



Historical and Current Perspectives on Bone Marrow Transplantation for Prevention and Treatment of Immunodeficiencies and Autoimmunities

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ABSTRACT

Primary immunodeficiency diseases often fully meet the definition of "experiments of nature." Much of the expanding understanding of the lymphoid systems and immunologic functions generated in recent years has been derived from studying patients with primary, generally genetically determined immunodeficiency diseases, as well as other relatively rare secondary immunodeficiency diseases. Increasing knowledge of immunologic defenses, their interacting cellular and molecular components, the evolving details of sequential stages of cellular differentiation, and the nature and control of the cellular and molecular interactions in immunity have now made it possible to define precisely many primary immunodeficiency diseases in full molecular genetic terms. With this wealth of scientific information based on experimental and clinical research, incredible advances have also been made in using bone marrow transplantation (BMT) often as a curative treatment for immunodeficiency, some 60 to 70 other diseases, leukemias, lymphomas, other cancers, and a rapidly expanding constellation of metabolic diseases or enzyme deficiencies. Also, progress in applying allogeneic BMT to prevent, treat, and cure complex autoimmune diseases, primary immunodeficiency diseases and certain forms of cancers, is considered. Further, mixed BMT (syngeneic plus allogeneic) that establishes a form of stable mixed chimerism has also been employed in animal experiments, which revealed that BMT can be used to treat not only immunodeficiency diseases, but also systemic and organ-specific autoimmune diseases, eg, diabetes and erythematous lupus-like diseases. Moreover, performing BMT in conjunction with organ allografts, eg, thymus or pancreatic transplants, has successfully prevented rejection of these allografts, sometimes without recourse to long-term irradiation or toxic chemical immunosuppressive agents. A crucial role for stromal cells in cellular engineering has now also been realized in animal models as a means of preventing graft rejection and promoting full and persistent reconstitution or correction of genetically-based diseases. With all of these achievements, BMT promises continued dramatic and impressive new approaches to clinical and scientific research and reveals an attractive strategy for the treatment and prevention of many currently intractable human diseases. If these achievements can be extended to larger outbred animals and humans, BMT may set the stage for induction of improved immunologic tolerance and for developing treatments for additional intractable human diseases in the 21st century.

INTRODUCTION: EXPERIMENTS OF NATURE—THE BEGINNINGS OF CELLULAR ENGINEERING

As first pointed out by Harvey [1] and reiterated by McQuarrie [2], clinical investigators have at their disposal powerful weapons with which to supplement tools available from the basic sciences to contribute to the understanding of the mechanisms of disease. Among patients, particularly those with unusual diseases, may be found "experiments of

nature," which permit a generation of new and useful knowledge applicable far beyond the disease implications of the patients studied.

It seems quite unimaginable today that when I began my work in immunology in 1944, we knew very little about the cells or molecules involved in immune defenses or about the function of major organs now known to be crucial to immunologic development and the function of the major immune components, eg, thymus and spleen. I have had the privilege to live and work at a time when I could contribute to the development of much new knowledge in several different fields of immunology [3]. The development of our

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understanding is occurring so rapidly and with such impressive precision that it is difficult for modern-day immunologists to keep abreast of all aspects of the expanding understanding of immunologic function. But this explosion of immunologic knowledge has taken place during my lifetime as well as the lifetime of others who are still working productively in this exciting field.

Lessons learned from each of the forms of primary immunodeficiencies have repeatedly shown that patients with primary, generally genetically determined immunodeficiencies, are, indeed, most revealing “experiments of nature.” Such patients, so vulnerable to infection, provide a crucial testing ground for developing understanding of the interrelationships between structure, differentiative influences, and function. The ability to correct by means of organ, cellular, and molecular engineering the defects that characterize patients with primary immunodeficiency diseases and in this way to prevent the infectious and malignant diseases that destroy such patients is a substantial witness to the fact that the new knowledge of immunobiology has pragmatic value. Thus, these “experiments of nature” led my colleagues and me to analyze with increasing depth how humans can exist usually free of infection while actually living in a veritable sea of potential invaders—microorganisms such as bacteria, fungi, parasites, and viruses with which humans and mammals share their ecological niche.

PRIMARY IMMUNODEFICIENCIES

We know that the primary immunodeficiency diseases usually result from innate genetic defects of the immune system and its development. As a consequence, recurrent protozoal, bacterial, fungal, and viral infections of varying severity; autoimmunities; and malignant diseases ensue. Thus, patients with immunodeficiency diseases have often become important and sometimes crucial teachers of immunobiology: witness the bisection of the microbial universe, the lymphoid system universe, and even the immunologic universes just from the analysis of the antibody deficiency that feature the X-linked form of agammaglobulinemia now known as Bruton’s disease [4]. Indeed, when thoroughly studied, almost every one of the primary immunodeficiencies have shed significant new light on the importance of immunologic functions. In the past decade, even the genes and molecular bases for several forms of human primary immunodeficiency diseases have been identified. Some of these genes have already been cloned and their function and expression characterized. This better understanding of the molecular basis of primary immunodeficiency disorders has led to improvement of available therapies—eg, bone marrow transplantation (BMT), cord blood transplantation [5-7], stem cell transplantation [8,9]—and to new treatment strategies, including gene therapy [10,11], which have already been discovered and developed as effective treatments of immunodeficiency diseases

Herein we consider 5 of the primary immunodeficiency diseases—X-linked agammaglobulinemia (XLA), X-linked hyperimmunoglobulin (Ig)M syndrome (XHIM), X-linked severe combined immunodeficiency disease (XLSCID), adenosine-deaminase syndrome (ADA), and DiGeorge syndrome (DGS)—and reflect on the contributions each of them has made to our modern concepts of immunology.

The basic philosophy underlying the recognition of the remarkable power of rare forms of disease as teachers of modern biology and medicine was long ago recognized and described in a letter written in 1657 by William Harvey [1] just 6 weeks before he died to a physician he addressed as Jan Vlaskfeld, “the distinguished and accomplished physician at Harlem.”

It is even so. Nature is nowhere accustomed more openly to display her secret mysteries than in cases where she shows traces of her workings apart from the beaten path; nor is there any better way to advance the proper practice of medicine than to give our minds to the discovery of the usual law of nature, by the careful investigation of cases of rarer forms of disease. For it has been found in almost all things, that what they contain of useful or of applicable, is hardly perceived unless we are deprived of them, or they become deranged in some way. The case of the plasterer to which you refer is indeed a curious one, and might supply a text for a lengthened commentary by way of illustration. But it is in vain that you apply the spur to urge me, at my present age, not mature merely but declining, to gird myself for any new investigation. For I now consider myself entitled to my discharge from duty. It will, however, always be a pleasant sight for me to see distinguished men like yourself engaged in this honorable arena. Farewell, most learned sir, and whatever you do, still love.

Thus, according to Harvey, this beautiful prose summarizes the influence that experiments of nature have exercised as the great teachers of truths in medicine [1].

Bruton’s [4] discovery of agammaglobulinemia—and a remarkable interplay between the clinic and laboratory—opened the door to an impressive increase in our understanding of the role played by lymph nodes, germinal centers, and plasma cells in the bodily defense. The first patients with this disease were found to bisect the microbial universe, the immunologic universe, and the lymphoid cellular universes in a most dramatic way. These patients lacked plasma cells and the precursors of plasma cells now called B lymphocytes, but they had normal cell-mediated immunities [12-17]. This disease, described for the first time almost 58 years ago by Colonel Ogden C. Bruton [4], is now a classic form of immunologic deficiency. A disease of X-linked inheritance, XLA is now known to be attributable to many different mutations of tyrosine kinase—Bruton’s tyrosine kinase (*Btk*) gene. The gene was discovered in 1993 by 2 independent groups. Bruton tyrosine kinase is a member of the Tec family of cytoplasmic protein tyrosine kinases. These kinases are essential to the growth of B-cell precursors and their development into mature B cells and plasma cells, which is why there are no circulating B cells in patients with XLA [18,19]. More than 300 different mutations in the *Btk* gene have been identified, but there has not been any correlation between the type of mutation and the phenotype of the disease. Children with XLA or deficient Ig production are protected against infection during the first few months of life by maternally transmitted IgG antibodies. Thereafter, they are susceptible to recurrent bacterial infections with encapsulated organisms such as *Haemophilus influenzae*, *Streptococcus pneumoniae* and, less frequently, *Staphylococcus aureus*, which are often manifested during the second half of the first year of life

in these patients. The most characteristic finding in infants with XLA is the arrest of B lymphocyte development in the peripheral blood and lymphoid tissues [20,21]. Bruton reported the case of an 8-year-old boy who had suffered since an early age from recurrent bacterial infections. It has been noticed by several investigators, including ourselves, that the boy described by Bruton may not have had what we now recognize as XLA [22-24]. However, certain patients with XLA, as confirmed by mutation analysis, have demonstrated a relatively mild disease phenotype similar to Bruton's case [4,25-27]. In a joint study, Bruton et al. [28] reported additional patients in whom the diagnosis of XLA seems indisputable. More importantly, however, is that Bruton's original report inspired a great number of investigators, including ourselves, to look for patients with antibody or cellular immunodeficiencies. This search turned up numerous patients who were identified not only with XLA, but with other forms of primary immunodeficiency as well [3,29].

X-Linked Hyper IgM Syndrome

XHIM is a rare inherited immune deficiency disease. This disease was first described in 1961 and appeared to be an X-linked syndrome that resembled XLA clinically. XHIM patients, however, were found to have normal or elevated serum concentrations of IgM but no IgA and little or no IgG or IgE [30,31]. Male patients with XHIM syndrome have a clinical history of pyogenic infections that resembles that encountered in male patients with XLA [32]. They also have a high frequency of autoimmune disorders and cancers [33]. In addition, these patients, for unknown reasons, are susceptible to infections other than those due to high-grade encapsulated bacteria that plague the patients with XLA. XHIM patients may particularly experience infections due to *Pneumocystis carinii*. Patients with XHIM are also prone to exhibit apparent autoimmune diseases, eg, hemolytic anemia, thrombocytopenic purpura, and recurrent, often severe, persistent, and prolonged neutropenias. These disease entities are often observed with T-cell immunodeficiencies but are not often encountered in patients with XLA or other forms of agammaglobulinemia and defective antibody production. Despite lymphoid hyperplasia, which distinguishes XHIM from XLA, lymph nodes in XHIM patients are characteristically devoid of well-developed germinal centers [33,34]; these patients cannot switch normally from production of IgM antibodies to production of IgG, IgA, or IgE antibodies because they lack the molecular essence of the switch mechanism, CD40 ligand. This disease has now been found to be attributable to mutations in the CD40 ligand (*CD40L*) gene [35-39]. The ligand for CD40 is a membrane protein present on activated T cells that has the capacity to induce a characteristic B-cell proliferation and differentiation to plasma cells. Several mutations of the *CD40L* gene were reported to be responsible for defective class switching of B cells in X-linked immunodeficiency with hyper IgM [40], resulting in failure to mount normal secondary antibody responses to T cell-dependent antigens, accounting for the increased susceptibility to infections [33]. Further work is required to explain the high frequency of autoimmune disease and prolonged episodes of neutropenia and susceptibility to opportunistic infections, including those due to *Pneumocystis carinii* pneumonia and lymphoproliferative complications that occur in patients with XHIM. If these patients live long enough, they

are likely to develop lymphoid malignancies that appear to involve especially cells of the B-cell series. These patients have recently been fully reconstructed immunologically by BMT from a major histocompatibility complex (MHC)-matched sibling donor, and their genetic abnormality has also been fully corrected by BMT in recent experiments [41].

X-Linked Severe Combined Immunodeficiency Disease and Adenosine Deaminase Deficiency

Hyper IgM syndrome can occur either in an X-linked form or in several autosomal recessive genetic forms. Unlike the X-linked form, the autosomal recessive form is not attributable to an abnormality of the CD40 ligand switch mechanism. Patients with XLSCID have a collection of abnormalities of the gene for interleukin (IL)-2R γ , which may also act as a receptor for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-2R γ c. XLSCID causes susceptibility to infection attributable to deficiencies of both cellular and humoral immunity early in life, often in the first days or weeks of life. Among different variants, SCID has also been found to be caused by a deficiency in the ADA enzyme in which lymphotoxic metabolites prevent normal development of T cells as well as B cells and plasma cell accumulation. The in-depth understanding of the biochemical and molecular basis of this disease has allowed the development of a number of experimental therapies for ADA-SCID. Patients with XSCID have decreased numbers of T cells but are able to produce B cells. They fail to exhibit normal proliferative responses to phytohemagglutinin and also exhibit hypogammaglobulinemia and failure of antibody production, although B cells are often present but probably in a diminished number [42]. Before 1968, patients with XSCID always died of infections within the first and second years of life. However, the achievement of immune reconstitution following the first successful allogeneic BMT (allo BMT) [43-46] marked the beginning of an era that has unlocked the door to future successes with BMT as a treatment for immunodeficiencies and paved the way to successful gene therapy, often thought of as the ultimate treatment for inherited immunodeficiency diseases [11,47]. SCID of the X-linked type and that associated with ADA deficiency were thought to offer ideal targets for the first clinical application of gene therapy [48] because SCID is a lethal condition that is, in many cases, curable by alloBMT.

Over 2 decades, identification of ADA deficiency, which severely impairs immunity, has provided the molecular basis for approximately 40% of autosomal recessive cases of SCID. In this inherited disorder, early descriptions of clinical and pathologic manifestations in ADA deficiency in SCID patients were based on studies of children diagnosed as having classic SCID with marked developmental delay and failure to thrive [49]. Because of the original means of identifying patients to be tested for ADA deficiency, the initial cases seemed clinically and immunologically indistinguishable from patients with other forms of classic SCID. However, they exhibited an autosomal recessive inheritance. These ADA-deficient patients had a disease of neonatal onset usually characterized by lymphopenia, absence of both cellular and humoral immune function, and deficiency of both T and B lymphocytes, which led to infections with bacterial, viral, or opportunistic pathogens, eg, *Pneumocystis carinii* [49]. ADA is the most extensively studied of all the congenital

immunodeficiency diseases because it was the first immunodeficiency disease in which the molecular defect had been fully identified [50] and was the first disease in which the responsible gene had been cloned [51-54]. ADA deficiency was thus described as the underlying problem treated in the first apparently only partially successful gene therapy [47] because of a number of characteristics that have made it a particularly attractive candidate for gene therapy. The involved gene was isolated independently in 1983 by 3 groups, providing investigators almost a decade to gain experience by investigating the gene, its function, and its regulation [51-54]. A landmark finding by Fischer and coworkers in Paris [11] recently described a more dramatic and complete cure of immunodeficiency using a technique similar to that thought to be effective in ADA-deficient patients by Blaese and Culver [47] and Anderson [48]. The French team's results demonstrate clearly that in patients with XLSCID, a selective advantage was conferred on both T- and natural killer (NK) lymphocyte progenitors, which enabled full-blown development of mature and functioning T and NK lymphocytes [55] as well as reconstitution of B lymphocytes, immunoglobulin, and antibody synthesis. Thus, this has become an era of gene therapy with exciting developing technology that will allow for treatment of various congenital and acquired genetic disorders as well as infectious diseases. Successful clinical trials in the next century should make gene therapy an efficacious, accepted approach, revolutionizing the treatment of numerous diseases—including immunodeficiencies, certain cancers, and other genetic diseases—and translating scientific knowledge into clinical reality [10,56,57].

Correction of DiGeorge Syndrome by Thymus Transplantation

DGS was first considered a rare disorder attributable to failure to develop the thymus and other derivatives of the third and fourth pharyngeal pouches, which explains the association of hypoparathyroidism, malformations of the aortic arch, and facial abnormalities. These patients were found to have hypoplasia of both the thymus and the parathyroid glands. This disease was recognized and first described by Angelo DiGeorge, a Philadelphia endocrinologist [58]. This "experiment of nature" became a major subject of investigation for geneticists and immunologists. No sooner had Cooper et al. [59-62] and Warner et al. [63] presented their findings differentiating the separate origins of 2 distinct lymphoid systems [64], than DiGeorge [65] described the complex set of developmental anomalies that provide the human counterpart of the Minnesota animal experiments that in human, as in the chicken [59] and the rabbit [66], separate central lymphoid organs control the maturation of humoral and cellular immune functions. DiGeorge recognized that survival of these athymic patients was limited by overwhelming susceptibility to infection due to failure to develop thymus-dependent immunocompetence, which is characterized by failure to develop a T cell-dependent immunity. Therefore, it was natural to consider the possibility of correcting this clinical immunologic anomaly by transplantation of thymus, as had been so successful in correcting the immunodeficiency of neonatally thymectomized mice [67,68]. In 1995, Markert et al. [69] successfully showed that using discarded postnatal thymic tissue grafts that were partially MHC-

matched with the recipient permitted maturation of normally functioning T cells identified in peripheral blood. Antigen-specific T-cell responses were also developed and B-cell function normalized in the DiGeorge patients. More recently, Markert et al. [70] have corrected 2 patients with the most complete form of DiGeorge thymic aplasia by transplantation of MHC-matched thymus grafts from unrelated donors when a portion of MHC-matched thymus was obtained at surgery for congenital heart disease. Typically, DGS patients have severely impaired production and function of T cells, decreased lymphocytes in the deep cortical areas of the lymph nodes, and increased susceptibility to viral infections. They also exhibit an increased risk of autoimmune diseases and virus diseases such as parainfluenza, adenovirus, and rotavirus, which have caused morbidity in DGS patients [71,72]. The initial apparent success of thymus transplantation from several analyses proved spurious, but MHC-matched allogeneic thymus transplantations did correct the deficiency in producing T cells in DGS patients. Recent studies also showed that the immune reconstitution was due to restitution of ability to produce new T cells and to generate T cell receptor recombination excision circles (TREC) within the thymus [73]. TRECs are the episomal circular DNA excision products of the T cell-receptor gene that are not replicated with cell division and therefore thought to be a marker for cells that have recently emigrated from the thymus. In 1 patient, there was also evidence of thymus function and CD45RA + CD62L + T cells more than 5 years after transplantation [70]. Thus, "experiments of nature" in the clinic provided crucial questions of fundamental nature to be taken to the laboratory and a testing ground for the usefulness of the developing understanding of lymphoid development. Cleveland et al. [74] and soon thereafter August et al. [75] achieved apparent impressive correction of immunologic function in children with DGS by transplantation of an embryonic thymus.

FIRST SUCCESSFUL CURE OF HUMAN DISEASE BY BMT

The cellular basis of immunodeficiencies thus seemed to have been greatly clarified. Strategies to correct by "cellular engineering" some of those primary immunodeficiencies of humans occupied much of our scientific and clinical attention. These new insights were soon followed by attempts to correct clinical immunodeficiency using transplantation of competent immunologic tissue. Based on the anomalies in XLA, DGS and SCID, we reasoned that SCID should be correctable by transplantation using either bone marrow or fetal liver as a source of normal lymphoid progenitor cells. As a first effort along with Richard Hong, we performed a fetal liver transplantation in a young child with autosomal recessive SCID [76]. Soon we had our first potential BMT recipient. Dr. Jerome L'Heureux, a pediatrician in Meridian, Connecticut, had heard me discuss BMT and contacted me regarding a child he was treating for SCID. This 4-month-old boy came from a family in which 11 male children had died from XLSCID. The infant already had had 3 or 4 bouts of pneumonia and most certainly would have succumbed to lethal infection soon thereafter. We were relieved to find that the little boy matched relatively well at the MHC with one of his sisters. So, in 1968 with our clinical research team, we cured a hereditary and lethal disease, XLSCID, for the very first time

by alloBMT [43-46]. However, the child was not perfectly matched with his sister, who was the best donor available but mismatched at the AMHC locus [77] due to a crossover event in the family. Consequently, he developed a severe graft-versus-host reaction that led in turn, to severe aplastic anemia. Instead of attempting to eliminate the bone marrow graft (as others suggested) that all recognized to have cured the manifestations of primary immunodeficiency, we took steps to apply BMT for the very first time to cure also severe aplastic anemia [45,46,78,79] using a second marrow transplant. This resulted in full immunologic, lymphoid, and hematopoietic reconstitution. The boy's red blood cell type switched from his genetic A type to his sister's group O. Indeed, all of his hematopoietic cells that could be made to divide exhibited a female karyotype. He became completely well and has remained healthy and immunologically vigorous for more than 33 years. An aspect which seemed perplexing at first is most interesting. After the initial transplant, this boy developed a sensitivity to mumps antigen, which has persisted to the present time, suggesting that, perhaps, a peripheral T-cell immunity system derived from the sister was included in his immunologic makeup. Twice he was intimately exposed to chicken pox but failed to develop the disease because his sister, whose marrow cells corrected his immunodeficiency, had already become immune to this disease. These findings, though inconclusive, suggest that the first case of successful transplantation of peripheral immunocompetent cells and reconstitution with stem cells had been achieved in this child. These 2 BMTs were the first successful alloBMTs in the world and they cured 2 different otherwise fatal diseases. The first cured SCID, and the second sustained that cure and also cured severe aplastic anemia in the same child [43-46,78,79].

EARLIER EXPERIMENTS WITH BMT USING MOUSE MODELS

Allogeneic BMT After Lethal Total Body Irradiation Has Been Used to Cure Organ-Specific and Systemic Autoimmune Diseases

Our understanding of the immunologic processes has recently been increased markedly by analysis in experimental animal models of immunologic abnormalities and by continued study of human diseases. With Ikehara [80] we had shown that autoimmune diseases appear to be attributable to genetic abnormalities of stem cells and had found that allogeneic but not syngeneic BMT can correct and cure many autoimmunities using marrow from MHC-identical donors. This might be a preferable approach to treatments of autoimmunities as well as immunodeficiencies. In humans, however, alloBMT across MHC barriers often fails, frequently due to graft-versus-host reaction arising from contamination of blood marrow with T cells and rejections based on graft-versus-host rejection; or to hematopoietic (BMT) competition [80]. Onoé et al. [81] showed that in autoimmune-prone mice, no such problems arise with the alloBMT when the bone marrow used comprises bone marrow cells that have been completely depleted of T cells and if myeloablation and immunosuppression of the recipients have been adequate. The rationale for using BMT for prevention and treatment of autoimmune diseases is that we had found that the autoimmunities apparently reside in the

hematopoietic stem cells (HSCs). Fully alloBMT can often cure these systemic as well as organ-specific autoimmune diseases whereas syngeneic BMT regularly failed to cure these genetically-based autoimmune diseases [80,82].

Complex BMT—Prevention and Treatment of Organ-Specific Autoimmune Disease by BMT Plus Organ Allografts

Diabetes mellitus is a heterogeneous disorder, and its pathogenesis remains an enigma. Type I diabetes is insulin-dependent and of juvenile onset. Nonobese diabetic (NOD) mice provide a splendid model of type I diabetes mellitus. In our earlier experiments with Ikehara and his group, we demonstrated that alloBMT can be effectively used to treat systemic autoimmune diseases in (NZB × NZW)F1-specific pathogen-free mice without inducing graft-versus-host reaction, provided that bone marrow cells of young BALB/c *nu nu* donor mice or T cell-depleted marrow (TCDM) cells have been used [82].

These observations prompted us to examine whether or not insulinitis as an organ-specific autoimmune phenomenon can also be treated by allogeneic or syngeneic BMT. In the first study [83], we showed that the lymphocytes infiltrating into the islets are T cells and that alloBMT can, after lethal total body irradiation (TBI), be used to treat this insulinitis and prevent overt diabetes. The newly developed T cells in the allogeneic bone marrow recipients were found to be tolerant to cells with both donor- and host-type MHC determinants, suggesting that BMT may ultimately be developed as a component of treatment of type I diabetes in humans.

BMT for Type I Diabetes Mellitus

With Ikehara, we performed a combined transplantation of fetal or newborn pancreas plus allogeneic bone marrow because we know that organ allografts are accepted if the organ and the bone marrow are from the same MHC-matched donor and transplanted at the same time [84]. NOD mice that had already developed overt diabetes accompanied by destruction of all islet β cells were lethally irradiated and then reconstituted with allogeneic BALB/c bone marrow cells. The pancreatic tissues from fetal or newborn BALB/c mice were then engrafted under the renal capsules of NOD mice that underwent BMT. Three months after transplantation, the mice exhibited normal correction to glucose levels and a correction to normal responses in the glucose tolerance test. As neither insulinitis nor rejection occurred, we had succeeded in treating type I diabetes by the combined transplantation of pancreas and bone marrow, which worked very well. Hyperglycemia was reversed and eliminated, deficient glucose tolerance curves were corrected, and circulating insulin levels increased from low to normal ranges [85].

Complex BMT—Successful BMT Plus Bone Grafts Were Used to Prepare Mixed Chimeras in Autoimmune Combinations That Otherwise Resisted Stable Chimera Formation

The HSCs of MRL/lpr mice are abnormally radioresistant to TBI. Even after successful BMT, these mice improve initially but regularly suffer progressive relapse of their lymphoproliferative syndrome and redevelop autoimmune disease after conventional BMT [86]. We have recently found

that there is an MHC restriction between HSCs and stromal cells as well as between lymphoid cells [88]. To prevent recurrence of autoimmune diseases in MRL/lpr mice, Ikehara's group carried out both BMTs and bone grafts to replace not only hematopoietic cells, but also stromal cells with donor stromal cells. When bones have engrafted along with BMT, donor-derived stromal cells present in the engrafted bones migrate into the recipient bone marrow and the latter are then replaced with both donor-derived stromal cells and donor-type hematopoietic cells. Based on these findings, attempts were made to prevent the recurrence of autoimmune diseases, such as systemic lupus erythematosus and rheumatoid arthritis, in MRL/lpr mice by the transplantation of both bone marrow cells and bone grafts. Thus MRL/lpr mice had TBI (8.5 Gy) and then were reconstituted with C57BL/6 bone marrow cells plus bone grafts. These mice were given a new long-term lease on a healthy life and were also prevented from experiencing recurrence of the autoimmune or lymphoproliferative disease [87]. Using another chimeric-resistant combination (DBA/2 → C57BL/6), Hisha et al. [88] confirmed that chimeric resistance can be overcome by BMT plus bone grafts when co-grafting DBA/2 donor bones from which the hematopoietic cells had been removed but in which stromal cells remained. In another study, Nakagawa et al. [89] showed that allogeneic bone marrow cells plus bone transplantation enable the complete prevention and correction of joint disease in male NZB/Kn mice, which spontaneously develop severe inflammatory polyarthritis at the age of 4 months, followed by gait disturbances and progressive swelling and ankylosis of the joints of the paws. Our findings indicate that BMT alone did not cure this experimental arthritis. But when BMT plus bone transplants were given to the NZB/Kn mice, their severe rheumatoid arthritis was also cured [87-89].

Complex BMT and Other Transplantations to Cure Autoimmune Diseases—BMT With Fetal Thymus Transplantation

Old congenic MRL/+ mice develop both pancreatitis and sialoadenitis. These symptoms are similar to those in human patients with Sjögren's syndrome. It is well known [90] that in humans, the success rate of BMT in patients older than 45 years is low. Recently, it has been shown [91] that the low success rate of curing this experimental type of Sjögren's disease by BMT was due to the aging of the thymus and that either bone marrow or fetal HSCs plus fragments of adult or fetal bone plus embryonal thymus grafts can be successfully employed to treat the late-onset Sjögren's-like autoimmune diseases in MRL/+ female mice. A Sjögren's-like syndrome that occurs in these aging old MRL/+ mice could not be cured by lethal TBI plus conventional BMT alone. However, if BMT plus a fetal thymus transplantation plus bone transplantations were all used, the Sjögren's syndrome appeared to be completely cured. This combination of BMT plus bone plus fetal thymus transplantation represents a potentially important new strategy for cellular engineering that can possibly cure the experimental form of Sjögren's syndrome and, thus, possibly cure a complex autoimmune disease that could not be cured by BMT alone. This approach needs to be considered in the present context [91].

Haploidentical BMT for Occlusive Coronary Vascular Disease

In some ways, the hemolytic anemias are the best studied autoimmune diseases of humans. In some instances, the actual antibodies are known precisely. In other instances, evidence of antibody reacting with the red blood cells and participation of complement have been demonstrated. Mice of the New Zealand Black (NZB) strain regularly develop autoimmune hemolytic anemia plus hyalinizing renal lesions similar to the lesions observed in lupus erythematosus. When NZB mice are mated to normal New Zealand White (NZW) mice, the F1 offspring (NZB × NZW)F1 combination or B/W mice develop elevated titers of antinuclear autoantibodies, anti-double stranded DNA (anti-dsDNA), and anti-cardiolipin autoantibodies. These mice die early in life from progressive glomerulonephritis. They also may exhibit accelerated coronary artery disease [92-94]. Both the advanced and progressive renal disease and the coronary artery disease were corrected and cured by successful BMT. In another study [95], we used reciprocal haploidentical BMT to determine whether the occlusive coronary vascular disease (CVD) of male (NZW × BXSB)F1 (W/B)F1 mice is treatable or transferable as a component of the systemic autoimmunity arising from abnormalities in hematopoietic marrow. TCDM of male W/BF1 mice was transplanted into autoimmune-resistant B6C3F1 mice. CVD developed only in otherwise healthy mice with systemic autoimmunity causing the coronary vascular lesions and the glomerulonephritis. These results indicate that murine lupus-associated CVD may be a reflection of systemic autoimmunity that is transferable by TCD BMT to nonautoimmune recipients. Further, these findings show that similarities in the pathogenesis of human and murine lupus-associated CVD exist [95].

BMT for Prevention of Crescentic Glomerulonephritis Disease

Rapidly progressive crescentic glomerulonephritis frequently occurs concomitantly with certain forms of vasculitis, including Wegener's granulomatosis and small vessel polyarteritis nodosa [96-98]. Kinjoh et al. [99] working in our laboratory bred a new inbred strain of mice by breeding that used selection for crescentic glomerulonephritis to express a highly lethal form of crescentic glomerulonephritis and also small vessel polyarteritis nodosa. These mice, when treated by BMT from BALB/c donors, showed inhibition of and even reversal of the polyarteritis nodes and a crescentic glomerulonephritis after transplantation of bone marrow from the BALB/c donors. The mice of this new SCG/Kj strain also showed autoantibodies similar to antineutrophilic cytoplasmic autoantibody that were suppressed after BMT from healthy nonautoimmune donors [100].

Mixed BMT for Induction of Immunologic Tolerance

Ildstad et al. [101] found that chimeras that received transplants of mixed TCDM from both allogeneic and syngeneic donors can fully reconstitute hematopoietic and immunologic function after supralethal TBI; these mixed bone marrow chimeras do not express the immunologic deficits observed after TBI plus BMT with fully allogeneic bone marrow. Reflection on these findings caused El-Badri

et al. [102] to extend Ildstad's research by showing, frequent survival with stable mixed chimerism and normally vigorous, functioning immune systems in C57BL/6 mice that received transplants of TCDM from both BALB/c allogeneic donors and C57BL/6 syngeneic donors that differed from each other across the entire MHC barriers. These stable mixed chimeras did not have the immunodeficiencies that are regularly observed in fully allogeneic chimeras [102].

Mixed BMT as a Well-Tolerated Cure for Autoimmunities

Wang et al. [103] in my laboratory then tested the possibility of developing BMT further to prevent autoimmune diseases and reconstruct the full immune functions of irradiated mice at the same time by transplanting mixed TCDM from both allogeneic normal healthy autoimmune-resistant donor mice and syngeneic autoimmune-prone donor mice into lethally irradiated autoimmune-prone BXSB recipients. Mice of this BXSB strain develop a spontaneous and highly lethal lupus-like illness, and the disease is worse in males than in females [104]. Acceleration of disease phenotype in males is due to a Y chromosome gene termed the Y chromosome autoimmune accelerator (*Yaa*). The function of these gene is not known except that when expressed in a susceptible strain it is able to cause disease [105]. Subsequently, in 1999, Wang et al. [106] attempted to prevent autoimmune diseases and, at the same time, to reconstruct full immunity functions of irradiated autoimmune mice by transplanting mixed TCDM from both the allogeneic autoimmune-resistant donor and the syngeneic autoimmune-prone donor marrow into lethally irradiated BXSB recipients. This approach of creating stable mixed chimerism proved to be a highly successful treatment of numerous manifestations of autoimmune disease. The concomitant regularly fatal chronic renal disease was corrected and could be both prevented and cured in the BXSB mice [103,106].

Osteoblasts as Facilitators of Allogeneic Stem Cell Engraftment

Because interaction of stem cells with the bone marrow stromal microenvironment is required for development, maturation, and differentiation of HSCs and because bone transplants have been found to facilitate BMT in some combinations, El-Badri et al. [107] tested bone progenitor cells, osteoblasts, as facilitators of allogeneic stem cell engraftment. Osteoblasts, purified from donor murine long bones, were cotransplanted with marrow stem cells into fully allogeneic mouse strains. The mice that received transplants demonstrated long-term survival, were free of disease, and were entirely engrafted with cells of both the allogeneic donor and syngeneic donor strains. Thus, bone progenitor cells or osteoblasts were found to represent an essential component of the stromal cell population that can facilitate engraftment of marrow stem cells in an allogeneic environment. In recent experiments, El-Badri et al. [108] extended their investigations to evaluate the capability of highly purified preparation of HSCs from healthy donors to prevent the development of glomerulonephritis and vasculitis in an autoimmune-prone mouse strain. Initially, pure allogeneic HSCs failed to engraft in the (NZW × BXSB)F1 (W/B)F1 mice that received transplants whether HSCs were transplanted alone or with

osteoblasts. To promote engraftment, the investigators gradually expanded the number of allogeneic HSCs to 2-5 million cells and with this change successfully engrafted and reconstituted the lymphohematopoietic systems of the transplant recipients. Histologically, the mice that received transplants were free of the glomerulonephritis and the vasculitis of the heart and the lung that is characteristic of mice of this strain [108].

Future Experiments Using Mouse Models

Because Ildstad and colleagues [109] concluded that lethal preparative measures would not be acceptable for preparations to treat autoimmune diseases, we plan now to use a more gentle method of producing stable mixed chimerism, which was described by Sharabi and Sachs about 10 years ago [110] to achieve mixed marrow transplantation and mixed hematopoietic chimerism without lethal irradiation. The protocol [111] utilizes monoclonal antibodies to the mature T-cell subsets (CD4 and CD8) to eliminate the T cells, which otherwise resist engraftment of allogeneic bone marrow. This treatment permits engraftment with a very low dose of whole body irradiation of 3 Gy plus 7 Gy local x-irradiation to the thymus or even no radiation if very large quantities of donor bone marrow are used. Other diseases that we are approaching using this gentle manipulation will include diabetes type I in NOD mice; the atherosclerosis of ApoE^{-/-} knockout mutant mice that have not been cured by BMT or stem cell transplantation (SCT); and the senility-accelerated disease in SAM-P mice that is accompanied by and driven by autoimmunities.

IMMUNE TOLERANCE MECHANISMS

Immune tolerance can be defined as the enduring acceptance of a viable organ or tissue graft from a genetically different individual without the need for permanent immunosuppression [111,112]. Various attempts have been made to induce persistent and complete tolerance across MHC barriers to ultimately be applied to organ transplantation in humans. Tolerance as produced by BMT involves 3 mechanisms: clonal deletion, anergy, and cellular mechanisms of immunosuppression. Associated with these proposed mechanisms are a number of treatment protocols which have been correlated with varying degrees of successful tolerance induction. Clonal deletion, which occupies the main part of self-tolerance, is induced by reconstituting lethally irradiated recipients with donor hemolymphoid cells [113]. We have previously reported [84,85,114] that successful organ allografts can be achieved by carrying out BMT in conjunction with organ allografts, thus creating stable mixed chimerism. It has recently been noted that potent and persistent tolerance may be induced using clonal anergy mechanisms (absence of signals from co-stimulatory molecules in an interaction between host T cells and antigen-presenting cells) and clonal suppression mechanisms (the term *clonal suppression* can reflect the concept that immunosuppressive treatment involves alteration of nonspecific regulator cells leading to death in some effector cell populations and a lack of responsiveness in others) [115,116].

Morita et al. [117] in Ikehara's laboratory in Osaka, Japan, have established donor-specific tolerance across MHC barriers to skin allografts by portal venous (PV) injection of

allogeneic cells without using any chemical immunosuppressants and/or irradiation. Recently, the mechanism by which the donor-specific tolerance is induced after PV injection has been found to be due to induction of clonal anergy in the CD8⁺ T cells of the recipients [118]. This tolerance appears to be maintained by suppression of the function of donor-reactive T helper (Th)1 cells via a dominant influence of their Th2 counterpart [116]. These investigators have previously found that even when injected intravenously (IV), bone marrow cells accumulate mainly in the host liver but not in the bone marrow or spleen. Zhang et al. [119] examined the fate of the injected allogeneic spleen cells and the migration patterns of allogeneic spleen cells, bone marrow cells, and thymocytes after their administration via different routes. They showed that using the PV route suppresses donor-specific rejection, although the preimmunization of recipients with allogeneic thymocytes via the PV route induces a rapid removal of subsequently IV injected donor spleen cells. These findings indicated that the allogeneic HSCs trapped in the liver may play a crucial role in the induction and maintenance of the tolerance induced by the PV route. Based on the above findings and using an initial PV injection of spleen cells or bone marrow cells plus IV injection of hematopoietic bone marrow cells followed by application of skin allografts, we presented a single-day protocol for the induction of potent and durable immunologic tolerance across MHC barriers that may prove more applicable to organ transplantation in humans than would a protocol that requires several days to complete [117].

With the establishment of a single-day protocol, Morita et al. modified this method and applied it to pigs [120] and guinea pig-to-mice xenogeneic transplantations [121]. Allogeneic bone marrow cells of donor pigs were injected using the PV route. The pigs accepted donor allografts, autologous grafts, and third-party grafts at the same time. One hundred percent of skin allografts were achieved, confirming that this strategy can overcome MHC barriers and, even in pigs, produce donor-specific tolerance over the allogeneic barriers, although an immunosuppressant such as cyclosporin A or tacrolimus is necessary. The combination of PV-induced tolerance using a single-day protocol plus pilot skin grafts could be a great advantage to organ transplantation in humans [120,121].

THE FUTURE OF XENOTRANSPLANTATION

At the entrance of a new era in which tolerance can be produced by creating stable mixed macrochimerism and clinical xenotransplantation, the transplantation of organs and tissues between animal species is becoming a valid option, owing primarily to the severe shortage of allogeneic donor organs. In addition to the ability to increase the availability of donor organs, xenotransplantation offers great advantages over allogeneic transplantation, including the ability to genetically engineer the donor xenograft and the resistance of some xenografts to infection by human viruses [122]. Xenotransplantation was first performed in the 1960s in several patients suffering from renal failure who received kidneys from chimpanzee donors [123-125]. Six of 7 patients who received the kidney transplants immediately demonstrated physiological activity and despite treatment with

immunosuppressive agents, acute rejection occurred. Five patients died from over-immunosuppression. One kidney functioned normally for 6 months. Starzl et al. [126] observed early graft function in kidneys transplanted from baboon donors. These patients received immunosuppressive agents and local irradiation to prevent acute rejection. However, as in the previous study, most patients died from sepsis related to the immunosuppression. The longest surviving patient's kidney functioned for almost 60 days. These 2 studies showed that the most current immunosuppressive therapies are unable to control acute xenogeneic rejection and were, in fact, too toxic. The discovery of cyclosporine and tacrolimus prompted investigators to reattempt xenotransplantation. The pig is the most suitable donor animal for humans because these animals are anatomically and physiologically sufficiently similar to humans, although xenografts used for human transplantation are normally destroyed by the host within minutes by hyperacute xenograft antibody complement-based rejection, despite the use of immunosuppressive agents [127]. An improved understanding of the immune recognition and rejection of xenografts has resulted in new techniques for producing stable mixed chimerism and xenogeneic tolerance. Considerable progress has been made in our understanding of the molecular genetic basis of the rapid hyper-acute antibody-mediated rejection mechanisms that occur in xenogeneic organ rejection [128]. However, the prompt and vigorous immune response to xenogeneic tissue still remains a significant barrier to clinical xenotransplantation [121]. The use of partial conditioning regimens to promote engraftment of xenogeneic HSCs, the development of donor-specific tolerance, and solutions to the remaining hurdles in clinical application may eventually make xenotransplantation in humans a clinical reality [122].

ALLOGENEIC VERSUS AUTOLOGOUS OR SYNGENEIC BMT

In contrast to the impressive success of alloBMT for treatment, our preclinical results do not reveal comparable promise for autologous or syngeneic BMT for treatment of mice that may already have developed systemic autoimmune disease. The findings in autoimmune-prone mice support the ultimate application of BMT from allogeneic donors not prone to develop systemic autoimmune diseases as an approach to prevent or treat life-threatening autoimmune disease [80,82,87,129-131].

Allogeneic Versus Syngeneic BMT

In genetically determined autoimmune disease in animal models, the superiority of alloBMT over syngeneic marrow transplantation has been repeatedly demonstrated in our experiments. We have never carried out autologous BMT to test its potential value for preventing or treating autoimmune diseases in mice. However, syngeneic BMT in these autoimmune models has never been followed by effective treatment or prevention of disease. By contrast, alloBMT from autoimmune-prone mice produces genetically determined autoimmune disease after intensive irradiation, with or without additional chemotherapy [132].

Autoimmunities of NZB, B/WF1, BXSB, MRL/lpr, W/BF1, and NOD mice have each been caused when BMT or stem cell transplants from these autoimmune-prone

strains are given to strains of mice that do not ordinarily develop or express any autoimmune disease. Such transfers of disease by stem cells were first seen by Ikehara et al. [133] and Sardiña et al. [134] with NZB donors. Kirzner [135] extended these findings using stem cell preparations to produce the characteristic autoimmunities and disease from (NZW × BXSb)F1 (W/B)F1 hybrid donors to B6C3F1 nonautoimmune recipients. Kirzner demonstrated for the first time the prevention [136] of degenerative CVD in autoimmune-prone male W/BF1 mice by the transplantation of haploidentical TCD marrow or HSC preparations. Closer examination of the progression and development of autoimmunity in a previously nonautoimmune strain, such as B6C3F1, may provide additional information concerning the relationship between autoimmunity, which arises from a genetic predisposition, and the effect of host influences, including thymic education, induction of tolerance, and other yet-to-be-defined influences on the engrafted hematopoietic and immunologic cellular systems.

Diseases Not Cured by Lethal TBI + Syngeneic or Autologous BMT or Stem Cell Transplantations

There have recently been reports on the rapid recurrence or persistence of autoimmune diseases after lethal TBI plus syngeneic or autologous BMTs or stem cell transplantations in classic lupus (B/W)F1 mice; idiopathic thrombocytopenia purpura, multiple autoimmunities, and lethal renal disease in NZB mice; fulminating glomerulonephritis, occlusive CVD, and lethal coronary occlusions that were correlated with anticardiolipin antibodies in the (NZW × BXSb)F1 (W/B)F1 model; Sjögrens syndrome in MRL/+ old female mice; severe polyarthritis in NZB/Kn male mice; and type I diabetes in the NOD mice. None of these diseases can be prevented or cured after lethal TBI coupled with syngeneic BMT (personal observations). Although we have not attempted to prevent or treat organ-specific or systemic autoimmunities by autologous BMT, we believe the failure of syngeneic BMT to prevent or cure these diseases is sufficient. Another observation that supports the crucial role of alloBMT as the best approach to prevention or treatment of autoimmune disease is the demonstration that lethally irradiated strains of mice that do not naturally develop autoimmune diseases spontaneously can regularly be induced to develop autoimmune diseases by passively transferring the autoimmune disease in its appropriate expression by BMT from the autoimmune-prone mice to the autoimmunity-free mice [82,129].

Mixed Allogeneic Plus Syngeneic BMT and Stable Mixed Chimerism

We have found that mixed allogeneic plus syngeneic BMT offers an effective and well-tolerated—and often dramatic—approach to treating high-risk and often lethal autoimmune, renal, and cardiovascular diseases in mice. The possibility of applying these approaches to humans deserves consideration. Further application of the gentler, kinder methods to produce stable mixed macrochimerism seems especially attractive as an option to achieve regular and durable immunologic tolerance in allotransplantation and even xenotransplantation of organs and tissues in humans, as well as in experimental animals.

CONCLUSION

The “experiments of nature” that pointed the way to the discovery of the role of the thymus, the germinal centers, and the plasma cells in the immune system led to the development of a most important approach to treating disease—cellular engineering. Thus, we believe that the future of cellular engineering continues to be bright. Fortunately, technical hurdles such as those confronting gene therapy today are not insurmountable. With much ingenuity and several essential discoveries, gene therapy will follow, at first with halting steps, then a brisk walk, and, ultimately, a full run, when gene therapy protocols are applied to transplantation much sooner than some think.

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