Xenotransplantation: where are we today?

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Xenotransplantation has retained its topicality since the resurgence of interest in the mid 1990s. Work continues in many scientific fields to overcome the obstacles. However, as old problems are solved, new ones present themselves for solution. In this brief review we discuss the need for xenotransplantation, issues relating to physiological compatibilities, current ideas on how to overcome the initial aggressive hyperacute rejection (HAR) and the later immunological difficulties as well as the risks and ethical issues.

THE CRISIS IN SUPPLY OF ORGANS FOR TRANSPLANTATION

For many patients with end-stage cardiac, respiratory or hepatic failure the only chance of survival is a transplant. In renal failure, although long-term dialysis is an option, transplantation offers better survival[1] and quality of life[2] as well as economic advantages[3].

The major difficulty facing the transplant community currently is the shortage of organs for transplantation, and strategies to increase the supply include the deployment of specialist donor liaison nurses, the use of so-called marginal donors (whose organs would never previously have been considered for transplantation) and the encouragement of live donation. Consideration has even been given to altering the basis of cadaveric donation, which at present proceeds only when the permission of relatives, actively sought, is granted. The 'presumed consent' approach (whereby objectors must actively register their wish to opt out) is now lawful in several countries and has increased the cadaveric donation rate.

However, even with the successful implementation of all these initiatives the number of donations would still be insufficient. In 1991 there were 4815 patients on the transplant waiting list, but in 2000 the number waiting for solid organ transplants was 6823 (renal 6154, cardiothoracic 494, liver 175). Over the same period the number of donors actually fell, from 934 to 845[4] (Figure 1).

Can we solve the problem by reducing the demand for transplantation? If we look only at the kidney, five groups of diseases accounted for about 70% of new starts on renal replacement therapy in 1992, according to the large registry of the European Dialysis and Transplantation Association–European Renal Association[1]. Diabetes mellitus and renovascular disease are actually becoming more prevalent, and, of the other three (glomerulonephritis, polycystic kidney disease, pyelonephritis), only the prevalence of the last is decreasing. Improvements in managing these patients are unlikely to reduce the need for renal transplantation, although screening programmes for diabetes may improve matters in the longer term. There is even less potential to decrease the demand for the transplantation of other organs, when one considers the minority of patients that are offered this option in cardiac and respiratory disease.

If we are unable, therefore, to solve the problem by increasing the level of donation or reducing demand we will have to look elsewhere. Increasing attention is being paid to other sources of organs for human transplantation. For heart failure implantable mechanical devices are being tried as are biomechanical support devices for other failed organs[5]. There is also much interest in cloning and stem cell differentiation research for tissue replacement[6]. However, these strategies are still much removed from the clinic. The greatest chance of providing an early solution is offered by xenotransplantation.

THE EARLY DAYS OF XENOTRANSPLANTATION

The xeno in xenotransplantation is derived from the Greek for foreign or strange. Donor-recipient combinations can be classified, as was initially done by Calne, into 'discordant' (where transplant between species results in a rapid, hyperacute rejection) or 'concordant' (where rejection occurs at a pace similar to that of allotransplantation). We know that this difference is essentially due to the presence or absence of preformed antibodies.

Blood transfusions from animals to man were performed in England and France from the early seventeenth century. These were the first clinical attempts at xenotransplantation. Solid organ transplants, attempted in the 20th century, had one or two successes, again mainly in concordant transplants. Reemtsma and colleagues reported patients surviving up to nine months after kidney transplants from a chimpanzee[7]. They also showed that acute cellular rejection could be reversed by high doses of steroids. By contrast, organ transplants from non-primates have had little success, graft survival being measured in hours or

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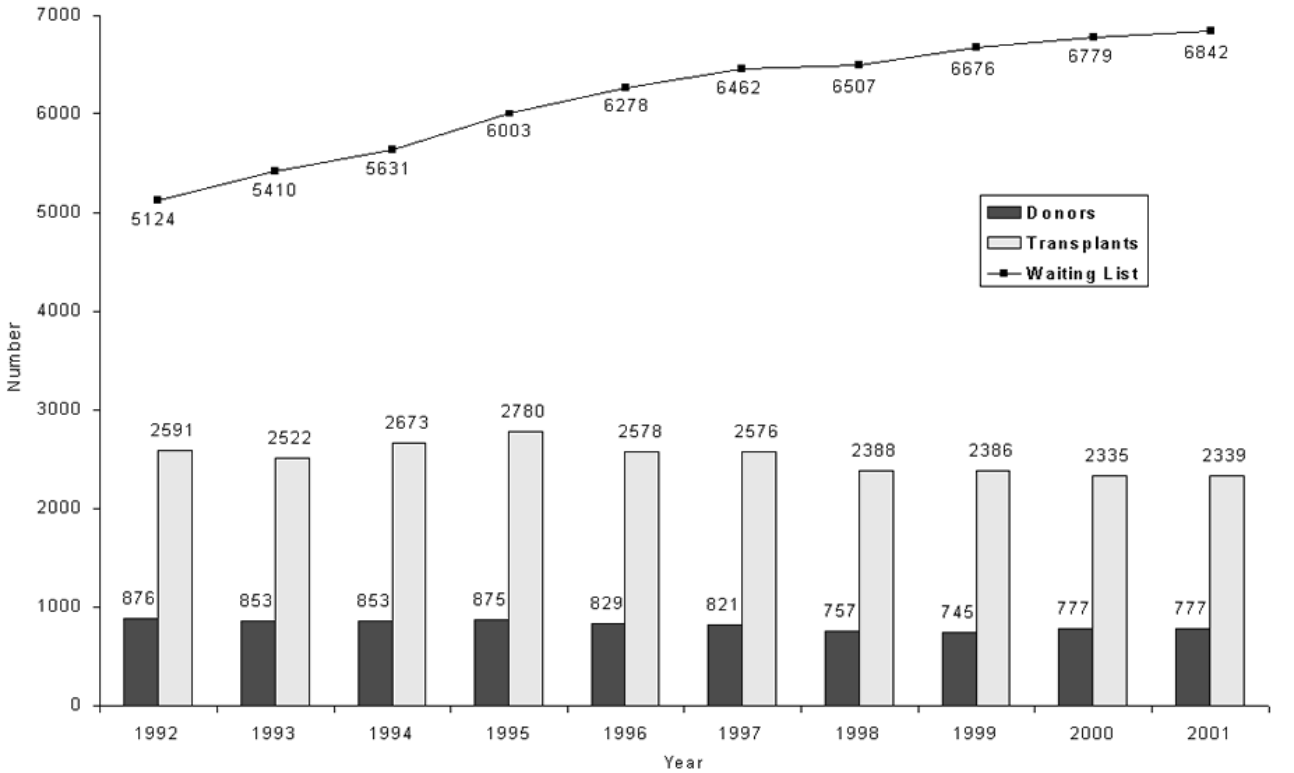

Figure 1 **Numbers of cadaveric donors and transplants in the UK and Republic of Ireland, 1991–2000 and patients on the waiting list at 31 December.** Statistics prepared by UK Transplant from the National Transplant Database maintained on behalf of transplant services in the UK and Republic of Ireland [www.uktransplant.nhs.uk] (Reproduced by permission)

minutes. With cells the picture is somewhat more promising: pig hepatocytes have been incorporated into extracorporeal liver assist devices, and non-primate tissue has also been used with varying success in treatment of diabetes[8] and parkinsonism[9].

IS THE PIG THE ANSWER?

While from numerous perspectives, non-human primates may be the preferred source of organs, they have several disadvantages: there is little experience in breeding these animals in captivity, and the cost of doing so would be large; they reproduce slowly; many of the relevant species are endangered (posing a conservation issue); and little work has been done on the genetic modification of such species. Most importantly, we should not discount the worries concerning inter-species transmission of infectious diseases: the more closely related the donor and recipient species, the more likely this is to arise. Finally, their very similarity to man poses ethical and moral dilemmas.

The present trend in research is towards use of the pig as donor. Transgenic pigs have been available for some years, and recent 'knock out' pigs[10] have been generated by nuclear transfer techniques. This means that we are now capable of removing or adding proteins to and from potential donor animals—a luxury clearly unavailable in allotransplantation. Added to that, the pig is bred for slaughter and its use should not generate the objections that arise with non-human primates. We already have extensive knowledge of husbandry conditions, and studies have shown the possibility of producing pigs with little or no infectious diseases[11]. Because of their phylogenetic distance from man, the likelihood of cross-species transmission of infections is less. We shall return to this last point later.

CAN XENOTRANSPLANTATION WORK?

Physiology

Two issues with pig organs are size and longevity. The heart or kidney of a young pig, when of suitable size for donation, may still have potential for rapid growth; whether this will happen we do not know. Also, the natural lifespan of a pig is only some fifteen years, and nothing is known about ageing in xenotransplanted organs. What of hormonal factors? That porcine insulin can achieve glucose homoeostasis in man has long been known, but not all porcine hormones are effective across the species barrier. For example, porcine renin does not cleave human angiotensin[12] and porcine erythropoietin does not stimulate human erythropoiesis. The liver produces over two thousand

proteins, and it is clear that many of these will have incompatible or absent function across the species barrier. This makes hepatic xenotransplantation less promising than that of other organs.

Recent work with organs from transgenic pigs has allowed long enough survival to study the *in vivo* physiological compatibility of porcine organs[13]. Porcine renal xenografts in a primate model were able to sustain plasma electrolyte homoeostasis for as long as the grafts survived[14], though not all the human kidney's functions were reproduced.

Another issue is temperature. The body temperature of pigs is roughly 39°C, whereas human body temperature is about 37°C[14]. The functional implications of this for the activity of porcine enzymes at this lower temperature remain unclear.

Hyperacute rejection

Until recently research focused on the phenomenon of hyperacute rejection. This, characterized histologically by the rapid onset of oedema, haemorrhage and vascular thrombosis, is caused by the presence of preformed antibodies, and occurs within minutes of transplantation.

The gal epitope

Xenoreactive natural antibodies (XNA) are similar to those produced naturally against blood group antigens. The epitope, which is the principal target of these antibodies, is the non-reducing trisaccharide group, galactosyl α-(1,3)-galactosyl β-1,4-*N*-acetyl glucosaminyl, commonly referred to as the gal epitope[15]. Man does not possess this epitope, because of the absence of the enzyme that generates it. Higher primates therefore recognize the gal epitope as 'non-self' and generate an immune response to it. Human beings are exposed to the antigen through the gut (the gal epitope is present on various microbes[16]) and generate anti-gal antibodies. XNA produce their effects primarily through activating complement, via natural killer (NK) cells[17] and by altering the phenotype of the endothelium. Research has so far focused on reducing the impact of XNA.

Prevention of the anti-gal response in recipients

One approach has been to deplete xenograft natural antibodies by means of affinity columns, extracorporeal perfusion of excised organs or plasmapheresis. Unfortunately, anti-gal returns to normal levels within a few days[18]. Attempts have been made to prevent the anti-gal response through the use of a α-gal toxin[19] to eliminate the plasma cells capable of producing this antibody. Results have been encouraging in the mouse model but its efficacy in higher models is still to be evaluated.

Complement

The main pathway through which the xenograft natural antibodies cause hyperacute rejection is the activation of complement and the consequent activation of the endothelial cells. Endothelial cells subsequently secrete various cytokines and platelet activating factor, and change from generating an anticoagulant milieu to a procoagulant one, causing thrombosis, haemorrhage and, quite rapidly, infarction.

One approach suggested to circumvent this is complement depletion with cobra venom factor and this has been shown to increase graft survival in rat-to-primate and pig-to-primate models[20]. An alternative strategy involves C1-inhibitor (C1-INH), the only physiological inhibitor of the first step in complement activation. Overexpression of this inhibitor has been shown to prevent hyperacute xenograft rejection *in vitro*[21] and *in vivo*[22]. Unfortunately, both techniques would deprive the body of the benefits of a functional complement system. Hence, the generation of donor organs that express complement regulators only locally has been pursued.

In view of the putative species incompatibility of porcine complement regulating proteins, pigs have been generated which express human complement regulators. *In vitro* work showed that expression of these molecules protects cells from complement-induced lysis[23]. Three human membrane-bound inhibitors of complement function have been expressed in pigs—CD55 (decay accelerating factor), CD46 (monocyte chemoattractant protein) and CD49. These have been shown to increase graft survival in pig-to-primate renal and cardiac transplants[24]. This work, using both transgenic organs and immunosuppression, led to graft survival of up to 78 days: 4 of the 9 animals survived for more than nine days with intact kidney grafts[25].

The Hanover group combined the two approaches outlined above, with encouraging results. Using a pig-to-primate kidney model with hDAF transgenic donor organs and postoperative immunosuppression, they found that episodes of acute vascular rejection were treated either with boluses of cyclophosphamide and steroids or with the same regimen supplemented by a three-day course of C1-INH. In all animals, one or more episodes of acute vascular rejection were observed. When, in 4 animals, C1-INH was added to the standard antirejection treatment regimen, acute vascular rejection was successfully reversed in six out of seven episodes[26].

Other approaches

The ultimate cause of xenograft destruction in hyperacute rejection is thrombosis. Thrombin inhibition has been shown to prolong graft survival. Research is currently directed towards the expression of anticoagulant molecules on the endothelial cell to produce a local anti-thrombotic

effect. Two groups have shown *in vitro* that the expression of these molecules on the cell surface can change the phenotype towards an anticoagulant one[27,28]. Our group is trying to develop an *in vivo* model for this approach. The difficulty is possibly compounded by species incompatibility: porcine tissue factor pathway inhibitor and porcine thrombomodulin may be ineffective in preventing the human coagulation cascade[24].

Glycosyltransferase transgenes

Miyagawa *et al*. have produced both mice and pigs transgenic for the human β-D-mannoside β-1,4-*N*-acetylglucosaminyltransferase. Overexpression of this gene reduced expression of the gal epitope with a consequent reduction of complement-mediated and NK-cell lysis by up to 40% in the transgenic group. Immunohistochemistry with normal human serum as a source of XNA confirmed a reduction in the level of antigenicity. This has also been demonstrated *in vivo* in a pig-to-cynomolgus-monkey cardiac model[29]. A similar approach has been used to produce pigs transgenic for α1,2 fucosyltransferase[30]. This approach decreases α-gal expression by about 70%. But will this be enough?

Knock-out pigs

The complete prevention of expression of the epitope will only be achieved by the production of knock-out animals. A homozygous mouse strain with disrupted α1,3 galactosyl transferase genes has been generated. The mice lack the ability to synthesize α-gal epitopes and are capable of producing low amounts of the natural anti-gal antibody, although repeated immunization with the gal epitope yields anti-gal titres and specificity comparable with those observed in man[31]. Knock-out pigs have recently been created[10,32], and data from these animals are keenly awaited.

The work summarized above suggests that hyperacute rejection can be eliminated or controlled through various techniques. But what about immunological processes that occur days and weeks after the transplantation?

Acute humoral xenograft rejection

The next barrier to be surmounted is acute humoral xenograft rejection (AHXR), also known as delayed xenograft rejection. The main histopathological features of AHXR are endothelial swelling or disruption, vascular thrombosis with blood extravasation and interstitial oedema[33]. This normally arises within 24 hours of transplantation and progresses to destroy the graft over the next few days. The initial response is mediated by IgM, principally but not exclusively specific for the gal epitope, with a subsequent increase in IgG levels[34]. The presence of these xenograft natural antibodies alone leads to the production of a procoagulant state and eventually to disseminated intravascular coagulation[35]. These complications generally develop despite the best available measures for depletion of xenograft natural antibodies, inhibition of complement activation and suppression of T-cell and B-cell mediated immune responses. The mechanisms underlying the disseminated intravascular coagulation and thrombotic microangiopathy associated with delayed xenotransplant rejection remain unclear. AHXR is the least well understood of the early phases of xenograft rejection.

Preventing acute humoral xenograft rejection

Approaches to prevention of AHXR have included depletion of anti-gal antibodies through the use of an immunoaffinity column for extracorporeal immunoadsorption of plasma[36]. Robson *et al*. have shown that use of synthetic low-molecular-weight thrombin inhibitor can prolong survival, enhance function of the explanted organ, and improve histological features at the time of rejection[37]. Cooper's group in Boston have used soluble synthetic gal sugars or bovine serum albumin conjugated to multiple gal molecules to deplete the primate bloodstream of the anti-gal antibodies[38]. However, as yet no definitive therapeutic intervention for AHXR has emerged.

Cellular rejection

Thus far we have discussed the consequences for the xenograft of its interactions with preformed antibodies. Although products of the adaptive immune response, xenograft natural antibodies result from stimulation by cross-reactive antigens that happen to be present on the flora of the recipient. In addition, we have considered how the innate immune system, with its limited set of predefined specificities and responses, which do not change (adapt) in

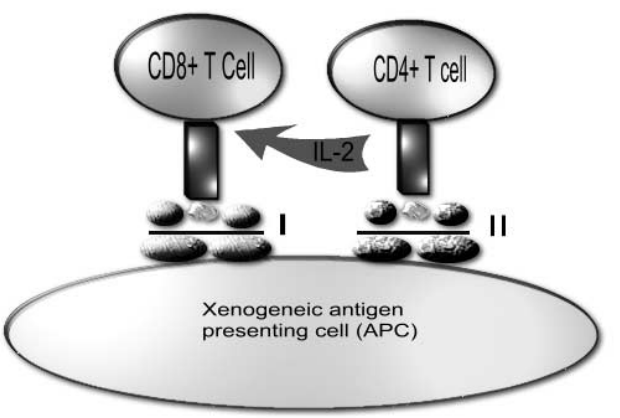

Figure 2 **Direct xenorecognition.** Xenogeneic APC presents peptide to the recipient $CD4^+$ T cell via the xenogeneic MHC class II molecules. This results in the production of interleukin 2 (IL-2) by the $CD4^+$ T cell. IL-2 acts on the $CD8^+$ T cell which itself recognizes xenogeneic peptide presented via the MHC class I on the xenogeneic APC

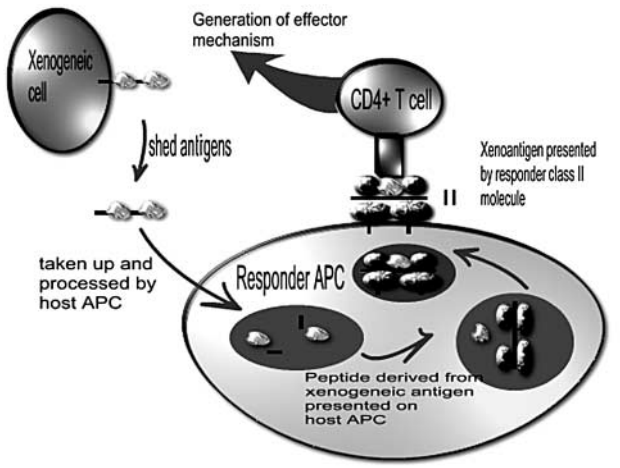

Figure 3 **Indirect xenorecognition.** Shed antigens from the xenogeneic cell are taken up by the responder antigen presenting cell (APC) to be presented to the $CD4^+$ T cell via MHC class II molecule, which results in generation of the effector mechanism

response to antigen exposure, has the potential to damage xenogeneic tissue. However, xenografts also interact with the adaptive immune system and stimulate their own specific immune response. In alloresponses, the immune system is able to recognize allogeneic MHC molecules directly by engaging T cell receptors with the MHC molecules (Figure 2). The direct xenoresponse is probably of comparable magnitude to the direct alloresponse. Thus it would require at least comparable levels of suppression[39].

However, it is known in allotransplantation that allogeneic MHC molecules, like any proteins, can be phagocytosed, broken down into peptides, presented on recipient type MHC molecules and generate an immune response. This is referred to as 'indirect' allorecognition (Figure 3). This also occurs in xenotransplantation. However, there are many more peptide differences between different species than between different members of the same species. Hence, the potential for indirect *xeno*geneic responses is much greater than for indirect *allo*geneic responses. This might need more immunosuppression than required in an alloresponse—perhaps more than is clinically acceptable. Given that such indirect responses appear to be increasingly important with time in allotransplantation, this may prove to be a major stumbling block in xenotransplantation.

One possible answer to this is to attempt to induce donor-specific non-responsiveness, or 'immunological tolerance'. Much effort has been expended in trying to generate tolerance by haematopoietic chimerism. The term mixed chimerism refers to the coexistence of donor and recipient haematopoietic cells. The development of a protocol to generate a stable state of mixed chimerism without subjecting the recipient to a toxic myeloablative regimen has been the focus of much research. Initial protocols involved the non-specific elimination with antibodies of pre-existing mature donor-reactive T cells and NK cells. More recently, models have been developed in which it appears possible to inactivate and eliminate only donor specific T cells while leaving the remaining T cell repertoire essentially intact, by use of co-stimulatory blocking reagents to induce peripheral clonal deletion after bone marrow transplantation. After the peripheral immune system has been eliminated, donor stem cells are infused intravenously, and engraft in the bone marrow compartment of the recipient where they coexist with recipient stem cells and give rise to cells of all haematopoietic lineages. Within the thymus, T cells deemed to be potentially self-reactive are deleted. This process is at least in part mediated by cells seeded from haematopoietic progenitor cells originating from the bone marrow. In mixed chimeras, haematopoietic cells from both the recipient and the donor locate to the thymus and hence mediate the elimination of both host-reactive and donor-reactive T cells.

The induction of mixed haematopoietic chimerism has been shown to lead to stable tolerance in allogeneic and closely related xenogeneic combinations[40]. Early data suggest that this may also be possible in a highly disparate pig-to-mouse model[41].

Cosimi *et al*. have induced tolerance to allotransplanted kidneys in monkeys by use of mixed haematopoietic chimerism[42]. The tolerance persists even after cessation of immunosuppressive therapy. This work is not only applicable to xenotransplantation but could also be of great benefit in allotransplantation where conventional immunosuppression leads to complications such as infection and malignancy. The small numbers of patients who have been given marrow and a kidney from the same donor have shown robust tolerance[14].

Another approach to inducing T cell tolerance is by transplanting pig thymic tissue into the recipient primate. This approach has been successful in the pig-to-mouse model[43].

The main concern regarding this approach is the risk of graft-versus-host disease—an attack by donor immune cells on the recipient's tissues. However, it remains one of the most exciting areas of research activity.

MICROBIOLOGICAL RISK

The risk of transmission of infectious agents across the species barrier is a major anxiety about this whole approach of xenotransplantation. Many such agents can be eliminated from the pig herd through scrupulous husbandry methods. Such methods include the sterilization of both feed and drinking water and the elimination of all mammalian

protein from the feed to prevent prion infection. Unfortunately, this does not eliminate the risk of transmitting viruses whose DNA is integrated into the nucleus of transplanted cells, such as porcine endogenous retroviruses (PERVs).

There is a long history of using porcine valves and insulin in the treatment of disease without generating any infectious complications. However, this represents experience in a cell-free situation. *In vitro* studies suggest that PERV can, in fact, infect human cells[44]. However, *in vivo* studies of 160 patients who have been exposed to living porcine cells or tissue have shown no evidence of PERV transmission; thus such transfer must be at least a rare event[45]. *In vivo* organ transplants in mice can generate transmission of PERVs. But whole-organ transplants, in patients who are likely to be immunosuppressed, have yet to be assessed.

The risk must be assessed both on an individual basis (the risk of infection versus the benefit of a viable organ) and for the public in general—spread of a new pathogen throughout the population. We have already witnessed the disaster over the transmission of bovine spongiform encephalopathy in humans, and HIV is thought to have originated in monkeys.

It is impossible to prove a negative—that a novel pathogen could never be transferred from pigs to man as a result of xenotransplantation. And any clinical development of xenotransplantation must be accompanied by rigorous and lifelong microbiological monitoring of recipients.

CONCLUSIONS

Although headway has been made in overcoming the initial hurdle of hyperacute rejection through modulation of the local immune response, we now have to deal with the other aspects of the immune system. Current work is mainly directed towards the production of transgenic and knock-out pigs. Alongside this is the exciting possibility of inducing tolerance through mixed haematopoietic chimerism.

Anxiety over the risk of infection may be diminished by data confirming the lack of transmission in well-controlled experiments, or by the identification of pig strains incapable of transmitting PERVs. However, there will always be concerns that experiments have failed to exclude transmission of pathogens with a very long lag time and the transmission of pathogens as yet unknown. Xenotransplantation does offer a way to meet the shortfall in organs available for transplantation, though the results may be inferior to those of allotransplantation: the greater immunological incompatibility, with need for stronger immunotherapy, could mean lower life expectancy and shorter graft survival. Against all these issues, xenotransplantation offers the potential to make available functional solid organs, on tap, to patients who at present have little or no chance of receiving a transplant.

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