹ compared the rejection of primary allografts with infections associated with delayed hypersensitivity (e.g., tuberculosis). The MHC-restricted mechanisms of this kind of immune response were unknown at the time. Once they were recognized, it was obvious that immune rejection of infected cells or tumors was the physiologic equivalent of allograft rejection.⁵⁶

Because these largely intracellular infectious agents may be noninjurious, a high priority is avoidance of excessive immunologic tissue damage. Otherwise, immune destruction of widely disseminated pathogens (e.g., hepatitis B or C virus) can kill or disable the host.^{55, 57} To prevent this, means exist that can temper or terminate the immune response. These are the same mechanisms (i.e., clonal exhaustion or depletion and immune indifference) that have been exploited for successful allotransplantation (see previous discussion).

Cytopathic Antigens and Xenografts

In contrast to the graded and highly targeted response to noncytopathic pathogens, the full resources, first of the innate and then of the specific (adaptive) immune system, are urgently mobilized to quickly and completely eliminate the damaging parasites without regard for destruction of host tissues by immunologic mechanisms.^{35, 55, 57, 58} The first line of defense is dominated by interferons, macrophages, gamma-delta T cells, and "natural" killer cells. In addition, B cells whose receptors are maximally cross-linked may be activated directly without T-cell help.

The antigenic signal issued by an infectious invader to B cells comes from its densely arranged and ordered repetitive epitopes, sometimes aided by lipopolysaccharides or by other unknown means.⁵⁵ In addition, nonspecific or less-specific effector mechanisms, such as complement, interleukins, and phagocytes, are promptly involved. The specific T-cell and B-cell immune responses then usually control cytopathic infections definitively.

These are the same mechanisms, predominantly those of innate immunity, that are responsible for the hyperacute rejection of discordant xenografts, and of allografts transplanted to ABO-incompatible or highly

sensitized recipients.⁴³ The best-characterized signal on the cells of discordant xenografts is the terminal residue Gal- α -(1,3)-Gal.^{6, 13} This antigen is chemically similar to ABO antigens and is found on numerous bacteria, protozoa, and viruses.¹²

Human complement regulatory proteins have been transfected into pigs^{7, 23, 52} in an effort to prevent clinical hyperacute xenograft rejection. This results only in temporary delay of xenograft destruction.^{1, 14} The reason is that the other mechanisms of innate immunity promptly cause inexorable rejection. Thus, additional genetic manipulation is required, whereby antigens are eliminated or equivalent human genes are introduced. The realistic objective is not to completely avoid the cell-mediated rejection of adaptive immunity but rather to change the epitope pattern to one that will be recognized by the immune system as a noncytopathic (i.e., allo-) antigen.

CHANGING THE XENOGRRAFT PROFILE

Gene-knockout procedures have not yet been done in the pig; however, using molecular technologies, some of which already have been shown to be applicable in pigs, Osman and colleagues²⁹ in Australia have been able to reduce cell surface expression of the Gal- α -Gal gene product in cultured African green monkey fibroblasts (so-called "COS cells") to negligible levels. As a first step in these staged experiments, the COS cells, which normally do not express the Gal- α -Gal epitope, were transfected with the Gal cDNA. Now presenting a Gal- α -Gal target, the transfected COS cells were vigorously lysed in human serum (Fig. 5).

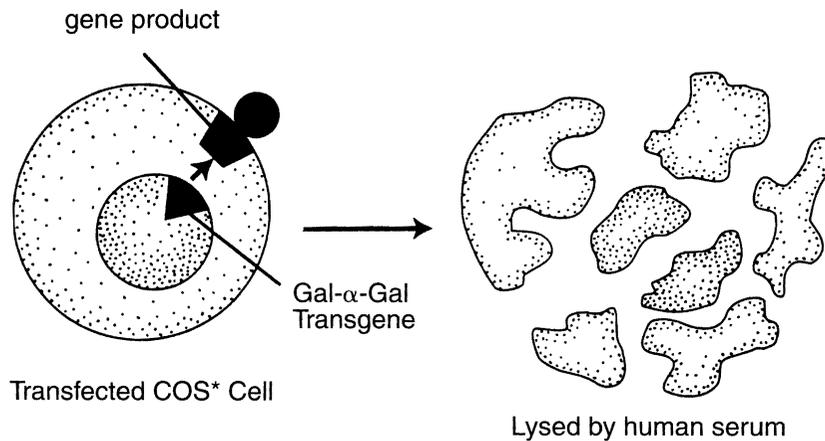


Figure 5. COS cells are lysed by human serum after their transfection with the Gal- α (1,3) Gal gene (see text). *African green monkey fibroblast (From Starzl TE, Rao AS, Murase N, et al: Will xenotransplantation ever be feasible? *J Am Coll Surg* 186:383-387, 1998; with permission.)

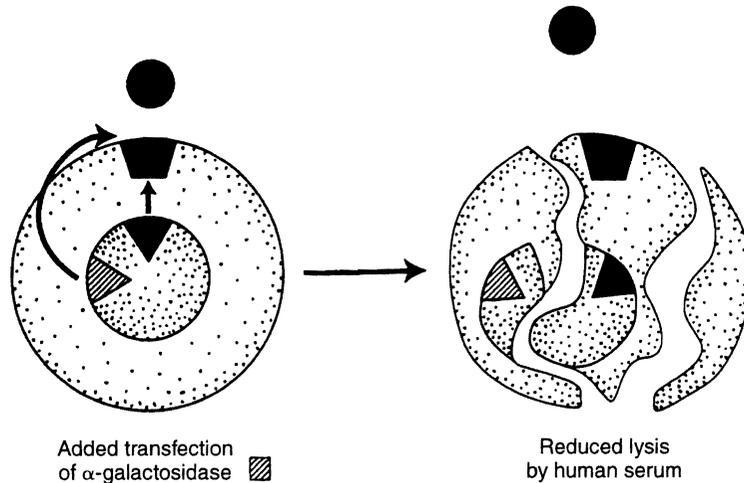


Figure 6. Insertion of α -galactosidase gene diminishes but does not eliminate the lysis shown in Figure 5. (From Starzl TE, Rao AS, Murase N, et al: Will xenotransplantation ever be feasible? *J Am Coll Surg* 186:383–387, 1998; with permission.)

The anti-Gal lysis was reduced but not eliminated by transfection of the altered COS cells with human α -galactosidase, which cleaves off α -linked galactosyl residues of the target epitope (Fig. 6). Because this exposes subterminal saccharides (i.e., *N*-acetyl lactosamine) to which there also are “natural” human antibodies, lysis is only reduced; however, the additional insertion of an α -1,2-fucosyl transferase gene resulted in the substitution of Gal- α -Gal with the nonimmunogenic H substance (i.e., the universally tolerated O blood group antigen). Thus, the double transfection (galactosidase plus fucosyl transferase) completely eliminated complement-mediated lysis of the COS cells (Fig. 7).

The α -galactosidase gene has not yet been transfected in pigs, but this has been accomplished with the α -fucosyl transferase gene by Logan and associates of the Nextran Corporation in collaboration with Sharma and colleagues at Duke University.³¹ Stable double transfection in pigs may be a realizable objective.

SUMMARY

In both transplant and infectious circumstances, the immune response is governed by migration and localization of the antigen. If the antigenic epitopes of transgenic xenografts are sufficiently altered to avoid evoking the destructive force of innate immunity, the mechanisms of engraftment should be the same as those that permit the chimerism-dependent immunologic confrontation and resolution that is the basis of allograft acceptance.

In addition to “humanizing” the epitopes, one of the unanswered

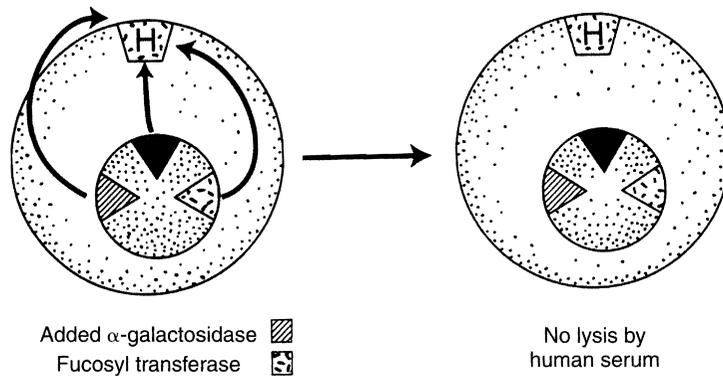


Figure 7. Additional insertion of the α -1,2-fucosyl transferase gene converts the xenogeneic Gal- α (1,3) Gal antigen to the H (O, or universal donor) antigen and eliminates lysis (see text). (From Starzl TE, Rao AS, Murase N, et al: Will xenotransplantation ever be feasible? *J Am Coll Surg* 186:383–387, 1998; with permission.)

questions is whether the species restriction of complement described in 1994 by Valdivia and colleagues⁵¹ also necessitates the introduction of human complement regulatory genes in animal donors. Because the liver is the principal or sole source of most complement components,^{43, 51} the complement quickly is transformed to that of the donor after hepatic transplantation. Thus, the need for complementary regulatory transgenes may vary according to the kind of xenograft used.

Much evidence shows that physiologically important peptides produced by xenografts (e.g., insulin, clotting factors, and enzymes) are incorporated into the metabolic machinery of the recipient body.⁴³ To the extent that this is not true, xenotransplantation could result in the production of diseases that are analogous to inborn errors of metabolism.

In the climate of pessimism that followed the failures of baboon to human liver xenotransplantation in 1992–1993,^{40, 43} it seemed inconceivable that the use of even more discordant donors, such as the pig, could ever be seriously entertained; however, this preceded insight into the xenogeneic and allogeneic barriers that has brought transplantation infectious immunity to common ground.³⁵ With this new insight and the increasing ease of producing transgenic donors, the goal of clinical xenotransplantation may not be so distant.

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