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Clinical Trials and the Projected Future of Liver Xenotransplantation

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Abstract. The trial and error of the pioneering xenotransplant trials over the past three decades has defined the limitation of the species used. Success was tantalizingly close with the chimpanzee, baboon, and other primates. The use of more disparate species has been frustrated by the xenoantibody barrier. Future attempts at clinical xenotransplantation will be hampered by the consideration of the species of animals and the nature of the organs to be transplanted. On one hand, primate donors have the advantage of genetic similarity (and therefore potential compatibility) and less risk of immunologic loss. On the other hand, pig donors are more easily raised, are not sentient animals, and may be less likely to harbor transmissible disease. It is recognized that the success of xenotransplantation may very with different organs. Because it is relatively resistant to antibody-mediated rejection, the liver is the organ for which there is the greatest chance of long-term success. Consideration of using xenotransplants on a temporary basis, or as a "bridge" to permanent human transplantation, may allow clinical trials utilizing hearts or kidney xenografts. Issues on metabolic compatibility and infection risks cannot be accurately determined until routine success in clinical xenotransplantation occurs. Based on a limited experience, the conventional approaches to allotransplantation are unlikely to be successful in xenotransplantation. The avoidance of immediate xenograft destruction by hyperacute rejection, achieved using transgenic animals bearing human complement regulatory proteins or modulating the antigenic target on the donor organ, is the first step to successful xenotransplantation. The ability to achieve tolerance by establishing a state of bone marrow chimerism is the key to overcoming the long-term immunologic insults and avoiding the necessarily high doses of nonspecific immunosuppression that would otherwise be required and associated with a high risk of infectious complications. Xenotransplantation faces criticism that is strongly reminiscent of that leveled against human-to-human transplantation during the late 1960s and early 1970s. Yet with persistence, the field of human-to-human transplantation has proved highly successful. This success was the result of a stepwise increase in our understanding of the biology of rejection, improvements in drug management, and experience. It is possible that xenotransplantation may not be universally successful until further technologic advances occur; yet cautious exploration of xenotransplantation appears warranted to identify those areas that require further study.

Organ transplantation has become an effective means for treating patients with end-stage organ failure. This achievement can be largely attributed to the development of advanced technical skills and the availability of new immunosuppressive agents, such as cyclosporine and tacrolimus. Patients undergoing organ transplantation experience excellent likelihood of survival with good quality of life. This success has increased the demand for organ transplantation, and thus an estimated 75,000 Americans suffering from end-stage organ failure currently await or receive a life-saving organ transplantation each year. However, almost 10% of patients awaiting transplantation die because of the lack of availability of human organs each year [1]. Despite heightened public awareness to address the need for organ donation, there appears to be little prospect of increasing donation to meet the current needs.

It is widely anticipated that the only means of addressing this shortage is by the development of artificial organs or utilizing organs from species other than humans (or both) and has inspired concerted research efforts in the field of xenotransplantation. Although artificial organs may become reality with future developments, their ability to replace complex organs, such as the liver, is probably years away. Without the benefit of artificial support of patients with liver, heart, or lung failure, transplantation of functioning organs is the only alternative to death. Thus xenotransplantation represents the most promising alternative to the current organ shortage, especially with an increased understanding of rejection mechanisms of both allografts (organs transplanted across the same species) and xenografts (organs transplanted across different species).

Recent developments in understanding the barriers to successful xenotransplantation have led to the application of novel drugs to manipulate the immune system. Along with the concept of microchimerism applied to xenotransplantation and bolstered by the utilization of genetically modified donor animals, application of new bioreagents to enhance microchimerism holds promise for future attempts at clinical xenotransplantation.

Xenograft Immunity

When organs are transplanted across closely related species (e.g., baboon to human), such xenotransplants are referred to as *concordant*. Organs implanted across widely divergent species (e.g., pig to human) are termed *discordant*. These two terms also characterize the extent of difficulty that exists in achieving successful organ transplantation across these barriers [2]. It is much easier to achieve xenograft acceptance across concordant combinations than with discordant combinations. A number of animal

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models have been developed for both discordant and concordant xenotransplantation, but long-term successes have been limited, for the most part, to concordant combinations. The lack of high-titer preformed xenoantibodies in the concordant combinations has avoided the almost immediate destruction of discordant xenografts, which has been difficult to prevent or treat. Nevertheless, even with the discordant combinations significant prolongation of xenograft survival has been reported utilizing antibody depletion [3] or removal of complement [4, 5]. These animal models of xenotransplantation have been used to learn more about the types of rejection and the drugs that can be utilized to reduce the threat of rejection. Combinations of drugs, each targeted at different limbs of the rejection pathway, have been shown to prolong xenograft survival significantly [6].

Across concordant barriers a major concern is cell-mediated immunity [8], a response similar to that encountered after allotransplantation and therefore somewhat controllable by the use of immunosuppressive drugs. In fact, in rodent models of xenotransplantation, indefinite survival of concordant liver xenografts using standard doses of immunosuppressive agents has been reported [6]. In addition, historic attempts of primate-to-human transplants revealed that prolonged concordant xenograft function may be achievable [8]. What then is limiting the pursuit of concordant xenotransplantation? Several issues have been raised against utilizing primates as donors, including "humanization of primates," limited availability, donor size incongruity, and the theoretic risk of transmitting infectious agents [9, 10]. These concerns have prompted a quest for alternative sources of animals for clinical xenotransplantation. It is widely anticipated that a species discordant with humans (e.g., pigs) may offer a source of organs for xenotransplantation.

Pigs are available in sufficient quantities, have anatomy and physiology similar to that of humans, and can be bred under conditions where they can be genetically modified. These factors have prompted the consideration of this species as a source for clinical xenotransplantation, although organs from discordant species are confronted with a formidable barrier: hyperacute rejection mediated by naturally occurring antibodies (preformed xenoantibodies) present in the recipient [11]. Because of the difficulty of controlling this form of rejection, novel approaches are required to overcome this barrier to successful discordant xenotransplantation. In addition, other unknown factors, including infection risks and metabolic compatibility, have not been addressed.

One strategy to prevent hyperacute rejection is removal of preformed antibodies from the recipient's blood prior to transplantation by plasmapheresis (nonspecific antibody removal) [12] or immunoabsorption (specific xenoantibody removal) [13]. Although this approach has been utilized in ABO blood type-incompatible human-to-human transplants with some success [14], its application to xenotransplantation has been limited owing to the rapidity with which the preformed xenoantibodies are produced, resulting in the restoration of xenoantibody levels leading to hyperacute rejection. To date, little success has been achieved in the prevention of xenoantibody synthesis using cytotoxic drugs, and alternative approaches must therefore be taken.

Preformed xenoantibodies play a vital role in mediating hyperacute rejection, but they are not the effector molecules responsible for the observed damage in discordant xenografts. Antibody binding to the xenograft results in activation of complement, most of which is manufactured in the recipient's liver [15]. These proteins exist in an inactive form but are activated when antibody binds to the target cells, resulting in damage to the cell. The importance of complement in the pathophysiology of antibody-mediated rejection is shown in studies in which complement is depleted. Cobra venom depletes the C3 and C5 components, resulting in paralysis of the complement system. Adachi and coworkers were able to obtain prolongation of discordant xenograft survival with the addition of cobra venom factor along with cyclosporine and an antiplatelet agent [4]. Other evidence of the importance of C5 in the process of xenograft hyperacute rejection is the ability of a sesquiterpene compound with anticomplement activity to prolong xenograft survival. K76 is though to block the C5 step of complement activation, and it accelerates the degradation of C5b [16]. Administration of 200 mg/kg to rats undergoing heterotopic guinea pig heart transplantation (discordant combination) resulted in marked prolongation of survival: from 8 minutes to more than 8 hours [17].

Cells express naturally occurring proteins (regulators of complement activation) on their surface that help modulate the effects of various complement-activated components. The molecules are thought to provide an intrinsic mechanism to limit the amplification of complement activation. Homologous restriction factor (CD59) and decay accelerating factor (DAF: CD55) are two proteins that have been described as mediators of complement activation. CD59 is thought to act by inhibiting the insertion of C9 into MACs, thereby aborting the terminal attack sequence of complement activation [18]. DAF limits the generation of classic and alternative complement pathway convertases [19]. The importance of these modulators have been demonstrated by experiments that have enhanced expression of these proteins by gene transfection [20]. The activities of these complement modulators are though to be species-specific and help to explain the phenomenon of "homologous species restriction" [21]. This phenomenon is most easily seen when addition of homologous complement to susceptible target cells does not effectively cause lysis, whereas addition of heterologous complement leads to effective cell lysis. Thus following pig-to-human xenotransplantation activated human complement is not inactivated by the complement-inhibitory proteins found on pig cells.

Cell damage occurs by activation of other inflammatory pathways as well. Reactive oxygen metabolites, prostaglandins, and cytokines can be generated by the degradation products of complement activation. Polymorphonuclear leucocytes and macrophages are attracted to the site of inflammation because of the presence of the C5a fragment, which results in the release of lysosomal enzymes and resultant cell damage. C3b enhances adhesion of these cells to damaged cells and enhances binding of platelets, which may lead to degranulation and release of vasoactive substances, such as serotonin and histamine, both of which increase vascular permeability.

Thrombosis of the microvasculature is enhanced by the loss of membrane-associated heparan sulfate from the endothelial cell [22]. Heparan sulfate proteoglycan is present in the endothelial cell layer of normal vessels and helps maintain a local anticoagulant environment by activation of antithrombin III, an inhibitor of thrombin generation. The release of tissue factors from injured cells promotes thrombosis.

Previous Clinical Xenotransplantation Efforts

The shortage of neonatal hearts for treatment of severe cardiac anomalies prompted Bailey et al. at Loma Linda University to transplant a baboon heart into a human neonate with a hypoplastic left ventricle in 1983 [23]. Although the immunosuppressive regimen included cyclosporine, the heart was eventually rejected by antibody-mediated mechanisms 20 days after transplantation. No further attempts at xenotransplantation were done for almost a decade. Within an 8-month period in 1992–1993, three attempts at transplantation of liver xenografts were performed in the United States. The liver appeared to be a logical starting point because of the known relative resistance of the liver to antibody-mediated rejection [24] as compared to the heart and kidney. Two baboon-to-human liver transplants were performed at the University of Pittsburgh [8, 17] and one pig-to-human liver transplant at Cedars–Sinai Medical Center in Los Angeles [25].

Pig-to-Human Liver Xenotransplantation

The recipient of a pig liver, a 26-year-old woman with accelerated liver failure due to autoimmune hepatitis, progressed to grade 3–4 coma. The patient was treated to remove preformed anti-pig antibodies using a combination of plasmapheresis and specific antibody removal by passing her blood through a set of pig kidneys. The immunosuppression was based on cyclosporine, cyclophosphamide, prostaglandin E1, and azathioprine. The liver was placed in a heterotopic position. The liver appeared to function for 20 hours as demonstrated by an initial decrease in serum lactate and presence of bile. However, the ammonia level did not fall, and by 20 hours after transplantation several clinical parameters worsened; the patient died 26 hours after transplantation from brain death.

The liver appeared to have undergone extensive infarction from hyperacute rejection despite lowering the antibody titer using plasmapheresis and pig kidney perfusion. Titers of xenoantibodies had risen rapidly to pretransplant levels by the time of her death. These findings are consistent with a rapid reaccumulation of xenoreactive antibodies after inital immunoabsorption [12].

Baboon-to-Human Liver Xenotransplantation

The clinical courses of the two baboon-to-human liver transplant patients are summarized. The first patient was a 35-year-old man who had previously undergone splenectomy following a motor vehicle accident. He was found to be human immunodeficiency virus (HIV)-positive. The second patient was a 62-year-old man. In both patients the principal complications of chronic hepatitis B were poorly controlled edema, fatigue, ascites, encephalopathy, and gastrointestinal bleeding. Because of the nature of their liver disease they were not considered candidates for human liver transplantation, but because of the severity of complications xenotransplantation was considered an experimental option.

Both patients experienced uneventful intraoperative courses following reperfusion of the transplanted baboon liver, and the clinical impression was one of immediate function of both xenografts. Immunosuppression utilized a combination of tacrolimus, steroids, prostaglandin E1, and cyclophosphamide. The first patients awoke promptly after transplantation and was extubated after 17 hours. The liver function tests returned toward normal levels by the second posttransplant week, and the transaminases returned to normal within the first week. The second patient never regained a level of consciousness that permitted weaning from the ventilator. In addition, the quality of the liver function in the second patient was suboptimal, with persistent hyperbilirubinemia during the entire postoperative course. Nevertheless, in both patients there was evidence of an adequately functioning liver mass, that is, normalization of the coagulation with normal prothrombin time, correction of the hyperammonemia, normal arterial ketone body ratio (a manifestation of hepatic energy stores), and clearance of serum lactate.

A number of liver biopsies were performed throughout the posttransplant course (73). The earliest postperfusion biopsies (4 hours after perfusion) revealed mild antibody-mediated insult. Immunofluorescence revealed binding of immunoglobulin with complement deposition, but no endothelial injury or platelet aggregation was seen. Polymorphonuclear cell infiltration and natural killer (NK) cells were seen in these early biopsy specimens. In the first patient a biopsy specimen obtained on day 12 revealed Kupffer cell hypertrophy, mild centrilobular hepatocyte swelling, and cholestasis with a mild mononuclear portal and perivenular infiltrate. This infiltrate was predominantly T cells and was consistent with mild cellular rejection with minimal antibody injury. Later biopsy specimens were remarkably free of rejection, either antibody or cellular. Mild cholestasis was noted in some of these later specimens. In the first patient the final antemortum biopsy was taken on the 64th posttransplant day and revealed obvious bile infarcts, with mural necrosis of segments of the septal bile ducts. It is conceivable that the unrecognized biliary stasis syndrome was an atypical manifestation of rejection. In the second patient a biopsy specimen obtained on the fourth posttransplant day revealed indirect evidence of antibody-mediated rejection, and a subsequent splenectomy was performed. Although both patients eventually died from infectious causes the baboon livers were able to sustain life for 70 and 26 days, respectively.

New Concepts

Until recently, an organ transplant (allograft or xenograft) was considered a defenseless entity vulnerable to rejection by the recipient immune system. In 1992 Starzl and colleagues demonstrated the ubiquitous presence of donor bone marrow-derived cells in human transplant recipients bearing functioning allografts for as long as 30 years after transplantation and termed this phenomenon *microchimerism* [26, 27]. It is postulated that the interaction of two coexisting donor and recipient cell populations leads to a mutual down-regulation of both recipient and donor immune systems (i.e., specific inactivation of the recipient immune system to donor tissue and vice versa). Although the concept of down-regulating the recipient immune response (to prevent rejection) is intuitive, the requirement for down-regulating the donor immune response transferred by the transplanted organ may not be as obvious. It has long been recognized that an activated donor immune response can lead to clinical syndromes, such as is seen after bone marrow transplantation, with the development of graft-versus-host disease and profound immune suppression leading to infectious complications. Verification of these findings by extensive laboratory research in animals has led several groups to undertake clinical trials to enhance microchi-

merism using donor bone marrow and hopefully promote full acceptance of transplanted human organ (i.e., tolerance). It is generally believed that higher levels of microchimerism are associated with a greater likelihood of achieving tolerance. Recently a number of novel growth factors that enhance the growth of bone marrow cells have been utilized in animal models of transplantation and were demonstrated to augment the level of microchimerism [28, 29]. Both species-specific and species-nonspecific growth factors have been used, and the availability of both classes of growth factors is important, especially in the realm of allo- and xenotransplantation.

How can we apply the concept of microchimerism to xenotransplantation? If stable microchimerism of xenogeneic donor bone marrow can be accomplished in a human, subsequent transplantation of a xenograft is more likely to be accepted. In addition, there is evidence that a radiosensitive bone marrow-derived population in the liver is important to the acceptance of that liver after transplantation [30]. Lethal irradiation followed by xenogeneic bone marrow reconstitution, leading to the development of a chimeric donor liver xenograft, may lead to diminished immunogenicity and decreased severity of rejection following transplantation. This point was suggested in rodent studies using irradiated mouse donors reconstituted with rat bone marrow in which the survival of the chimeric xenograft was significantly enhanced [31].

Humans have preformed antibodies to pig tissue, which would lead to hyperacute rejection of the transplanted xenograft. A unique approach has been taken that entails expressing human complement inhibitory proteins on pig cells; this has been achieved by generating genetically modified pigs that carry the genes for human complement inhibitory proteins, either human CD59 or DAF [32, 33]. Organs obtained from these transgenic pigs enjoy prolonged survival when transplanted across discordant barriers into primates, suggesting that the human complement-inhibitory proteins inserted genetically into the pig organ can overcome hyperacute rejection [34]. This was shown in the marked prolongation of xenograft survival in heterotopically transplanted transgenic pig hearts into unmodified baboons.

Future Clinical Trials of Xenotransplantation

It is impossible to overcome hyperacute organ rejection, with the establishment of microchimerism and tolerance, using the current approaches to xenotransplantation. However, based on two seemingly independent strategies (i.e., donor bone marrow augmentation and transgenic donors), one hopes to approach the goal of successful discordant xenotransplantation in stepwise advances by first overcoming the hyperacute rejection barrier and then achieving stable chimerism. Only after these goals are achieved will clinical xenotransplant trials be feasible.

We anticipate that this goal will be achieved in a stepwise manner. The first step requires reliable establishment of chimeric transgenic pigs using xenogeneic human hematopoietic stem cell infusion with the use of specific or nonspecific hematopoietic growth factors. Using large-animal experimental models, primate bone marrow (human [28] and baboon [35]) has been administered to discordant recipients, many of whom subsequently developed evidence for donor primate microchimerism. Based on previous studies, it has been necessary to infuse primate bone marrow into fetuses or neonates as their functionally immature immune system is more amenable to manipulation than that in their adult counterparts. In addition, the number of donor cells in these recipients can be enhanced by utilizing hematopoietic growth factors, resulting in expansion of primate bone marrow-derived cells.

It has been previously shown that successful engraftment of allogeneic bone marrow leads to a proportionate replacement of bone marrow-derived cells in the tissue and organs of these recipients. This is the concept on which we base the utilization of organs from these animals for transplantation into primates, as we have shown that a bone marrow-derived, radiosensitive population in the liver is a primary target for rejection. The rationale for utilizing transgenic pigs as the recipient of primate bone marrow infusion is that their subsequent use as the donor (after confirmation of primate chimerism) can withstand the early onslaught of hyperacute rejection following xenotransplantation.

The last step in the prevention of discordant xenograft rejection will be the utilization of donor bone marrow as an adjunct to developing tolerance. Therefore donor pig bone marrow must be given to primates prior to organ transplantation, and survival of chimeric-transgenic xenografts can be utilized as the endpoint for measuring success. If this approach is determined to be feasible clinical trials can be considered.

Conclusions

Myths describing the use of animal organs to save humans have been recorded since antiquity. The hypothesis proposed here represents a logical step in xenotransplantation research. It builds on the area of allotransplantation, immunology, molecular biology, pharmacology, biochemistry, and bioengineering. Although artificial organ development remains a realistic goal, it is impractical for immediate needs and for complex organs such as the liver. Thousands of patients will die waiting for a donor organ unless practical alternatives are developed. We believe that this xenotransplant project will yield important new scientific information about the immune system and organ rejection, which will be invaluable regardless of whether the donor organ is animal or human.

Résumé

C'est grâce à un certain nombre d'essais par tâtonnement qu'on est arrivé à définir les espèces animaux utilisables pour la xénotransplantation. Les premiers résultats enregistrés avec le chimpanzé, le babouin et d'autres primates ont été très encourageants. L'utilisation d'autres animaux, les espèces plus disparates, ont été, par contre, des échecs en raison des problèmes immunologiques. Les essais de xénotransplantation cliniques à l'avenir seront limités selon le choix des espèces d'animaux ainsi que par le type d'organes à greffer. Si les primates ont l'avantage d'avoir une similarité génétique (et par conséquence une comptabilité potentielle) et les échecs au plan immunologique sont moindres, le cochon présente d'autres avantages: on les élève plus facilement, leur sensibilité est restreinte et ils sont moins susceptibles d'abriter des maladies transmissibles. Le succès de la xénotransplantation diffère selon l'organe transplanté. En raison d'une résistance importante au rejet dû aux anticorps, le foie est un organe de choix qui a le plus de chances de succès à long terme. Utiliser la xénotransplantation comme greffe temporaire, une «greffe relais», dependant, en attendant de pouvoir faire une

greffe permanente, pourrait être la base du départ pour permettre de continuer les essais cliniques en ce qui concerne les xénogreffes du coeur ou du rein. On ne pourrait pas répondre à la question de compatibilité métabolique ou de risques d'infection avec précision avant que la xénotransplantation ne s'effectue avec succès de façon régulière. Basé sur une expérience limitée, les approches conventionnelles propres à l'allotransplantation n'amènent pas forcément aux succès dans le domaine de la xénotransplantation. Eviter le rejet hyper-aigu avec destruction immédiate de la xénogreffe, soit en utilisant des animaux transgéniques ayant des protéines régulatrices du complément humain, soit en modulant le cible antigénique sur l'organe donneur, représente le premier étape vers la réussite de la xénotransplantation. La possibilité d'établir une tolérance en créant un état de chimérisme de la moelle osseuse paraît être la clé pour vaincre les problèmes immunologiques à long terme et éviter les inconvénients d'une immunosuppression non-spécifique en ce qui concerne les complications infectieuses. La xénotransplantation n'est pas exempte de criticismes similaires à ceux qu'on a entendus concernant la transplantation d'homme à homme à la fin des années 1960 et début 1970. Pourtant, la persévérance dans la transplantation humaine a été couronnée de succès. Ce succès a été le résultat d'une meilleure compréhension progressive de la biologie du rejet, des améliorations dans la manipulation des médicaments et de l'expérience. Il est possible que la xénotransplantation ne soit pas un succès universel avant de voir venir d'autres progrès technologiques, mais continuer une exploration prudente semble justifiée pour définir les domaines d'exploitation qui méritent d'être poursuivis.

Resumen

Los ensayos pioneros de xenotrasplantes practicados en la pasadas tres décadas han definido las limitaciones de las diferentes especies que han sido utilizados. El éxito ha sido atormentadoramente cercano con el chimpancé, el mandril y otros primates. El uso de otras especies más disímiles se han frustrado por la barrera de los xenoanticuerpos. Los intentos futuros de xenotrasplante tendrán los impedimentos correspondientes a las especies de los animales y a la naturaleza de los órganos a ser trasplantados. Por una parte, los primates donantes tienen la ventaja de la similitud genética (y por consiguiente compatibilidad potencial) y menor riesgo de pérdida inmunológica. Por otra parte, los cerdos donantes son de más fácil crianza, no despiertan sentimientos afectuosos y probablemente son de menor riesgo en canto a enfermedades transmisibles. Se reconoce que el éxito del xenotrasplante varía según los diferentes órganos. Por su resistencia relativa al rechazo mediado por anticuerpos, el órgano aparece como el de mayor probabilidad de éxito a largo plazo. Sin embargo, la consideración de utilizar xenotrasplante en forma temporal, o a la manera de "puente" hasta un trasplante definitivo, puede hacer posible la realización de ensayos clínicos con xenotrasplantes de corazón o de riñón. Asuntos tales como la compatibilidad metabólica y los riesgos de infección no podrán ser determinados con certeza hasta cuando se logre éxito con el xenotrasplante clínico. Con base en una experiencia limitada, se piensa que los aproches convencionales del alotrasplante no serán exitosos en el xenotrasplante. El primer paso hacia el éxito con el xenotrasplante consiste en evitar la destrucción inmediata del xenoinjerto por el rechazo hiperagudo, lo cual es posible usando animales transgénicos portadores de protéinas reguladoras del complemento o por la modulación del blanco antigénico en el órgano donante. La capacidad para lograr tolerancia mediante el establecimiento de un estado de quimerismo de la médula ósea será la clave para superar los insultos inmunológicos a largo plazo y evitar las necesariamente elevadas dosis de inmunosupresión no específica que de otra manera se requieren y que se asocian con un alto riesgo de complicaciones infecciosas. El xenotrasplante se enfrenta a críticas que recuerdan aquellas elevadas ante los trasplantes de humano a humano por los años 1960s y 1970s. Y sin embargo, con persistencia, el avance con este tipo de trasplante ha probado ser altamente exitoso. Tal éxito ha sido el resultado de un incremento escalonado del rechazo biológico, de progresos en el manejo de las drogas y de la experiencia. Es posible que el xenotrasplante no llegue a ser universalmente exitoso hasta alcanzar mayores avances tecnológicos, pero se justifica su cautelosa exploración, con miras a identificar aquellas áreas para las cuales se requieran mayor estudio.

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