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Infectious Disease Issues in Xenotransplantation

ROUMIANA S. BONEVA,* THOMAS M. FOLKS, AND LOUISA E. CHAPMAN

HIV/AIDS and Retrovirology Branch, Division of AIDS, STD and TB Laboratory Research, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia 30333

INTRODUCTION

A number of diseases in humans can cause end-stage failure of an organ, necessitating transplantation. The transplantation of living organs, tissues, or cells from one human to another or between individuals within the same species is called allotransplantation. Currently, allotransplantation includes organs (e.g., kidneys, heart, lungs, liver, and pancreas) and tissues (e.g., bone marrow, bone, heart valves, eye tissue, and skin). The demand for human organs and tissues for transplantation far exceeds the supply both in the United States (U.S. Department of Health and Human Services, Transplant data 1988–1997; http://www.unos.org) and worldwide. For every person who benefits from an organ transplant, there are an estimated 5 to 10 other potential recipients for whom human organs are not available. In some cases, artificial organs can be used as a substitute for the failing organ, at least temporarily. Hemodialysis for renal failure and mechanical pumps to assist or even replace a failing left ventricle pending heart transplantation are examples of such artificial alternatives to organ transplantation.

Xenotransplantation, the transplantation of living organs, tissues, or cells from one species to another (e.g., from nonhuman primates [NHPs] or pigs to humans), is viewed as a potential solution to the existing shortage of human organs for transplantation. Xenotransplantation in humans is defined by the U.S. Public Health Service as any procedure that involves the transplantation, implantation, or infusion into a human recipient of either (i) live cells, tissues, or organs from a nonhuman animal source or (ii) human body fluids, cells, tissues or organs that have had ex vivo contact with live nonhuman animal cells, tissues, or organs. Xenotransplantation products are defined as live cells, tissues, or organs used in xenotransplantation. The term "xenotransplantation products" is now used in Public Health Service documents instead of the previously used term "xenograft" (U.S. Department of Health and Human Services, Guideline on infectious disease issues in xenotransplantation; http://www.fda.gov/cber/guidelines.htm).

Most xenotransplantation activity is still in the preclinical stage, involving basic research on technical developments and immunological response and research on infectious risk. Very few applications have proceeded to human experimentation and actual clinical trials (11).

Xenotransplantation has several potential advantages over allotransplantation (97): (i) it offers a virtually unlimited source of organs; (ii) scheduling is not dependent on the unpredictable availability of a donor human organ, allowing for both advance planning and the intentionally timed harvesting of an organ for immediate transplantation as well as immunologic pretreatment of the recipient if necessary; (iii) the risk of many infections can be reduced by allowing lifelong control of exposure of the source animals to potential infections and extensive prescreening prior to organ harvest; and (iv) some animal species are refractory to infection by certain viruses that persistently infect humans (e.g., human immunodeficiency virus [HIV] and hepatitis B virus), thus possibly providing a therapeutic advantage.

However, xenotransplantation is also associated with a number of concerns. These include immunologic problems (particularly the risks of hyperacute and acute rejection), the risk of xenogeneic infections, and many ethical, legal, and social concerns.

* Corresponding author. Mailing address: HIV/AIDS and Retrovirology Branch, Division of AIDS, STD and TB Laboratory Research, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Mail Stop G-19, 1600 Clifton Rd, N.E., Atlanta, GA 30333. Phone: (404) 639-1024 (branch); (404) 639-0220 (direct). Fax: (404) 639-1174. E-mail: rrb5@cdc.gov.

As with allotransplantation, the success of xenotransplantation will depend on an ability to balance sufficient immunosuppression (to ensure lack of rejection) with the avoidance of infection. While whole-organ xenotransplantation (because of the genetic discordance between the donor species and humans) may require significant immunosuppression, similar to that needed for allotransplantation, some cellular applications (such as encapsulated porcine pancreatic islet cells for treatment of diabetes or encapsulated porcine liver cells in artificial hepatic dialysis devices) may preclude the need for immunosuppression altogether.

MODERN HISTORY OF XENOTRANSPLANTATION

The modern history of xenotransplantation in the United States is generally thought to begin in 1963 to 1964, when Keith Reemstma transplanted kidneys from chimpanzees into six patients with renal failure (100). Although one recipient survived 9 months after the transplantation, the attempts of Reemstma and of others at solid-organ xenotransplantation (100, 114) had largely disappointing results. Several attempts at solid-organ xenotransplantation have received wide coverage in the lay media. For example, in 1985, Leonard Baily transplanted a baboon's heart to a baby born with hypoplastic left heart syndrome (Baby Fae) (9), hoping that the newborn's undeveloped immune system would tolerate the xenograft. The child survived less than 4 weeks, and subsequent press coverage was very negative. In the early 1990s, Thomas Starzl transplanted baboon livers into two patients with HIV infection and advanced hepatitis B, who survived 27 and 70 days (5, 115). Heavy immunosuppressive therapy led to fatal infections in both patients. Since then, clinical trials attempting to sustain the lives of pediatric patients in fulminant cardiac failure by implanting a baboon heart xenograft "bridge" pending the availability of a suitable human donor organ have been proposed (79). Pig organ transplantation has also been attempted without much success. For example, a pig heart was transplanted into a patient with Marfan syndrome in 1990 in Eastern Europe and lasted about 24 h (J. Czaplicki, B. Blonska, and Z. Religa, Letter, J. Heart Lung Transplant. **11:**393–397, 1992).

Most lay media coverage has addressed controversial and ultimately unsuccessful attempts at solid-organ xenotransplants. However, the most imminently promising xenotransplantation applications include a variety of proposals to use cellular xenotransplantation products or extracorporeal perfusion devices. All of these avoid vascular anastomoses, thus diminishing the stimulus for immunologic rejection, and several use various modes of immunoprotection that may preclude the need for exogenous immunosuppression. Past and current clinical trials have attempted to ameliorate refractory pain in terminal cancer patients by using bovine adrenal preparations (16), to improve the symptoms of Parkinson's disease and Huntington's disease by implanting fetal porcine neuronal tissue [27, 106; J. M. Schumacher and O. Isacson, Letter, Nat. Med. **3:**474–475, 1997; M. St. Hillaire, K. Shannon, J. Schumacher, P. Starr, R. Penn, C. VanHorne, O. Isacson, and J. S. Fink, Abstract, Neurology **50**(Suppl. 4)**:**A80–A81], to biologically "dialyze" patients with hepatic failure, for whom human donor livers are not immediately available, by performing extracorporeal perfusion of their blood over porcine hepatocyte preparations (60), or to treat sepsis by hemoperfusion through pig spleen (88). Clinical trials attempting to functionally "cure" diabetes by implanting porcine pancreatic islet cells have also been conducted (41, 46, 101). Thus, xenotransplantation, and exposure of humans to its risks, is already well under way around the world. The efforts to regulate the use of xenotransplantation in the United States are only one part of ongoing international activities.

BARRIERS TO SUCCESSFUL XENOTRANSPLANTATION

Immunologic rejection remains the primary hindrance to successful xenotransplantation. However, advances in immunosuppressive therapy, genetic manipulations of source animals, and other approaches to immunologic modification have increased our potential to overcome hyperacute rejection (97). The immunologic reactions are reviewed later briefly, mainly to clarify how their suppression is related to increased risks for xenogeneic infections. Other barriers to xenotransplantation arise from ethical and legal issues associated with the use of animals as donors, but they are not addressed in this review. A major concern associated with xenotransplantation, and the primary focus of this paper, is the potential to introduce infections across species barriers. Infectious diseases passed from animals to humans under natural circumstances are called zoonoses. The risk that human recipients will be infected with recognized zoonotic agents can be reduced to negligible levels by appropriate selection of source animals and adequate screening of the source animal and the transplantation product prior to the xenotransplantation (28; U.S. Department of Health and Human Services, http://www.fda.gov/cber/guidelines.htm). However, the breaches of normal host defenses inherent in xenotransplantation may enable infectious agents that are unable (or poorly able) to infect humans under normal circumstances to cause infections. "Xenozoonoses" and "xenoses" are proposed appellations for such xenogeneic infections (28, 36, 37, 76). The risk for such infections would presumably increase with both increased immunosuppression and possible introduction of potentially pathogenic microorganisms through the xenotransplantation product, bypassing the normal defensive mechanisms.

ANIMAL SOURCES FOR XENOTRANSPLANTATION

Nonhuman Primates

The immune barrier to acceptance of xenotransplantation products is directly proportional to the phylogenetic distance between the source animal and the human recipient. Xenotransplantation products from NHPs, especially chimpanzees which are phylogenetically most closely related to humans, carry the least immunologic liability. From this viewpoint, NHPs would be the most logical species choice as donors of organs and tissues for xenotransplantation.

However, the use of xenotransplantation products from NHPs also presents special challenges. Some scientists believe that the very phylogenetic proximity that facilitates immunologic acceptance of an NHP xenotransplantation product may also enable infectious agents endemic to NHPs to infect humans as well (4). This latter concept, however, remains hypothetical.

Examples of devastating human diseases that have entered human populations from NHP sources include HIV-1 from chimpanzees (42) and HIV-2 from sooty mangabeys (25), Marburg virus from African green monkeys (112), and cercopithecine herpesvirus 1 (B virus) from macaques (18). However, nonprimate species have also transmitted lethal diseases to humans (e.g., Nipah virus encephalitis from pigs, hantavirus pulmonary syndrome from mice, and anthrax from cattle) (22, 30, 95). The scientific data addressing whether the infectious risk to humans from one species (e.g., NHPs) exceeds that from other species are very incomplete (15).

Regardless of whether infectious agents endemic to NHPs would have an a priori advantage in establishing infections in humans, current standards of NHP husbandry increase the risk that persistent viruses they harbor may go unrecognized. NHPs currently in captivity are generally only one or two generations removed from the feral state. Although attempts to develop specific-pathogen-free (SPF) colonies of NHPs for research purposes have produced some small colonies of animals free of a limited number of viruses, these husbandry methods remain crude compared to those developed for SPF pigs. The long duration between birth and sexual maturity, the prolonged gestational period, the limited litter size (generally one infant per gestation), and the complex social requirements of NHPs markedly increase the time and financial costs needed to develop colonies that are as infection free as those of industrially developed SPF pigs. Indeed, it is questionable if such standards could ever be achieved with these species. As a result, the existing populations of endemic microbial flora remain less well defined and the potential to limit and/or define lifelong exposures to infectious agents remains less feasible for NHPs compared to domesticated species such as pigs.

NHPs are infected with persistent viruses that cause no apparent disease in the species in which they are endemic but can cause devastating disease in humans or other NHPs. Examples include cercopithecine herpesvirus 1 (B virus), which is relatively silent in macaque monkeys but causes fatal encephalitis in humans and other primate species (18, 56), simian hemorrhagic fever virus, which is silent in Patas monkeys but causes dramatic and fatal hemorrhagic disease in macaques (24), and simian immunodeficiency virus (SIV), which is silent in sooty mangabeys but causes fatal immune deficiency disease in macaques (25). Many of the viruses transmitted to humans from NHPs are of little concern in the xenotransplant setting, since they are either not present in the species considered for xenotransplantation products (e.g., B virus, which is endemic only in macaque species [130]) or can be eliminated by limiting the geographic origin and lifelong contacts of potential source animals (e.g., Ebola, Marburg, monkeypox, and rabies viruses) as well as by pretransplantation screening of the source animal and the colony from which it is chosen (68, 77).

However, the temporal proximity of captive NHPs to the feral state raises concerns that these species may be more likely than pigs and other domesticated animals to harbor one or more persistent viruses that produce clinically silent and therefore unrecognized infections. In addition, NHPs are recognized to harbor at least two retroviruses that would be difficult or impossible to eradicate from source colonies, simian foamy virus (SFV) and baboon endogenous retrovirus (BaEV).

Latent SFV provirus is ubiquitous in naturally infected African green monkeys, where viral replication can be observed in the oral mucosa. Thus, unlike other retroviruses, SFV is copiously present in oral secretions and spreads easily from primate to primate without sexual or parenteral exposure (34). As a result, SFV infection is virtually always present among captive NHPs. Therefore, attempts to develop SFV-free breeding colonies to produce source animals for xenotransplantation products would require significant financial and temporal investment and might still prove unsuccessful.

Phylogenetic analysis of SFVs have demonstrated species-specific clusters, arguing that specific SFVs have coevolved with specific primate species. Despite the species-specific nature of SFV, sporadic cross-species infections of humans occupationally exposed to NHPs have been documented with SFV from baboons, African green monkeys, and possibly chimpanzees (52, 107). The significance of such human infections remains undefined. Although SFV was first isolated from a nasopharyngeal carcinoma in an African man (2), no etiologic association between the infection and the carcinoma was determined, and it has been speculated that the identified virus may have been a contaminant. No evidence of either SFV-associated disease or human-to-human transmission has been identified in association with any of the other six SFV-infected humans reported in the literature to date (52, 107).

A large variety of Old World monkeys and apes, including the baboon, carry simian T-lymphotropic virus (STLV) (23, 43, 81), which is closely related to the human T-lymphotropic virus type 1 (HTLV-1). HTLV-1 infection is associated with lymphoproliferative disease and with myelopathy (69). STLV, being highly lymphocyte associated, could be easily transmitted via transplantation. An exogenous type D simian retrovirus (SRV), found in Asian macaques, causes immunodeficiency (63), and SRV sequences have been found in retroperitoneal fibromatosis tumor tissues (44). SIV, common among African green monkeys, has been also found in baboons, providing evidence for simian-to-simian cross-species transmission in the wild (59).

Baboons, the primary NHP species candidate for xenotransplantation product sources, have an endogenous retrovirus (BaEV), several variants of which have been described (126, 128). In contrast to the exogenous simian retroviruses discussed above (SIV, STLV, SRV, and SFV), which are transmitted horizontally from one host to the next by infection, BaEV is an endogenous retrovirus. Endogenous retroviral genes are present in the genomes of all mammals adequately studied to date (92). Species considered as potential source animals for xenotransplantation, such as baboons, pigs, and cows, all carry fully expressible endogenous retroviruses. Hypothesized to represent "fossil remnants" of exogenous retroviruses once capable of causing active infection in the host species, endogenous retroviruses usually remain as incomplete retroviral DNA remnants embedded into the cellular genome of host animals. Many of these endogenous retroviruses are no longer capable of causing active infection in the host but can express infectious virus (92). Some of these expressed viruses, such as BaEV, are xenotropic, meaning that they are able to infect cell lines from other species (e.g., human cell lines) in the laboratory (57, 74). Since endogenous retroviruses are present in the genome of every cell and cannot be removed at present, all xenotransplantation products from any species may

contain benign genomic DNA that, on transfer into a human host, may express infectious retrovirus capable of creating active persistent infection. Whether such infections could occur in xenotransplantation product recipients and whether they would cause disease or be capable of being transmitted among humans has been the subject of much concern and scientific inquiry.

Current diagnostic assays are capable of assessing the presence of BaEV infection in recipients of living baboon tissue (50, 51). Studies on a recipient of one baboon xenotransplantation product have found no evidence of BaEV infection to date (M. Michaels, J. Hilliard, S. Deeks, P. Gupta, W. Heneine, D. Pardi, C. Kaufman, C. Rinaldo, K. St. George, L. Chapman, T. Folks, Y. Colson, P. Volberding, and S. Ilstad, Abstract, J. Acquired Immune Defic. Syndr. Hum. Retroviruses **14:**S3, 1996).

Simian virus 40 (SV40) and a closely related human virus, JC virus, are both small DNA viruses (family *Papovaviridae*, subfamily *Polyomavirus*) which typically establish persistent but usually inapparent infections in their natural hosts. SV40 was first isolated from rhesus monkey kidney cells used for polio vaccine stocks (45). A generational cohort of children seroconverted to SV40 after being exposed to contaminated polio vaccine (108). Polyomaviruses have the ability to induce tumors when introduced into certain foreign hosts (62). The human neurotropic polyomavirus, JC virus, induces tumors but not productive infection when inoculated into nonhuman primates (45). Therefore, concerns exist that these SV40-infected humans may be at an increased risk of cancer. However, studies of such an association have been inconclusive to date (35, 54, 58, 86, 119). These issues remain controversial and underscore the uncertainty about the long-term significance of human infection with apparently innocuous persistent simian viruses.

Concerns that the use of xenotransplantation products from NHPs may pose special risks have caused some scientists to call for a moratorium on the use of any primate xenotransplantation products (8). The U.S. Food and Drug Administration has identified these potential risks as a safety concern in need of additional research and special consideration. (Food and Drug Administration, Federal Register; http://www.fda.gov/cber/gdlns/xenoprim.txt).

Other issues also limit the potential use of NHPs as a source of xenotransplantation products. Many people consider the phylogenetic proximity of NHPs to humans and their intellectual and social complexity to merit special ethical consideration (84). In particular, the use of chimpanzees, the species most closely related to humans, in research or therapeutic applications is complicated by their status as an endangered species. Finally, even if husbandry techniques could be refined to the point that "clean" source colonies were a reality and the ethical concerns were adequately addressed, the supply of captive NHPs is and will remain inadequate compared to the demand for organs and tissues for transplantation. On this basis alone, some have argued that the minute risk of introducing new infections into human populations cannot be justified for a measure that cannot significantly narrow the gap between the need for and availability of donor tissue (4). For the above-mentioned reasons, future xenotransplantation trials will probably be conducted with cells, tissues, or organs from pigs.

Swine

Pigs attain an adult size that makes organs potentially compatible with those of an adult human. The life expectancy of pigs (around 30 years) also is compatible with a duration acceptable for xenotransplantation products for use in adult humans. Despite the obvious phylogenetic discordance, biological products from pigs have been used effectively for therapy of several human diseases (e.g., porcine insulin in the treatment of diabetes and porcine factor VIII in the treatment of hemophilia). The early sexual maturity, relatively short gestational period, and large litter size make the relatively rapid development of large SPF pig breeding herds logistically and functionally feasible. The widespread societal acceptance of the use of pigs for food and other products presages a broad social acceptance of their use for medical purposes. Additionally, many believe that because pigs and humans have coexisted closely for thousands of years, pigs pose lower infectious risks than NHPs as sources of donor organs for humans. NHPs and humans have also coexisted closely in Africa and Asia, but even in these settings NHPs have remained feral, not constricted, animals. In contrast to the situation with NHPs, husbandry methods for the generating and monitoring of SPF pig herds are well developed and in active use. However, concerns remain that anatomic and physiologic differences between humans and pigs may severely limit their usefulness for all but a few clinical conditions. Additionally, the greater phylogenetic distance between humans and pigs results in more significant immunologic challenges. Finally, pigs have been the source of significant zoonotic infections of humans, including fatal cases of "swine influenza" attributed to the swine influenza virus (124, 132). The recent epidemic of encephalitis in Singapore and Malaysia caused by Nipah virus (95) is another example of the potential risks of transmission of infections from swine to people. Because pigs are the most likely source of xenotransplantation products, the risks of infection with pig viruses are of major concern and are being extensively studied.

Like baboons, pigs carry an endogenous retrovirus that has generated much interest in relation to xenotransplantation. Porcine endogenous retrovirus (PERV) is a xenotropic retrovirus, also capable of infecting human cell lines in vitro.

In 1970, Breese described the presence of C-type particles in porcine cell lines for the first time, and in the following year, Armstrong et al. documented that these C-type particles were produced by the pig kidney cell line PK15 as well as by two other pig kidney cell lines, IB-RS2 and SK6 (6). Later, Strandström et al. demonstrated type C (viral) particles from a cell line established from a leukemic pig (118). Moening and Todaro further characterized the virus and showed that normal pig liver contains DNA homologous to the PK15 viral genome (80, 127), indicating that pigs have endogenous type C viral information. It was speculated that the virus was transmitted not only genetically but also horizontally (127). Later, it was shown that six different porcine cell lines spontaneously begin to release type C viruses after long-term propagation in vitro. Two of the viral isolates could replicate in the pig cell line ST-Iowa (65). The ST-Iowa cell line was not shown to release

the virus itself, but it could be infected with the C-type virus. In 1985, Suzuka et al. characterized a virus isolated from a swine malignant lymphoma cell line (Shimozuma-1) as a porcine retrovirus of type C appearance by electron microscopy. This virus has been referred to as porcine retrovirus Tsukuba-1 (120, 121).

Detailed infectivity studies on PERV have been conducted in the last 5 years. Patience and colleagues studied the infectivity of PERV from two porcine cell lines, PK15 and MPK (miniature pig kidney) (93). The supernatant from PK15 cells was found to infect pig (ST-Iowa), mink (lung mv-1-lu), and human kidney 293 cell lines, while the MPK supernatant was infective only for the pig cells. Cocultivation of X-irradiated PK-15 cells with human cells (diploid fibroblasts and B- and T-cell lines) resulted in their infection as well. Some of the infected human target cells (293 cells and HT 1080 cells) became productively infective. The infectious titer of PERV obtained from the human 293 cells was even higher than that obtained from porcine PK-15 cells. Human peripheral blood mononuclear cells (PBMCs) could also be infected by PERV, but only nonproductively (93).

By analyzing the PERV envelope gene, Le Tissier et al. classified the human-tropic PERV isolated from the PK15 cell line as PERV-A and PERV-B (64). Comparisons of the transmembrane region of the envelope protein of PERV-A and PERV-B have shown 92% amino acid identity to one another and 63 to 66% identity to the corresponding region from gibbon ape leukemia virus, feline leukemia virus, and Friend murine leukemia virus. Both PERV-A and PERV-B have been found in multiple tested pig breeds and were expressed in all pig tissues tested, including heart, spleen, and kidney tissues. Both variants were found to be capable of infecting human 293 cell lines. Although some polymorphism existed, eight proviral fragments were common to all pigs tested (64).

In all of the above studies, infectivity was achieved with PERV released from established porcine cell lines (6, 93, 118, 127) but not with PERV from primary or secondary pig cells (6). Wilson et al. showed that after mitogenic stimulation, primary porcine cells—PBMCs from the Yucatan and the National Institutes of Health (NIH) miniature pigs—can also release type C retrovirus (PERV-Y and PERV-NIH, respectively) capable of infecting porcine ST-Iowa cells and human 293 cell lines (133). Coculture of mitogenically activated porcine PBMCs with pig or human cell lines resulted in the transfer and expression of PERV-specific sequences and the establishment of a productive infection. PERVs derived from porcine PBMCs were related to but distinct from PERV derived from the pig kidney cell line (PERV-PK). Sequence analyses of portions of the PERV *pol* gene, expressed in pig cell lines productively infected with PERV-NIH and PERV-Y, showed remarkable similarity: 99.8% identity to each other and very high similarity (95%) for this region to PERV-PK. PERV-NIH and PERV-Y, however, differed in their tropism: while both infected porcine and human cell lines, PERV-Y also infected mink cell lines (133).

Not only porcine cell lines but also primary porcine cells—aortic endothelial cells—have been shown to release infectious PERV capable of infecting human cell lines in the absence of mitogenic stimulation of the source cells (70). These observations also suggest that xenotransplantation products may be associated with some risk of PERV infection, but it is not clear whether the titer of expressed virus will be high enough to cause productive infection in recipients.

Akiyoshi et al. characterized the nucleotide sequence of PERV obtained from lymphocytes of miniature swine (PERV-MSL) and found the greatest nucleic acid sequence identity to the gibbon ape leukemia virus (GALV) and murine leukemia virus and high homology of *gag, pol*, and *env* to those of the Tsukuba-1 retrovirus (from the Shimozima-1 cell line) and the endogenous retrovirus of the porcine kidney cell line PK15 (PK15-ERV) (3). Most of the amino acid sequence differences between PK15-ERV and PERV-MSL were in the cell attachment region (gp70), which contains viral receptor-binding domains. Such differences would be expected to affect the host ranges and the antigenicities of these viruses (3). The Centers for Disease Control and Prevention was able to exploit the phylogenetic relatedness between PERV and GALV to develop a serological assay to detect anti-PERV antibodies by using GALV antigens as cross-reactants (73).

Using a vector transduction assay, Takeuchi et al. assessed the host ranges of three types of PERV: PERV-A and PERV-B (from the PK15 cell line), and PERV-C (from Shimozuma cell line and from MSL) (123). These three types of PERV have distinct *env* genes but have highly homologous sequences in the rest of the genome. Retrovirus vectors bearing PERV-A and PERV-B *env* genes had wider host ranges, including several human cell lines, while a vector bearing the PERV-C *env* gene infected two pig cell lines and only one human cell line. PERV-A and PERV-B receptors were identified on cells of multiple species—pig, human, mink, rat, mouse, and dog—but not on NHP cell lines. The latter fact suggests that NHPs may not be the best animal model to study PERV zoonoses (123). All PERV types could infect both human and pig cell lines. Thus, they may have the potential to replicate in xenotransplantation products and further to infect the human recipients.

Some investigators have noted the high homology of PERV-MSL to ape and murine leukemia viruses and have hypothesized that, like other C-type retrovirus, PERV may be capable of inducing leukemia in a receptive host (99; D. Onions, D. Hart, C. Mahoney, D. Galbraith, and K. Smith, Letter, Trends Microbiol. **6:**430–431, 1998). Lymphoma has been induced following experimental retrovirus-mediated gene transfer in primates (31), and murine leukemia virus-related elements have been expressed in a primate recipient of retroviral gene transfer (98).

Peripheral blood lymphocytes, skin, lymph nodes, and lung tissue samples from immunosuppressed baboons, which received PERV-releasing primary porcine aortic endothelial cells, were negative for PERV by PCR 12 to 24 months after the transplantation (71). However, these findings should be interpreted very cautiously as indicating a lack of evidence of infection because (i) that study showed no evidence that the transplanted aortic endothelial cells survived long enough in the baboons, (ii) the same researchers were unable to infect baboon lymphocytic cell lines in vitro in another study (72), and (iii) as mentioned above, PERV-A and PERV-B receptors could not be identified on NHP cell lines, suggesting that NHPs may not be a good model for PERV infectivity studies (123).

Recently, van der Laan et al. reported the first evidence of cross-species transmission of PERV in vivo after transplantation of pig pancreatic islet cells into combined immunodeficiency mice (L. J. W. van der Laan, C. Lockey, B. C. Griffeth, F. S. Frasier, C. A. Wilson, D. E. Onions, B. J. Hering, Z. Long, E. Otto, B. E. Torbett, and D. R. Salomon, Letter, Nature **407:**90–94, 2000). The pig pancreatic islet cells produced PERV that infected the 293 human cell line in vitro. After transplantation of pig islet cells into the NOD/SCID mice, PERV expression and limited infection of murine cells in several tissue compartments were detected (van der Laan et al., Letter). However, the PERV infection was not associated with symptoms or widespread viremia (D. R. Salomon, personal communication). The severe combined immunodeficiency and the absence of natural antibodies to the Gal-α(1-3)Gal epitope in mice are certainly contributing factors in achieving that infection (see "Natural Xenoreactive Antibodies" below). These observations suggest that an immunodeficient mouse model may provide useful data in the future.

To date, limited studies of humans exposed to pig cells and tissues have produced no clinical or laboratory evidence of PERV infection. In a study of two patients with short-term extracorporeal exposure to pig kidneys, no evidence of residual pig DNA or PERV infection was found (94). None of 10 diabetic patients who had received porcine fetal islets 4 to 7 years previously had evidence of infection with PERV despite concomitant immunosuppression and prolonged xenotransplantation product survival (6 months to 1 year in 4 of the patients) (53). Paradis et al. studied 160 patients (including the 2 renal patients [94] and 8 of the 10 diabetic patients [53] previously reported) with exposures to a variety of xenotransplantation products and found no evidence of PERV infection (88). However, the limitations of this study are that 100 of 160 patients had short-term extracorporeal exposures (spleen perfusions); only peripheral blood was tested, which may not be sensitive for latent retroviral infection; and the study was retrospective, and so many early samples were not available and selection bias cannot be eliminated. Identification of long-term persistence of microchimeric porcine cells in 23 of 100 persons who underwent spleen perfusion was an unexpected finding (88).

One potentially significant difference between xenotransplantation of organs (which requires the establishment of a vascular anastomosis between the graft and the host) and the use of bioartificial organs or encapsulated cells is that a membrane may separate the animal (porcine) cells in the bioartificial organ from the patient's circulation. Such a membrane may serve both as an immunological barrier and as a barrier to transmission of animal viruses. No data are presently available on the PERV infection risk from encapsulated porcine islet cells. Artificial liver dialysis devices, currently in use in clinical protocols, contain hollow-fiber membranes designed to allow the diffusion of certain molecules but not that of viruses. Experiments show that pore size, membrane composition, and duration of exposure influence the transfer of PERV from the intraluminal into the extraluminal space. Extraluminal samples from cellulose fibers with a 70-kDa molecular mass cutoff and polysulfone fibers with a 400-kDa cutoff (predicted pore size, 5 and 10 nm, respectively) could not infect human cell lines (293 cells), while extraluminal samples from mixed cellulose fibers with 200-nm porosity were infective. Thus, 70-kDa-cutoff cellulose fibers and 400-kDa-cutoff polysulfone fibers decrease the risk of viral exposure of patients during bioartificial liver therapy (85).

In summary, infectivity studies to date have shown that porcine cell lines and some primary porcine cells can release PERV. PERV from both porcine cell lines and primary porcine cells can infect human and some other mammalian cell lines in vitro and the nude mouse in vivo. PERV from porcine cell lines can also nonproductively infect primary human PBMCs. No studies have been published describing the ability of PERV from primary porcine cells to infect primary human cells, limiting the extent to which these findings can be extrapolated to the xenotransplantation setting. To date, no evidence of human infection with PERV has been documented in patients exposed to pig tissue, in spite of the presence of long-term PERV microchimerism in some patients.

OVERCOMING THE IMMUNOLOGIC BARRIERS

Immunologic rejection remains the major barrier to xenotransplantation. The stages of rejection, in order of temporal occurrence, are (i) hyperacute rejection (HAR), (ii) acute vascular rejection (also known as delayed xenograft rejection), (iii) acute cellular rejection, and (iv) chronic rejection. Detailed reviews of the nature and mechanisms of rejection can be found elsewhere (17, 89, 97). Here, we review mainly HAR because some approaches used to resolve certain immunologic problems of rejection may have the unintended side effect of facilitating the adaptation of animal viruses to human hosts. For example, human sera inactivated PERV grown in pig cells but failed to inactivate PERV after the virus was passaged through human cells once (93).

HAR occurs within minutes after the implantation of porcine organs into humans or Old World NHPs and results in acute injury to the vascular endothelial cells of the donor organ and intravascular thrombosis within minutes to hours after the exposure. It is mediated by two primary factors: (i) the binding of xenoreactive antibodies (naturally present in the primate recipients) to antigens on endothelial cells in the porcine graft and (ii) the uncontrolled activation of the complement system resulting from the incompatibility of complement regulatory proteins in the porcine graft with the complement system of the primate recipient (89).

Natural Xenoreactive Antibodies

The endothelial cells of all lower mammals (e.g., swine and New World monkeys) have an antigen on their surface, galactose-α(1-3)galactose sugar residue [Gal-α(1-3)-Gal, or α-Gal], that is absent from the cells of humans and other Old World primates (40, 90). Humans have lost the gene for galactosyltransferase, the enzyme that adds the α-Gal residue on cell membranes. This genetic loss occurred long ago, probably in Africa, since New World monkeys still have that gene. Humans and other Old World primates develop complement-binding, natural xenoreactive antibodies (NXA) directed against Gal-α(1-3)-Gal, presumably because they are exposed to the α-Gal antigen originating from bacteria in the gut. Just as anti-blood group A and anti-blood group B antibodies pose a strong humoral barrier to the transplantation of allogeneic organs or

blood, NXAs directed against Gal-α(1-3)-Gal pose a barrier to the transplantation of tissues from species possessing α-Gal into species lacking α-Gal. When endothelial cells of the swine organ come into contact with the recipient's blood, they are attacked by these complement-binding antibodies in a way similar to the acute hemolysis of donor erythrocytes in a mismatched blood transfusion. Antibodies directed against Gal-α(1-3)-Gal constitute the major fraction of circulating xenoreactive antibodies, and their depletion from the blood of primates can prevent HAR in xenotransplantation (66). Although little endothelium is present in porcine fetal islet-like cell clusters, their transplantation into immunosuppressed diabetic patients triggers a considerable increase in the anti α-Gal activity of the recipient's serum (41). Development of transgenic pigs in which the α-Gal epitope has been eliminated may allow the production of grafts with which successful long-term xenotransplantation could be achieved.

Prevention of HAR has been approached by attempts to (i) alter the organ source through using genetically engineered pigs (e.g., pigs that will lack the α-Gal epitope and/or will have an artificially added gene[s] for human complement-regulatory proteins (such as decay-accelerating factor) and (ii) alter the recipient by depleting naturally occurring antibodies directed against α-Gal (NXAs) prior to xenograft receipt. Either approach to overcoming HAR may also facilitate the potential for the human recipient to be infected by endogenous retroviruses from the pig xenotransplantation product (131).

Genetically Engineered Pigs

α-Gal knockout pig. Eliminating (knocking out) the single gene coding for the enzyme α(1-3)galactosyltransferase will result in lack of an α(1-3)-Gal epitope in the donor animal and could significantly diminish the risk of HAR. However, this has yet to be successfully accomplished in pigs. Mice, deficient for α(1-3)-Gal, have been developed and are being used for studying HAR in xenotransplantation models (125). Recently, cloning of pigs from cultured adult cells has been accomplished through a new, two-stage nuclear transfer procedure (I. A. Polejaeva, S.-H. Chen, T. D. Vaught, R. L. Page, J. Mullins, S. Ball, Y. Dai, J. Boone, S. Walker, D. L. Ayares, A. Colman, and K. H. S. Campbell, Letter, Nature **407:**86–90, 2000). Cultured cells are easier to manipulate, so this cloning technique may allow targeted disruption of the α(1-3)-galactosyltransferase gene and the future cloning of pigs lacking the Gal-α(1-3)-Gal epitope.

The characteristics of animal virus envelopes are influenced by the characteristics of the cells in which they replicate. If a virus replicates in a host cell (e.g., a pig cell) that contains α-Gal, the envelope of the virus budded from the pig cell will also express α-Gal residues. The binding of the anti-α-Gal antibodies in human sera to the α-Gal residues on the viral envelope will result in complement-mediated viral inactivation (virolysis). Thus, this viral inactivation occurs by the same mechanism as HAR, and, consequently, genetically modified porcine xenografts that do not express α-Gal on their cell surfaces may be more resistant to HAR. Viruses budding out of cells that do not express α-Gal on their surface may avoid complement-mediated lysis. Xenotransplantation products manufactured using material from transgenic rather than unmodified pig tissue would not express the Gal-α(1-3)-Gal epitope on either its cell surface or the envelope of the virus shed from these transgenic pig cells. Some authors have speculated that this virus would then be more readily able to infect human recipients and to spread among the human population (131; M. L. Gustavson and L. M. Steen, Letter, Transplantation **66:**939, 1998). Patience et al. have demonstrated that PERV from infected human cells was not inactivated by human serum complement, in contrast to PERV from porcine (PK) cell lines (90). Thus, use of these transgenic products may increase the risk that the recipient will be infected by PERV or other viruses of porcine origin (131).

Competitive glycosylation. The competitive glycosylation method aims to reduce the level of α-Gal expression on the source animal cells through competition of two enzymes for the same substrate. Pigs and many other mammals have an enzyme, α(1-3)-galactosyltransferase that places the terminal sugar residue, α-Gal, on glycoconjugates (131). Both humans and Old World primates lack this enzyme. Humans have an H histo-blood group antigen, which pigs lack. The enzyme H-transferase [α(1-2)-fucosyltransferase] is involved in the synthesis of this antigen. If the gene for this enzyme is introduced into the pig, the α(1-2)-fucosyltransferase and α(1-3)-galactosyltransferase compete for the same substrate (*N*-acetyllactosamine). This competition may reduce the presence of the α-Gal epitope on pig cells to only 10 to 20% of its original expression (103).

Inserting another gene, that for α-galactosidase, an enzyme which causes the dissociation of the α-Gal residue from the subterminal *N*-acetyllactosamine, results in about 70% down-regulation of α-Gal expression. Experimentally, the presence of the genes for both enzymes, α(1-2)-fucosyltransferase and α-galactosidase, has led to an almost complete absence of α-Gal expression in the pig cells (reviewed in reference 17), with similar implications for the envelope protein of PERV expressed from such cells.

Expression of human complement-regulatory proteins in the pig. The NXAs, are complement binding, and thus the classical complement pathway is involved in HAR. Complement-regulatory proteins are largely species specific, and those of pigs can block porcine but not human complement. The development of pigs expressing human complement-regulatory proteins such as CD46 (membrane cofactor protein), CD55 (decay-accelerating factor), and CD59 (protectin) on their vascular endothelium has been demonstrated to prevent HAR in primates undergoing transplantation with pig organs. These proteins inhibit steps in the complement cascade, and although the natural antibodies will still be bound to the α-Gal antigen, cell lysis would be prevented. Several transgenic pig herds have been developed expressing one (e.g., CD55[104]) or more genes encoding human complement-regulatory proteins.

Introducing human complement-regulatory proteins into genetically engineered pigs, however, might also have unintended consequences. Weiss has noted that CD46 and CD55 are also cell receptors for human viruses: CD46 for measles virus and CD55 for echovirus and coxsackie B picornaviruses. The possibility that animal measles-related viruses (morbilliviruses) may preadapt for human transmission in the CD46 transgenic pig is an important concern (131). The porcine xenotransplantation product may also become more susceptible to human

viruses, and the use of products from genetically engineered pigs, expressing antigens compatible to the human recipient, may facilitate direct infection of human recipients with PERV. Annual antigenic drift in human influenza viruses is thought to represent the outcome of "viral mixing" that occurs when avian and human influenza viruses traffic through swine (110). The concept proposed here is that a similar process may occur whereby the human xenotransplantation product recipient (who now contains living tissue from both humans and pigs) may serve as an efficient "mixing vessel" for viral reassortment.

REQUIREMENTS FOR SELECTION OF ANIMAL SOURCES

Public health guidelines have recommended that the animals to be used for graft procurement be from closed herds with barriers to introduction of infections and undergoing close herd surveillance (U.S. Department of Health and Human Services; http://www.fda.gov/cber/guidelines.htm). The animals should be screened microbiologically for traditional zoonoses and for pathogens with a broad host range. Animals infected with certain pathogens should be excluded. Currently suggested exclusion criteria for donor swine include the presence of (i) known pathogens of humans (e.g., *Mycobacterium tuberculosis* and rabies virus), (ii) known pathogens of immunocompromised human hosts (e.g., *Toxoplasma gondii* and *Salmonella* spp.), (iii) microbes similar to those recognized to be pathogens of transplant recipients (e.g., porcine cytomegalovirus and adenovirus), and (iv) viruses with high a capacity for recombination (e.g., parvovirus and rotavirus) (reviewed in details in references 36 and 37).

Breeding SPF animals and screening for specific pathogens markedly decreases the risk for transmission of known bacterial and viral infections through xenotransplantation. In addition, inadvertent bacterial xenogeneic infections transmitted to the recipient should be amenable to antibiotic treatment. Of special concern, however, remains the risk of introducing antibiotic-resistant microorganisms through the xenotransplantation product.

Currently, about 90% of the antibiotics used in agriculture are for growth promotion in general animal husbandry (61), and the quantities of certain antibiotics used in animals are hundreds to thousands of times larger than those of the equivalent antibiotics used in human medicine (134). This has contributed to the selection of drug-resistant bacteria (e.g., *Salmonella enterica* serovar Typhimurium type DT104, *Enterobacter, Campylobacter, Escherichia coli* O157:H7, and enterococci (61). The use of antibiotics in source animal colonies could result in the development of bacteria with antibiotic resistance patterns not anticipated on the basis of hospital epidemiologic monitoring. The risk that xenotransplantation products will serve as vectors for these antibiotic-resistant microbes could be reduced by decreased use of antibiotics in herd maintenance. The antibiotic resistance pattern of bacteria colonizing source animal herds should be carefully monitored. Source animals for xenotransplantation should be free from bacteria with unusual drug resistance patterns (U.S. Department of Health and Human Services; http://www.fda.gov/cber/guidelines.htm).

Prevention of transmissible spongiform encephalopathies (TSEs) in source animals is also important. Infectious agents that can cross the species barriers and present as overt clinical infections many years after the initial infection are of particular concern for the individual patient's health but are not a large public health concern because there is no evidence of person-to-person transmission. A good example of one such disease is the new variant of Creutzfeldt-Jakob disease (CJD), which has been associated with bovine spongiform encephalopathy (10). It is estimated that the new-variant CJD may have an incubation period of 10 to 30 years (129). The presence of an undetected disease with a long incubation period in a xenotransplantation source animal at the time of product procurement is a concern. However, for now, this risk appears to be rather hypothetical. Although experiments have shown that pigs can be infected with TSE parenterally (by injection), they have never naturally or even experimentally been infected by ingestion, the usual route (14). In addition, the requirement that animal sources for xenotransplantation products be procured from closed colonies with controlled feed sources minimizes the risks for introduction of TSE (U.S. Department of Health and Human Services; http://www.fda.gov/cber/guidelines.htm).

ALLOTRANSPLANTATION: LESSONS FOR XENOTRANSPLANTATION

Although infectious agents introduced specifically through xenotransplantation will be unique to that setting, the infectious-disease problems associated with immunosuppression in xenotransplantation will, to a certain degree, resemble those in allotransplantation. However, patients with animal grafts (xenotransplantation products) may need more powerful immunosuppression than those with allografts and thus may be exposed to greater risks.

Viral, bacterial, and fungal infections have been transmitted via allotransplantation of organs, tissue allografts (e.g., skin, cornea, and heart valves), and cells (such as islet cells, hematopoietic stem cells, and semen). Protozoan and worm parasites have been transferred via organ allotransplants. For example, hepatitis B virus, cytomegalovirus, herpes simplex virus, HIV-1, rabies virus, prions, *M. tuberculosis* and other bacteria, and fungi have been transmitted through bone allografts, cornea, heart valves, skin, dura mater, and pericardium transplantations (reviewed in detail in reference 32).

During identified periods after allotransplantation, recipients are at risk for immunosuppression-associated endogenous and exogenous opportunistic infections. These have been reviewed in detail by Fishman (36). Public concerns have focused mainly on viral pathogens. Cytomegalovirus is the most common cause of disease due to reactivation of latent viral infection. Herpes simplex virus, varicella-zoster virus, Epstein-Barr virus, and others are also significant pathogens in the setting of immunosuppression (91). Epstein-Barr virus, for example, is associated with a spectrum of B-cell lymphoproliferative diseases that develop some time after organ transplantation (48). Furthermore, persistent viral infections such as hepatitis B virus and HIV infections may progress more rapidly in immunosuppressed allotransplant recipients (26, 33, 111).

Isolated cells from a donor organ migrating to anatomically dispersed sites in the recipient have been identified in allograft

recipients (83, 116); this phenomenon is known as microchimerism. Persistent microchimeric Y chromosome cells (or fetal DNA) have been identified in mothers of male offspring as long as several decades after delivery (7, 12). These observations suggest that exposure to a graft can have a lingering influence on the recipient. Persistent microchimeric porcine cells have been identified as long as 8 years after exposure in persons whose blood has been ex vivo perfused through pig spleens (88).

Autopsies of the two HIV-positive humans with end-stage hepatic disease who received transplanted baboon livers (5, 115) revealed DNA sequences from two simian retroviruses—SFV and BaEV—in multiple tissue compartments. However, baboon mitochondrial DNA sequences were also identified in all these tissue compartments, suggesting that xenogeneic "passenger leukocytes" harboring latent or active viral infection had migrated from the xenotransplantation products to distant sites within the human recipients (5). These baboon liver recipients differ from the splenic perfusion patients described above in that the baboon livers remained in place as persistent sources of migrating michrochimeric cells. Nevertheless, the failure of the host immune response to eradicate michrochimeric baboon cells is noteworthy.

Taken together, these findings underscore the persistent duration of potential infectious risks associated with xenotransplantation even if the xenograft is rejected or removed or when the initial exposure is transient.

APPROACHES TO IDENTIFY XENOGENEIC INFECTIONS: POSTTRANSPLANTATION TESTING

As discussed above, one of the primary concerns about xenotransplantation is the emergence of new, unrecognized pathogens in the graft recipient. While prolonged clinical surveillance of graft recipients is designed to identify unexplained illnesses that may present after variable periods of clinical latency, this after-the-fact defensive strategy may not be adequate to protect the public health. Therefore, the laboratory must be ready with a repertoire of generic assays for detecting new agents that may emerge from a large and diverse pool of microorganisms. During the last decade, our ability to identify known agents has increased by several magnitudes, primarily because of the advancements in both detection specificity and sensitivity brought about by new molecular tools. Diagnostic assays for identification of PERV and BaEV and diagnostic algorithms that allow endogenous retrovirus infection to be distinguished from the endogenous virus genome in microchimeric animal cells have been developed. These include PCR assays for the identification of PERV and BaEV DNA as well as the detection of porcine or baboon mitochondria (evidence of microchimerism) (51, 122). Reverse transcriptase PCR (RT-PCR) assays capable of identifying PERV or BaEV RNA and Western blot assays intended to detect serologic evidence of antibodies to PERV or BaEV are also available (73).

Because so much about these specific assays has been discussed, the focus here will turn to new technologies currently being developed to identify unknown potential pathogens. Interestingly, the evolution of new areas of clinical medicine such as xenotransplantation and gene therapy requires that laboratory technology and methodology develop in parallel with these advances.

Cell culture has traditionally served as the standard method for identification of microorganisms, both known and unknown, although the optimal growth conditions for an unknown organism are always a guess. While culture remains a benchmark for identification, its associated labor, time, and failure rate will continue to be drawbacks to timely identification of potential unknown agents in a xenotransplantation setting. However, a new generation of developmental genomic and biochemical technologies show great promise for identifying unknown microorganisms amidst the tissues of the human host. Three well-defined and proven molecular methods are discussed here. Among them, broad-based PCR is considered first, since it can be used as a specific and nonspecific identifier.

It has been known for some time that groups of organisms have "fingerprint" sequences within their genomes. PCR can be utilized to amplify these sequences to classify a known organism into a specific group or to determine if one of the group members sharing the fingerprint sequence might be present. For instance, retroviruses carry sequences in their RT gene which are unique to that class of virus and can be identified by PCR. A generic primer set, called MOP (mixed oligoprimers), can be used to amplify a 90-bp fragment from any known retrovirus (109). Caution must be used, however, since these sequences are also found within human DNA polymerases. Thus, a first step is to convert RNA into cDNA before attempting to utilize these primers, making this approach to finding a retroviral adventitious agent useful only if the agent is expressing RNA. Another PCR-based test for retroviruses takes advantage of the function of the RT enzyme itself (49). Here, the RT enzyme of the unknown retrovirus is used to catalyze the RNA-to-cDNA step. By using a known RNA sequence which will be reverse transcribed if RT is present in the unknown sample, specific DNA primers can be made which will subsequently amplify the cDNA produced. A specific probe is then used to detect the amplified DNA. If the specific DNA can be amplified and probed, the test sample must have contained RT, providing positive evidence for the presence of a retrovirus. One still would not know the type of retrovirus, but since humans do not normally express retroviruses, it could be assumed that an exogenous retroviral agent is present.

Although retroviruses were used as an example for generic PCR detection, other microorganisms can also be detected this way quite simply, especially if their life cycle includes a DNA intermediate. For instance, repetitive DNA sequences in genes that code for ribosomal subunits of bacteria can easily be detected and used for classification (135). Obvious shortcomings of this approach include the fact that bacteria are part of the normal flora of the human host and may be mistaken as the causative or transmitted agent following xenotransplantation.

Another rapidly developing area is the use of subtractive technologies. Here, the RNA from expressed foreign genes is reverse transcribed into cDNA and subtracted by hybridization from the background of host (human) DNA. One such subtractive technology, known as representational difference analysis, takes advantage of the differences in expressed genes from the host and the agent (67). The presumed causative agent of Kaposi's sarcoma, human herpesvirus 8, was identified using that technology (19). When this method is coupled with a

protein expression technique, whereby the nonspecifically selected cDNA fragment is transcribed and translated, the resulting protein can be incorporated into an antibody identification assay. Thus, if a xenotransplant recipient has been infected or exposed to a foreign agent, as represented by the subtracted fragment, antibodies induced by the infection should react with the protein fragment. This would establish a link between the immune response of the individual and an unknown nonhost sequence.

All of the assays described above are dependent on the presence of a replicating or expressing agent. However, not all microorganisms will be so accommodating. We only need to look to chronic infectious diseases to understand how an organism might have been present at one time but is no longer present or is present in a latent state. Generally, an infectious agent will leave a trace of its presence, usually an immunological response, that can be detected to identify the microorganism. However, this is not as useful when the organism is unknown. Recently, a new technology has been developed that enables us to determine if something foreign is or has been present. By taking advantage of the transcriptional response machinery of the host, one can determine if a class of genes has been turned on or off following an infectious exposure (102). This method utilizes microarray technology, which permits the investigation of expressive changes in thousands of human genes. Steady-state human RNA production can be evaluated in the context of an invading organism which would provide a characteristic pattern of response indicative of the organism type. Just as we might examine the white blood cell compartment for changes in lymphocyte subsets or plasma cytokine profiles, we can now observe a greatly expanded number of RNA changes that the host might make in response to a foreign invader. Furthermore, the unique changes in the host response pattern to a "family" of pathogens might remain permanently regardless of the presence or expression level of the agent. This technology is, of course, in its earliest stage of development. However, one may begin to target certain families of genes, such as cytokines, chemokines, and other genes controlling host defense, for altered expression.

Applications of laboratory technologies for surveillance of zoonotic transmission of microorganisms following xenotransplantation will certainly parallel those being used in other rapidly developing areas such as chronic and infectious emerging diseases. We have already seen, for example in the hantavirus pulmonary syndrome outbreak, that a new virus (Sin Nombre virus) was accepted and confirmed as the etiologic agent by molecular identification before any virus isolation was achieved (22).

Biosafety is always a consideration for laboratories working with poorly characterized infectious agents. Biosafety level 2 (BSL-2) standard and special practices and containment equipment are needed to handle clinical specimens from recipients. For activities that may create bioaerosols or if unknown infectious agents isolated from xenotransplant recipient materials are under propagation, BSL-3 standard and special practices and containment equipment should be used in a BSL-2 facility to ensure further protection (28; U.S. Department of Health and Human Services; http://www.fda.gov/cber/guidelines.htm). These biosafety recommendations are similar to those for working with HIV.

Clearly, these new laboratory methods will enhance our ability to discover the transmission of unknown microorganisms before they can spread. Although there is much to learn, these methods are important new tools for addressing the public health concerns associated with clinical xenotransplantation research. As such, these methods and subsequent improvements are vital to the future of this promising research area.

CONCLUDING REMARKS

New diseases and newly discovered viruses and other pathogens will continue to "emerge." Recent examples include Sin Nombre virus (22), Nipah virus (95), porcine hepatitis E virus (75), and the porcine Menangle virus (20, 96). Viewed in this context, we must ask whether xenotransplantation will increase the risk of exposure to unknown or poorly studied viruses and other pathogens compared to (i) natural exposure to "emerging" infectious agents, (ii) allotransplantation, (iii) gene therapy, (iv) occupational risks, and (v) vaccines from nonhuman animal substrates.

Knowledgeable observers have argued that the risk to the public from xenotransplantation may be exaggerated compared to that from other types of ongoing exposures (82). Occupational risk of exposure to simian retroviruses among animal workers and researchers is one example of the opportunities for infections to cross species lines (52).

However, while we cannot control what happens in nature, we must work to prevent and control biosafety hazards associated with intentional therapeutic interventions (82). Concerns that therapeutic interventions may result in cross-species transmission of infectious agents are valid. Lingering concerns remain about the oncogenic potential of the inadvertent inoculation in the 1950s of a large cohort of children with polio vaccine manufactured using nonhuman primate cells and contaminated with adventitial SV40 (108). Although some epidemiologic studies have not found a significant increase in malignancies among the vaccine recipients to date (86, 119), these studies have some recognized limitations that may not allow them to detect an existing association. Other studies suggest that an associated risk may exist (35, 58), and thus the controversy continues. Xenotransplantation is an intentional exposure, and infections acquired as a result of it are, in that sense, iatrogenic—increasing the burden of responsibility for those who perform such transplants. Xenotransplantation may carry serious risks if it introduces a pathogen that could be transmitted between humans, especially one that causes disease after a long period of clinical latency.

Because these exposures occur under controlled circumstances, we can implement measures to minimize the risk of xenotransplantation-associated infections. It is expected that the xenotransplantation patients and their contacts will be monitored more closely than the average allotransplantation patient or the general population (U.S. Department of Health and Human Services; http://www.fda.gov/cber/guidelines.htm). Close monitoring of recipients should enable early identification of adverse outcomes, allowing for aggressive efforts to control infections that have occurred and prevent additional procedures that may result in new infections.

The risks of an infectious disease associated with xenotransplantation will be different for the individual recipients, their

immediate contacts, and the general public. The xenotransplant recipient will be at highest risk for any infection associated with the animal cells, tissues, or organs because (i) a potential pathogenic microorganism may be introduced directly into the patient's body, bypassing normal defensive mechanisms and (ii) the recipient's immune system may be weakened by immunosuppressive therapy. Still, this risk may be quite acceptable to many recipients, given the usually poor prognosis of end-stage organ failure (e.g., liver, heart, and lung failure) and the paucity of alternative therapeutic options. In contrast, the risk/benefit analysis for individuals with kidney failure, stable on dialysis, or with diabetes mellitus, treatable by insulin, will be quite different. For the recipient's immediate contacts, the risk will depend on whether any potential xenogeneic pathogen originating from the xenotransplantation product is transmissible among humans. If so, the ease of contagion and the modes of transmission will also determine the risk. However, we are really not able to predict how such putative xenogeneic pathogens would behave after transmission to a human host.

The major concern with xenotransplantation is to avoid the emergence of infections that may cause a threat to the public health. Stoye has rightly pointed out that the mere presence of a xenogeneic infectious agent in a xenotransplantation product is not necessarily associated with risk to either the recipient or the public (117). Using PERV as an example, he has pointed out that a whole chain of events is required for PERV to pose a public health risk: (i) infectious, human-tropic PERVs must exist (this condition has been met); (ii) such viruses must be present in the germ line of pigs bred for xenotransplantation (met); (iii) these viruses must be expressed in transplanted cells, tissues, or organs (demonstrated in vivo only in a mouse model of transplantation of porcine pancreatic islet cells); (iv) expressed PERVs must infect the xenotransplant recipient (unknown in vivo, but PERV infectivity has been proven for human cell lines in vitro); (v) replication and spread through the recipient must take place (unknown); and (vi) transmission to others must occur (unknown) (117). Even if a secondary transmission among humans occurs, the public health significance of such a xenogeneic infection will depend on the associated pathogenicity; e.g., the significance of a dormant or a transient and benign infection is quite different from that of a persistent or transient infection associated with a serious clinical course.

Onions et al. (Letter, Trends Microbiol.) have even suggested that in the longer run, any residual public health risk may be eliminated by the ability to vaccinate patients against PERV or eliminate infectious PERV in the donor pig through cloning and knockout technology. This hypothesis has not yet been thoroughly experimentally explored.

Allotransplantation has also contributed to the transmission of both infection and cancer (reviewed in reference 32). Transfer of biological materials between humans has contributed to the spread of HIV through transfusion of contaminated blood and blood products and transplantation of HIV-infected tissue or organ grafts (32). While allotransplantation and blood transfusion are minor contributors to the HIV pandemic, within certain subpopulations they have been major contributors, with devastating effects. Before a combination of HIV screening of donors and better inactivation techniques were able to provide a safer supply of factor VIII concentrate, more than half of the U.S. hemophiliac population became infected with HIV (39, 55). Thus, concerns exist about the potential for xenotransplantation recipients to be sources of occult xenogeneic infections through donation of biological materials. For this reason, the U.S. Public Health Service has recommended that xenotransplantation product recipients should not donate blood, organs, tissues, and other biological materials and that this be clearly represented in informational materials and consent forms for such donations (U.S. Department of Health and Human Services; http://www.fda.gov/cber/guidelines.htm). The wisdom of extending this ban to include other contacts of recipients is under discussion.

In summary, advances in immunology and immunosuppressive therapy, along with genetic engineering and cloning of animals, have enabled early clinical trials of cellular and tissue xenotransplantation and extracorporeal circulation devices. Discussion is under way on what preclinical bench works would justify consideration of clinical trials for revascularized whole-organ xenotransplantation (heart and kidney). Genetically engineered pigs, bred under special conditions, are currently envisaged as the major source of xenotransplantation products (organs, tissues, and cells). It is quite encouraging that, thus far, there has been no evidence for human infections caused by pig xenotransplantation products. However, the presence of xenotropic endogenous retroviruses in pigs and the clinical evidence for long-lasting porcine cell microchimerism in recipients are indications that the potential for xenogeneic infections cannot be discounted. Further trials should continue only under close clinical and laboratory monitoring for potential xenogeneic infections. The evidence that HIV probably resulted from a simian virus being transmitted across species lines into humans should serve as a caution to all scientists working in the area of xenotransplantation.

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