

Graft-versus-Host Reaction and Rejection after Experimental Small-Bowel Transplantation

Minireview based on a doctoral thesis

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INTRODUCTION

Since the first successful kidney transplantation in 1954 (108), developments in the field of organ transplantation have been rapid. A deeper understanding about the immunological reactions that occur after transplantation and the introduction of new immunosuppressive drugs have played a role in this development. Nowadays, hearts, lungs, livers, and pancreases are also transplanted successfully. However, small-bowel transplantation is still at an experimental stage, despite the fact that an obvious clinical demand exists. The small bowel appears to be one of the most difficult organs to transplant successfully. One reason for this being its specific immunology: because of the large amounts of lymphoid tissue present in the graft, not only rejection but also graft-versus-host reaction (GVHR) may occur after small-bowel transplantation.

There are two groups of patients who are in particular need of small-bowel transplantation; viz. (i) infants born with malformations such as atresia, aganglionosis or gastroschisis leaving a length of just a few centimeters of normally functioning bowel (52) and (ii) patients who, for one reason or another, have lost most of their small bowel, e.g. from mesenteric vascular diseases, volvulus, radiation enteritis, traumatic disorders or inflammatory diseases (138). All these conditions may lead to "short-bowel syndrome" - a state of malabsorption and malnutrition. Although life-long dependency on total parenteral nutrition may in some cases be well-tolerated, adverse effects are not uncommon. Major obstacles are catheter septicaemia and liver dysfunction (52, 138).

Small-bowel transplantation was first described as early as 1902 when Carrel anastomosed segments of the small bowel to the neck vessels of dogs (17) but it was not until the late fifties that the interest in this field increased. Lillehei, who is regarded as the pioneer of small-bowel transplantation, developed a technique for small-bowel transplantation in dogs. He investigated the effect of graft ischemia in autologous transplantation (97) as well as the fate of allogeneic grafts (98).

Despite his fundamental research work, Lillehei was not the first to perform a human small-bowel transplantation. In 1964, Deterling and colleagues in Boston

performed two transplantations, both in children, one with an ileal segment from a living related donor and one from a cadaveric donor; both of the grafts were lost within 48 hours (2). Three years later, Lillehei performed a small-bowel transplantation in a 46-year-old woman; this operation was also unsuccessful but, in contrast to the two transplantations performed in Boston, this case is carefully documented (99). Up until 1970 only five further attempts at small-bowel transplantation were performed (2, 32, 117, 118); the last one, performed by Fortner and colleagues in 1970, was the most successful with the recipient surviving for 76 days before dying of septicaemia (32).

As a consequence of these discouraging results, no further cases were performed until the introduction of cyclosporine A (CyA) about 15 years later. The first reported small-bowel transplantation using CyA for immunosuppression was performed in 1985 in Toronto with the patient surviving for 11 days (20). In 1987, a young patient in Pittsburgh received a small bowel as part of a complete multivisceral graft including stomach, small bowel, colon, liver, and pancreas and survived for more than six months (155). This case was soon followed by reports of successful small-bowel and small-bowel/liver transplantations from Kiel (27), London, Ontario (48) and Paris (43). Today about 100 small-bowel transplantations have been performed around the world, most of them in Pittsburgh (164).

Rejection

The major histocompatibility complex

One major problem encountered in solid organ transplantation is that of rejection. This immunological reaction occurs as a result of histoincompatibility between the donor and the recipient. These genetic differences were discovered as early as 1937 by Gorer, who transplanted tumours between various mice strains (41). The gene region coding for these "transplantation antigens" is called the major histocompatibility complex (MHC), regardless of species. In the human, it is named the "human leukocyte antigen" (HLA), in the mouse, "histocompatibility locus 2" (H-2), and, in the rat, "rat transplantation antigen 1" (RT1).

In the human, the genes coding for HLA are located on chromosome 6 and can be divided into class I, II and III, where the region coding for class III lies between those coding for the class I and class II genes. Both MHC class I and II consist of more than one locus. MHC class I molecules are named A, B, C and class II, DP, DQ, DR. For each loci there are several alleles and the MHC is thus extremely polymorphic. MHC class III codes for various molecules, e.g. substances involved in the complement system and tumour necrosis factor (TNF). The gene products of MHC class I are designated RT1A, Pa, F, E, G and C in the rat, whereas class II gene products are named B and D (37).

MHC class I antigens are expressed on most nucleated cells (59). MHC class II antigens are normally expressed on cells with functions within the immune system, such as B lymphocytes, antigen-presenting cells, macrophages, activated T lymphocytes and endothelial cells. Epithelial cells of various organs may also express these antigens (22) and, after stimulation with interferon- γ (IFN- γ), many other nucleated cells can express MHC class II antigens (55).

Cells of the immune system predominantly make selective use of the surface expressed MHC. Thus, T lymphocytes expressing the surface marker CD8 ("cytotoxic" T lymphocytes) recognize foreign antigens in association with MHC class I, and T lymphocytes expressing CD4 ("helper" T lymphocytes) recognize antigens in association with MHC class II.

Rejection types

Rejection can be divided into three sub-classes: (i) hyperacute rejection, (ii) acute rejection and (iii) chronic rejection.

Hyperacute rejection, which is antibody-mediated, may occur within minutes after revascularization of the graft and is a result of earlier sensitization against donor HLA, e.g. from previous grafting, blood transfusions or pregnancy (78).

The typical acute rejection is mediated by T lymphocytes and usually occurs within the first months after transplantation. The mechanisms involved are complex and still not fully understood. In a simplified manner, the process is usually described as starting with the presentation of an antigen by macrophages which, by means of secretion of interleukin-1 (IL-1), stimulates CD4-positive T lymphocytes, which then secrete interleukin-2 (IL-2) that activates CD8-positive T lymphocytes. The T lymphocytes proliferate and both subsets are capable of being cytotoxic when in contact with appropriate target cells. The process is, however, more complicated and includes a cascade of cytokines with both stimulatory and inhibitory functions on a variety of cells and there occurs not one single mechanism but a combination of events that leads to graft destruction.

Most investigations in this field have been performed in non-immunosuppressed animals, whereas patients transplanted naturally are treated with immunosuppressive drugs. Rejection occurring under immunosuppressive treatment is assumed to be driven by the same mechanisms as rejection occurring in untreated animals, although the evidence for this is so far incomplete.

The graft can be presented as foreign to the T lymphocytes of the recipient in three different ways: (i) by donor antigen-presenting cells expressing normal self proteins in association with MHC class I antigens, (ii) by donor antigen-presenting cells expressing processed recipient proteins in association with MHC class II antigens, and (iii) by recipient antigen-presenting cells expressing processed donor proteins in association with MHC class II antigens. Obviously, cross-reactivity plays a

major role in alloreactive responses; i.e. recipient T lymphocytes recognize an antigen not only in association with self MHC but also in association with donor MHC and today it is not known when and to which extent this phenomenon occurs.

Chronic rejection may jeopardize the long-term function of transplanted organs. The mechanisms involved are poorly understood, but several factors contribute to the development of chronic rejection that may lead to graft loss. The graft vessels are a main target, the vessel wall lesions resembling those occurring in atherosclerosis. Narrowing of arteries and arterioles occurs as a result of infiltration of the intima by mononuclear cells, smooth muscle cells and fibroblasts. Several cytokines and growth factors are involved as regulators in this process. The endpoint is structural changes in the parenchyma of the organ leading to a deterioration of graft function. (For review see 121.)

Graft-versus-host reaction (GVHR)

GVHR is a frequent well-known complication after bone-marrow transplantation, but may also occur after solid organ transplantation. The risk of GVHR increases with the amount of viable lymphoid tissue included in the graft, i.e. there is a greater probability of GVHR after small-bowel transplantation than after kidney transplantation. Incidents of GVHR have been described after the transplantation of small bowel (166), liver (131), heart-lung (70), and also after the transplantation of pancreas and spleen (24). The knowledge about this immunological reaction comes mainly from studies in bone-marrow transplantation.

The development of GVHR has been stated to require a histoincompatibility between the donor and recipient, presence of immunocompetent cells in the graft and inability of the host to reject the graft (9). Under certain conditions, GVHR may occur when there is no genetic difference between the donor and recipient (38, 126). Analogously, the severity of a GVHR is assumed to be dependent upon the immunogenetic disparity between donor and recipient, the number of immunocompetent cells existing within the transplant as well as the immunocompetence of the recipient.

GVHR may be acute or chronic and the number of clonable T lymphocytes transferred to the recipient has been found to correlate with the development of GVHR (75).

Acute GVHR affects mainly the skin, liver and gastrointestinal tract (151), the epithelial cells in these organs being the main target of the disease (114). The first sign of acute GVHR is often a skin rash that presents on palms, soles and/or ears and eventually progresses to total erythroderma. Symptoms from the gastrointestinal tract, e.g. nausea, diarrhoea, abdominal pain and paralytic ileus, or the liver, e.g. hyperbilirubinemia and elevations in alkaline phosphatase and aminotransferase

levels, often develop at a later stage. Acute GVHR is also associated with a profound immunodeficiency that leads to an increased susceptibility to infections (31).

Acute GVHR has been described to comprise two phases; one afferent and one efferent. The afferent phase results in T lymphocyte activation and consists of three steps; (i) antigen presentation, (ii) activation of individual T lymphocytes and (iii) clonal proliferation and differentiation. The efferent phase is complex and still poorly understood, but involves the release of several cytokines which have various effects on T lymphocytes, B lymphocytes, macrophages and natural killer (NK) cells and are also directly involved in destroying target cells (31).

The skin and liver are the main targets for chronic GVHR; the lungs, mucosal surfaces and salivary glands may also be affected. Symptoms from the gastrointestinal tract are, however, uncommon (147). The immune system is also affected in this case (31). Acute GVHR is not a prerequisite for the development of chronic GVHR but one or more episodes of acute GVHR increase the risk of developing a chronic GVHR (31, 147).

The mechanisms behind chronic GVHR are probably, at least to some degree, autoimmune (120); autoreactive T lymphocytes can be detected in the skin and chronic GVHR in many aspects resembles autoimmune diseases such as scleroderma (31).

Specific problems related to small-bowel transplantation

Transplantation of the small bowel presents special difficulties when compared with that of other organs. The small bowel contains large amounts of immunocompetent cells which may act both as antigenic stimuli that lead to rejection and as attacking cells causing GVHR (49). These immunological events, as well as the difficulties involved in monitoring rejection, will be discussed in further detail later.

The primary vessels of the intestine are relatively small, which may give rise to technical problems that eventually lead to thrombosis. The choice of vascular anastomoses may also be of significance with regard to metabolic and immunological complications. Venous drainage directly into the systemic circulation via the caval vein may, at least experimentally, lead to hyperammonemia and changes in the serum amino-acid profile (83, 136) and there is also evidence that portal drainage with venous anastomoses between the vein of the graft and the portal vein of the recipient may entail certain immunological benefits (84, 135).

Ever since the late sixties, it has been known that the transplanted liver can induce immunological tolerance (16). In certain rat strain combinations, the simultaneous transplantation of the liver can protect heart grafts from rejection (74); this phenomenon has also been described following combined small-bowel/liver transplantation (178). After BN to Lewis multivisceral transplantation, however, no protective effect of the liver on the small bowel could be observed - the small bowel

was rejected as severely as after transplantation of that organ alone (111). After human small-bowel and small-bowel/liver transplantation, the incidence of early, acute rejection has surprisingly been found to be lower in patients grafted with only the small bowel (166). Others have, however, proposed that simultaneous liver grafting really does have a protective effect (102).

The small-bowel mucosa is highly sensitive to ischemia (138) and preservation is thus an important part of small-bowel transplantation. At present, the optimal method of vascular and luminal preservation has not been established; several groups have compared different techniques (153) as well as different preservation solutions and have come to varying results (15, 119, 141). The preservation solutions in present use have not been developed with the specific aim of preserving the small bowel and conventional solutions should possibly be further modified in order to provide this sensitive organ with optimal protection.

The small-bowel mucosa acts as a barrier in preventing the passage of bacteria and toxins into the blood stream and, if the mucosa is damaged, there is risk of severe infections (50). Septicaemia is a major complication after human small-bowel transplantation (102, 166) and may initially develop as a result of an ischemic injury to the mucosa. Rejection episodes that damage the intestinal barrier can also lead to a translocation of bacteria from the lumen of the graft to the reticuloendothelial system of the host (50, 123). Furthermore, during GVHR, there is an increased risk of septicaemia due to the impaired immunological functions of the recipient (47). In addition, the use of potent immunosuppressive drugs may lead to the manifestation of opportunistic infections. Thus, the use of antibiotic drugs plays a very important part in the management of patients who have undergone small-bowel transplantation.

For small-bowel transplantation to be successful, it is necessary for the graft to be capable of mounting a normal immune response to the potentially harmful substances to which it is continuously exposed. Experiments in both rats (176) and pigs (3), where the transplanted intestine was challenged with bacteria and virus, indicate that a stable, non-rejecting graft can still function immunologically and produce almost normal levels of secretory IgA.

Experimental techniques

Previous investigations in the field of small-bowel transplantation were performed in large animal models, i.e. the dog (57, 97, 98) and the pig (60). In 1971 Monchik and Russell presented an auxiliary, heterotopic technique for small-bowel transplantation in the rat (110). The superior mesenteric artery and the portal vein of the graft are anastomosed to the recipient's aorta and caval vein, respectively, and the proximal and distal ends of the bowel brought out as stomas. This technique is still the most widely used in experimental small-bowel transplantation. Two years later, Kort et al. described a model for orthotopic small-bowel transplantation in the rat, where the

graft vessels are anastomosed to the aorta and the portal vein, the native bowel resected and the graft interposed end to end in continuity (84).

Heterotopic transplantation, which is better tolerated by the animal, is ideal for immunological studies but, when performing physiological studies, orthotopic transplantation is the method of choice (51, 91).

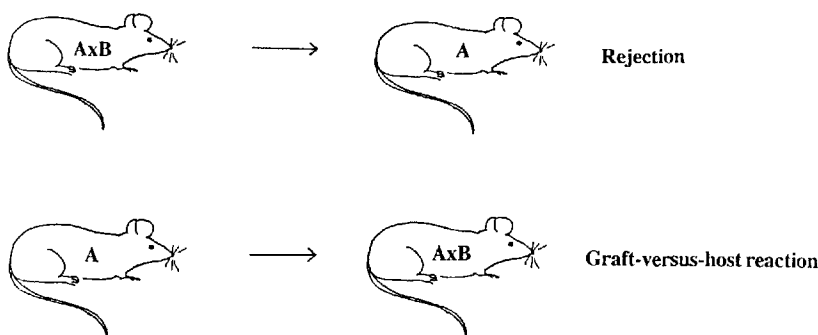


Figure 1. Semi-allogeneic (AxB to A) and semi-syngeneic (A to AxB) transplantation leading to rejection and GVHR, respectively.

In many respects, the rat is a suitable model for studying small-bowel transplantation. One of the reasons for this is the possibility to study rejection and GVHR as separate phenomena by using inbred parental strains and F₁-hybrids (figure 1). Transplantation of the bowel from a F₁-hybrid to a rat of the parental strain leads to a uni-directional rejection (semi-allogeneic transplantation), whereas transplantation from a rat of parental strain to a F₁-hybrid (semi-syngeneic transplantation) results in uni-directional GVHR (110).

Rejection after small-bowel transplantation

Acute rejection of the small bowel has been characterized mainly in untreated animals. The earliest morphological findings are an infiltration of the mucosa and submucosa by mononuclear and polynuclear leukocytes, cryptitis and the blunting of the villi. As the infiltration of cells progresses, signs of vasculitis and mucosal sloughing can also be seen. Finally, there ensues a complete destruction of the mucosa and the submucosa and a necrosis of the graft (49, 133). Macroscopically, acute rejection is seen as a distention of the graft, thickening of the intestinal wall, inflammation of the mesentery and a swelling of the mesenteric lymph nodes.

In chronic rejection, there is progressive destruction of Peyer's patches and mesenteric lymph nodes but, in contrast to acute rejection, the bowel wall does not seem to be the primary target (87). Infiltration and destruction of graft vessels have been described during chronic rejection under immunosuppressive treatment both experimentally (5) and clinically (42, 164).

When, as a result of rejection, the bowel loses its barrier function, bacteria are easily translocated from the intestinal lumen to the mesenteric lymph nodes of the graft and to the liver and spleen, thus increasing the risk for systemic infections (50). The therapeutic window for immunosuppression after small-bowel transplantation appears to be very narrow compared with that in other transplanted organs (44). Too weak an immunosuppression leads to rejection and/or GVHR, both of which in turn can give rise to infections, while excessively high doses of immunosuppressive drugs may lead to the manifestation of opportunistic infections as well as to the toxic side-effects related to the particular drug. With high doses of immunosuppressive drugs follows also an increased risk for tumour development.

With the introduction of CyA, the interest in small-bowel transplantation had been renewed, but this drug has not proven to be as effective in preventing rejection of the small bowel as it is in that of other organs. A short course of CyA can induce unresponsiveness to the grafted small bowel after semi-allogeneic (77, 173) as well as fully allogeneic (28, 146) small-bowel transplantation in the rat, although this is not possible in certain strain combinations (14, 62). However, in large animal models, the outcome of small-bowel transplantation under immunosuppression with CyA has, in most cases, been unsuccessful, although some long-term survivors have been described (for reviews see 124, 174).

The immunosuppressive drug FK 506 has, in certain models, proved superior to CyA (62, 94) while, in others, it has been less effective (13). Other new immunosuppressive drugs tested include 15-deoxyspergualin (DSG; 65), rapamycin (157, 158), RS-61443 (1) and prostaglandin E₂ (82).

In the occasional clinical cases performed before the advent of CyA, immunosuppression was induced by azathioprine, prednisone and anti-lymphocyte globulin (ALG; 2, 32). Thereafter, immunosuppressive treatment has been based on CyA in various combinations with azathioprine, steroids, and anti-thymocyte globulin (ATG; 27, 43, 48, 155) until FK 506 was introduced. In the more recent cases, FK 506 has been advocated as the immunosuppressive drug of choice, especially in combination with steroids (164, 166).

Monitoring rejection

Up until the present day, easy, safe and reliable methods of detecting rejection at an early stage after small-bowel transplantation do not exist. Such an obvious serum marker as creatinine in the monitoring of kidney function after kidney transplantation does not exist in the case of the small bowel. Histological evaluation of biopsies is still the golden standard for monitoring rejection after small-bowel transplantation. The biopsy technique has been shown by many investigators to have several drawbacks. The intestine is a large organ and rejection may be patchy (46, 133); in a human small-bowel transplant that was removed about two weeks after transplantation due to

rejection, the bowel displayed areas of full thickness necrosis interspersed with areas of normal mucosa (46). In order to obtain adequate specimens it is thus advantageous to use an endoscopic technique when taking the biopsies (166). In addition, there appears to be need for full thickness biopsies as biopsies of the mucosa alone will not reveal deeper pathological changes within the intestinal wall (5, 109, 133); full thickness biopsies are, however, associated with a risk of perforating the graft.

An alternative to conventional histological staining techniques in the analysis of biopsies is to use immunohistochemistry to detect altered levels of, for instance, MHC class II-expression (134) or brush border enzymes (140) that correlate to the development of rejection. Recently, the in situ expression of various cytokines and serine esterase B has been investigated in the intestine undergoing rejection (33).

Another measurement of rejection is to follow the absorption of actively transported substances from the intestinal lumen, where a decrease in absorption would point to bowel damage. Substances investigated in absorption studies include maltose (8), glucose (57, 116) and short-chain fatty acids such as lauric acid (152). Another technique is to inject chromium-labelled ethylene-diamine-tetraacetate (^{51}Cr -EDTA) into the intestinal lumen and to follow the renal clearance; increased urine levels of ^{51}Cr -EDTA, as a result of leakage through the intestinal wall, indicate rejection (45, 48). A decreased transmural potential difference due to damaged mucosal cells may also indicate rejection (95, 107). Finally, serum levels of N-acetyl hexosaminidase (104) and procoagulant activity (20, 76, 148) as potential markers of rejection have also been investigated.

Graft-versus-host reaction (GVHR) after small-bowel transplantation

The small bowel contains large amounts of lymphoid tissues located in the Peyer's patches, lamina propria, mesenteric lymph nodes and also in the intraepithelial compartment. These lymphoid cells, apart from acting as potent antigenic stimuli which can give rise to rejection, can, under certain circumstances, also induce GVHR. In this respect, the small bowel is unique among solid organs, although GVHR in rare cases, as mentioned above, has actually been described after liver-, heart/lung- and pancreas/spleen-transplantations.

Studies using T lymphocyte-deficient donors have demonstrated that the presence of mature T lymphocytes in the graft is a prerequisite for the induction of GVHR (77, 168).

The semi-syngeneic rat model is excellent for investigating the mechanisms involved in GVHR and also for screening new preventive or therapeutic modalities. After fully allogeneic transplantation, rejection is the dominant immunological reaction in untreated rats in most strain combinations. Under immunosuppressive treatment, temporary (28, 113) as well as lethal (13, 113) GVHRs have been seen. In large-animal models, several investigators have observed early deaths with enlarged

mesenteric lymph nodes and no histological evidence of rejection. The proposed explanation for these findings has been that the animals suffered from GVHR (19, 99). In contrast, Reznick et al. did not observe any signs of GVHR in the canine model and thus found rejection to totally dominate (128).

After small-bowel transplantation in humans, GVHR has occasionally been described. One of the earlier recipients of a small-bowel allograft was suspected to have died of septicaemia and GVHR; however, the diagnosis was not confirmed histologically (32). In one published case (20) GVHR may have contributed to the unsuccessful outcome. In 1992, Todo et al. reported on a case where, due to infectious complications, the immunosuppression had to be decreased, after which classical signs of GVHR appeared. The young patient's death was classified as a combination of septicaemia and GVHR (164, 166). Later, another paediatric recipient of a combined small-bowel/liver-graft died from a combination of GVHR and septicaemia (103). Grant et al. have reported transient, mild GVHR following small-bowel/liver transplantation (48).

In the rat, GVHR has been well characterized. The first visible signs comprise a redness of ears and paws and progress to dermatitis, hair-loss, diarrhoea and loss of weight. The lymphatic tissues of the graft and host are progressively depleted of lymphoid cells until the normal architecture of lymph nodes and spleen is lost. The native bowel display enteritis with progressive crypt necrosis and a shortening of the villi (137). The injuries to the recipient bowel are probably the main cause of death in animals that develop GVHR (25, 26). GVHR impairs both the humoral and the cell-mediated responses in the host (47), leading to a state of immunosuppression which, in combination with the use of potent immunosuppressive drugs, increases the risk of severe infectious complications after small-bowel transplantation.

Cohen et al. observed that untreated canine recipients of small-bowel allografts died of GVHR nine days after transplantation, while, when the grafts were irradiated with 150 rad, the survival times were similar but now the animals suffered from rejection. Irradiation with 50 rad prolonged the survival to 28 days (19). From these findings a balance between GVHR and rejection was proposed. Similar findings have later been described in the rat both with irradiated grafts (132) and with grafts treated with anti-lymphocyte serum (ALS; 14). Studies of bone-marrow transplantation also indicate that GVHR and rejection are inversely correlated and often mutually exclusive (34). Because of the lack of easy, standardized small-animal models, it has, however, been extremely difficult to study the association between GVHR and rejection after small-bowel transplantation experimentally. In most rat strain combinations, rejection in untreated animals is such an early event that an eventual GVHR has not been able to develop before the animals suffer from end-stage rejection.

Two different approaches have been adopted to prevent GVHR; (i) the reduction of the amounts of immunocompetent cells in the transplant before grafting or (ii) the diminishing of the immune reactions of the grafted cells by using immunosuppressive drugs. Irradiation of the graft (93, 110) and surgical removal of mesenteric lymph nodes (26, 122) are examples of the former category, as are pretreatment of the donor with ALS (28, 48, 142) or monoclonal antibodies such as OX8 reactive with CD8-positive cells (23, 143), OX19 reactive with CD5-positive cells (149), R73 raised against the T cell receptor (144) and OKT3 raised against CD3-positive cells (48, 155). The immunosuppressive drugs investigated include CyA (77), FK 506 (62), rapamycin (158), RS-61443 (145) and DSG (160).

Cell migration

In 1957 Snell propounded that cell migration occurred after organ transplantation (150). He suggested that donor lymphoid cells in the graft migrate to the regional lymph nodes of the host and there incite an immune response. This theory has later been supported by Larsen et al. who, on the basis of observations after cardiac allografting in mice, speculated that sensitization to allografts may occur centrally, i.e. in recipient lymphoid tissues, rather than peripherally, i.e. in the graft (88). In small-bowel transplantation, cell migration between graft and host is of special importance because of the potential threat of GVHR. In untreated animals, the percentage of donor cells in the recipient's spleen, mesenteric lymph nodes and Peyer's patches has been demonstrated to increase until day 4 after transplantation, whereas two days later there were no longer any detectable graft cells. Treatment with CyA for 7 days delayed the peak until day 7 and, on day 21, the donor cells had vanished. There was progressive infiltration in the small bowel graft by recipient cells (90, 92). A local chimerism appears to arise in the transplanted bowel; after twelve days, almost all cells in the lymphoid tissue of the graft were of recipient phenotype when FK 506 was used for immunosuppression (112). A similar replacement of donor lymphocytes by recipient lymphocytes has been observed after human small-bowel transplantation with donor intestinal epithelial cells remaining intact (68).

Lately, the theory has been put forward that a two-way cell migration between graft and host is associated with acceptance rather than rejection (112, 156). The background for this hypothesis is that, after liver transplantation, there occurs a similar local chimerism in the graft as that described after small-bowel transplantation; in addition, peripherally located donor cells have been detected several years after transplantation (156). After small-bowel transplantation in the rat, however, no systemic lymphoid chimerism was detected in recipients of permanently accepted grafts one year after transplantation (86). In this study, it was also demonstrated that the recipients remained immunocompetent and did not retain their grafts because of specific donor hyporesponsiveness. The in vitro proliferative response of

lymphocytes from peripheral lymph nodes of the recipient to both donor-specific and third-party antigens was normal, as was the rejection time of newly transplanted skin grafts.

Hyaluronan

Hyaluronan (HA), hyaluronic acid according to older nomenclature (4), is a linear polymer built up of the repeating disaccharide N-acetyl-glucoseamine-glucuronic acid. HA is synthesized by mesenchymal cells and is an important stabilizing constituent of loose connective tissue (21). A local accumulation of HA occurs in various organs undergoing inflammatory reactions, e.g. alveolitis (115) and myocardial infarction (167). In renal (53, 175) and cardiac (54) allograft rejection a progressive interstitial accumulation of HA has been demonstrated. Moreover, the increased levels of HA are proportional to the increased water-content of the rejecting organ. Since HA has a great water-binding capacity, it is thus suggested that the interstitial secretion of HA is responsible for the development of transplantation oedema. After experimental small-bowel transplantation increased amounts of HA have been observed in the lamina propria of the crypt area during rejection (170), but the increase is not as pronounced as it is in kidneys and hearts undergoing rejection, possibly indicating that HA is cleared into the intestinal lumen.

MC 1288

MC 1288 (20-epi-1 α ,25-dihydroxy-cholecalciferol; figure 2) is a recently synthesized vitamin D analogue which differs from 1 α ,25-dihydroxy-cholecalciferol (1 α ,25(OH) $_2$ D $_3$), the active form of vitamin D, by its altered stereochemistry at carbon 20. 1 α ,25(OH) $_2$ D $_3$ has been demonstrated to possess immunomodulatory properties both *in vitro* and *in vivo*. Monocytes, as well as activated T and B lymphocytes,

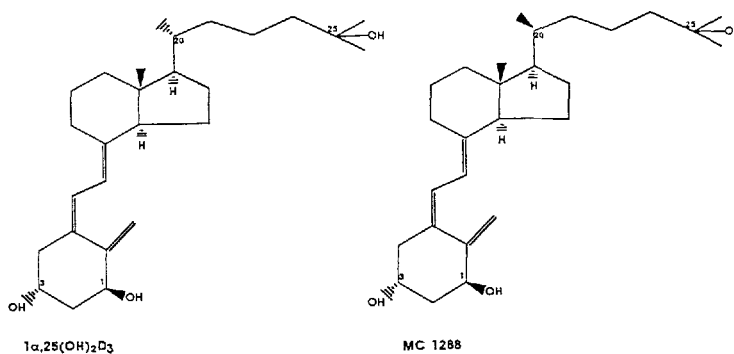


Figure 2. Molecular structure of 1 α ,25-dihydroxycholecalciferol (1 α ,25(OH) $_2$ D $_3$) and 20-epi-1 α ,25-dihydroxycholecalciferol (MC 1288). The single difference between the molecules is the stereochemistry at carbon 20.

express receptors for $1\alpha,25(\text{OH})_2\text{D}_3$ (7, 125) and antigen- or mitogen-activated T lymphocytes show decreased proliferation rates when incubated together with $1\alpha,25(\text{OH})_2\text{D}_3$ (129). The in vitro production of various cytokines e.g. IL-2 (96, 129), IFN- γ (127, 130) and granulocyte-macrophage colony-stimulating factor (GM-CSF; 163) is also inhibited by $1\alpha,25(\text{OH})_2\text{D}_3$. However, the therapeutic use of $1\alpha,25(\text{OH})_2\text{D}_3$ in the treatment of immunological disorders is impossible because of its deleterious effects on calcium metabolism. From this point of view, new vitamin D analogues have been synthesized in order to find substances which are more potent and/or less hypercalcemic than $1\alpha,25(\text{OH})_2\text{D}_3$.

In vitro MC 1288 is effective at much lower concentrations than $1\alpha,25(\text{OH})_2\text{D}_3$. In the inhibition of allogeneic T lymphocyte activation, as investigated in a mixed lymphocyte reaction, MC 1288 was nearly 300 times more potent than $1\alpha,25(\text{OH})_2\text{D}_3$, while, in a mouse thymocyte co-stimulatory assay, it was over 7,000 times more effective (10). Thus, MC 1288 has properties that might in time qualify it as a suitable immunosuppressive agent for controlling rejection and/or GVHR.

LS-2616

The substance LS-2616 (Linomide; figure 3) - a quinoline-3-carboxamide - has been shown to affect several immunological reactions. In models of autoimmune diseases (rheumatoid arthritis and systemic lupus erythematosus), treatment with LS-2616 leads to prolonged survival when using mice that spontaneously develop the disease (161, 162). In type II collagen-induced arthritis in mice and rats, there is an inhibition in the severity of the disease when treatment is started on the day of immunization, whereas there is an increased severity of the arthritis when the treatment with LS-2616 is initiated at the onset of disease (11, 79). LS-2616 has, in several mice models, been shown to possess antitumoral effects; it reduces the frequency of viable tumours and protects against metastases (58, 64, 72). LS-2616 has also been demonstrated to

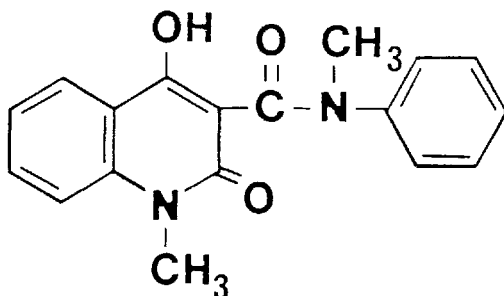


Figure 3. Molecular structure of LS-2616.

inhibit programmed cell death (40) and to prevent death in models of septic shock (39), both effects investigated in the mouse. Also, LS-2616 enhances the delayed type hypersensitivity reaction (159). Stimulatory effects on NK cells (71, 73) and macrophages (89) have been demonstrated. After human autologous bone-marrow transplantation, LS-2616 has been administered with the aim of inducing a graft-versus-leukemia effect. In these patients, increased numbers of NK cells and monocytes, as well as enhanced in vitro production of IL-1, tumour necrosis factor-alpha (TNF- α), and IFN- γ were observed (6).

Of interest in studying mechanisms of graft rejection is the finding that LS-2616 extinguishes the immunosuppressive effect of CyA in a heterotopic heart transplantation model (171). In the same model, LS-2616 also counteracts the immunosuppressive effects of prednisolone (36) and low dose DSG (35) completely. The mechanisms behind these effects have been suggested to be due to an ability to stimulate effector T lymphocytes (172). Subsequently, it has been proposed that LS-2616 could function as a suitable model for the testing of new immunosuppressive therapy regimens (165).

RESULTS

Monitoring rejection

In these experiments, the intestinal release of HA was measured in rejecting and non-rejecting animals in order to evaluate whether the release of HA into the intestinal lumen could function as a rejection marker. In non-rejecting animals (Lewis to Lewis), the concentration of HA, as measured on day 6, was stable over time (figure 4). However, in rejecting animals (Lewis x BN to Lewis), the recovery of HA during the first 10 minutes of perfusion was elevated and then started to fall until a steady state was reached after about 50 minutes.

As regards the first 10 minutes of perfusion, the HA levels were similar in rejecting and non-rejecting animals on day 2 (figure 5). During the development of rejection, there was a progressive increase in the recovery of HA on days 4 and 6, while there was merely a minor increase on day 4 and no further increase on day 6 in the non-rejecting controls. After immunosuppression with CyA (15 mg/kg) given for 20 days, the amount of HA in the intestinal lumen was comparable with that observed in the syngeneically transplanted controls.

Rejecting rats developed a palpable mass in the abdomen detectable on day 5 - 6. Histologically, there was mucosal sloughing on day 6, while lymphocytic infiltration was visible in some specimens on day 4 and was apparent on day 6. All controls were histologically normal.

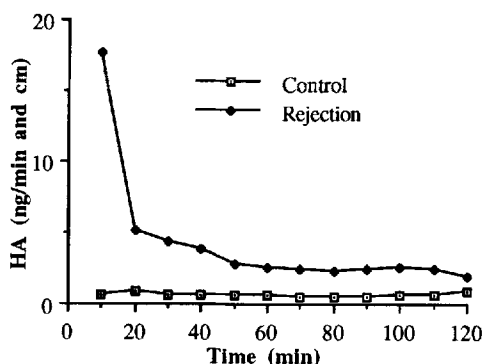


Figure 4. The time-dependency of the recovery of HA during intestinal perfusion day 6 following syngeneic (control) and semi-allogeneic (rejection) transplantation.

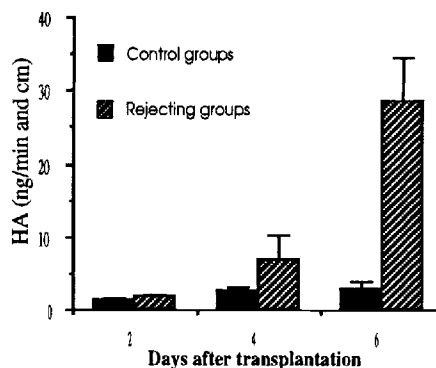


Figure 5. The recovery of HA during the first 10 min of intestinal perfusion on day 2, 4 and 6 after syngeneic and semi-allogeneic transplantation.

Prevention of GVHR

In this study, we investigated whether monoclonal antibodies administered via ex vivo perfusion of the vascular system of the intestine are able to reach target cells in mesenteric lymph nodes and the intestinal wall. Untreated rats and rats treated with monoclonal antibodies intravenously served as controls. In these experiments, OX8 was used as a model antibody. In all OX8-treated animals, a varying, but demonstrable, labelling of CD8-positive T lymphocytes was observed. The number of cells stained in the intestinal wall was usually similar, irrespective of whether the specimen was taken from the proximal, middle, or distal part of the small bowel.

After perfusion of the vascular system with an OX8-containing solution for 60 minutes, an almost optimal labelling of cells in both mesenteric lymph nodes and intestinal wall appeared to be obtained, since additional OX8 applied to the tissue sections did not seem to change the staining pattern (table 1). Twenty minutes of perfusion showed similar labelling of CD8-positive T lymphocytes in the intestinal wall but, in the mesenteric lymph nodes, somewhat fewer cells were labelled. Perfusion for 10 minutes resulted in patchy staining in both mesenteric lymph nodes and the intestinal wall.

Administration of OX8 intravenously one or four hours before the taking of specimens showed almost total labelling of CD8-positive T lymphocytes in mesenteric lymph nodes while the labelling was not absolute in the intestinal wall of certain animals. An intravenous injection 20 minutes before harvesting resulted in few labelled cells in both mesenteric lymph nodes and intestinal wall and the administration of the antibody 24 hours before harvesting gave weak staining of a limited number of cells.

Table 1. Binding of the monoclonal antibody OX8 to target cells in the intestinal wall and mesenteric lymph nodes following ex vivo or intravenous administration. The sections are graded arbitrarily for labelled cells on a scale from 0 to ++.

<u>Treatment with OX8</u>	<u>Small bowel</u>	<u>Mesenteric lymph node</u>
10-min perfusion	+	+
20-min perfusion	++	+ / ++
60-min perfusion	++	++
Intravenously, 20 min	+	+ / ++
Intravenously, 60 min	+ / ++	++
Intravenously, 4 h	+ / ++	++
Intravenously, 24 h	+	+

The ex vivo perfusion technique was evaluated and further refined in the one-way GVHR model, using the monoclonal antibody R73. In the strain-combination used (Lewis to Lewis x BN), untreated rats develop signs of GVHR on about day 7, starting with redness of ears and paws and progressing to dermatitis, secretion from nose and eyes, hairloss, diarrhoea, and loss of weight. Animals in the untreated group survived until day 14 (10 - 16; figure 6). Recipients of transplants from donors treated with a single, intravenous dose of R73 four or sixteen hours before procuring the bowel for transplantation showed a similar GVHR pattern and also survived for about 14 days. The administration of the antibody even earlier (24 hours before organ procurement) resulted in 3 out of 10 animals surviving indefinitely. These animals had temporary signs of GVHR in the form of erythema and dermatitis of the ears on days 8 - 18.

Ex vivo perfusion of the vascular system for 60 minutes resulted in survival times comparable with those of untreated animals. However, perfusion for 15 minutes combined with R73 incubation in the intestinal lumen resulted in 4 out of 11 animals surviving for about 19 days with a delayed onset of the GVHR. The remaining rats in this group were sacrificed without any visible signs of GVHR, 5 of them with extremely swollen abdomens and distended transplants. We suspected that this phenomenon was due to an extensive hypersecretion from the transplanted intestine and therefore decided to study the fate also of recipients of similarly treated, resected grafts. In this group, where 14 cm of the distal part of the intestine together with all mesenteric lymph nodes were grafted, 3 out of 8 animals survived indefinitely while 4 others showed significantly prolonged survival compared with untreated animals receiving resected grafts (figure 6).

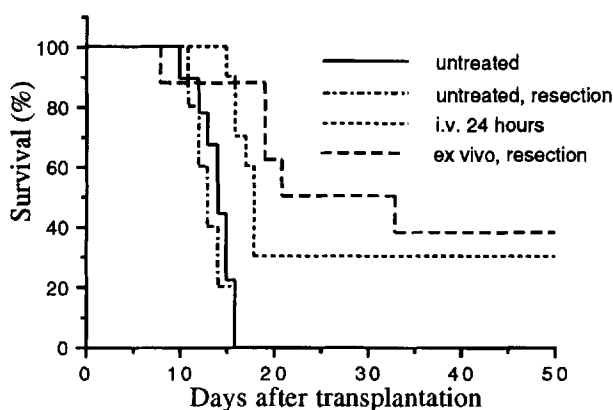


Figure 6. Survival times in recipients of untreated grafts, untreated, resected grafts, grafts treated with a single dose of R73 i.v. 24 h before organ procurement or resected grafts treated with R73 ex vivo.

Immunohistochemical staining of specimens taken at the time of transplantation, immediately after restoration of the blood flow to the transplant, revealed evenly distributed positive cells in the cortex, outer paracortex and medulla of mesenteric lymph nodes from grafts pretreated with a single, intravenous dose of R73 24 hours before grafting (table 2). In some specimens, there were also positive cells invading the germinal centres. Following ex vivo treatment (15 minutes perfusion combined with intraluminal incubation for 60 minutes), the same labelling of cortex and outer paracortex was obtained but no positive cells were detectable in the medulla and there were only single positive cells in the germinal centres. In the Peyer's patches of both intravenously and ex vivo treated intestines, there were positive cells in both T cell areas and germinal centres.

Table 2. Binding of the monoclonal antibody R73 to target cells in mesenteric lymph nodes and Peyer's patches of the transplanted bowel following intravenous or ex vivo administration. Areas are judged for labelled cells as either positive (+) or negative (-).

Treatment with R73	Mesenteric lymph nodes			
	Cortex	Paracortex	Medulla	Germinal centres
Intravenously, 24 h	+	+	+	+/-
15 min perfusion and 60 min lumen	+	+	-	single +

GVHR and rejection - mirror images?

This study was undertaken in order to investigate the effects of the immunomodulatory substance LS-2616 in a semi-syngeneic GVHR model (Lewis to Lewis x BN). LS-2616, 160 mg/kg administered via the drinking water with start on day 0, gave a survival time of 8 (7 - 11) days - that is 6 days shorter than that of untreated animals (figure 7). Four out of 7 animals thus treated showed typical signs of GVHR such as redness of ears and paws. When LS-2616 treatment was started on day 5 post transplantation, the GVHR pattern was similar to that of untreated animals but, in this group, the survival times varied between 9 and 41 days with a median of 12. Treating the donors for 5 days with continued treatment of the recipient from day 0 produced the same result as when the donors were untreated, viz. a survival of 7 (6 - 11) days.

Animals receiving small bowels from Lewis donors previously grafted with hearts from BN rats survived for 12.5 (6 - 13) days, most of them with a GVHR pattern comparable with that of untreated animals. Notably, 2 of these 8 rats showed a clinical picture resembling that seen after treatment with LS-2616 starting on days -5 or 0.

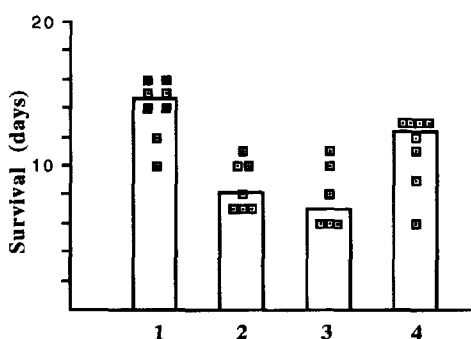


Figure 7. Survival after semi-syngeneic transplantation. Recipients were either untreated (1), treated with LS-2616 from day 0 (2), treated as group 2 combined with pre-treatment of the donor with LS-2616 for 5 days (3) or untreated, receiving grafts from sensitized donors (4).

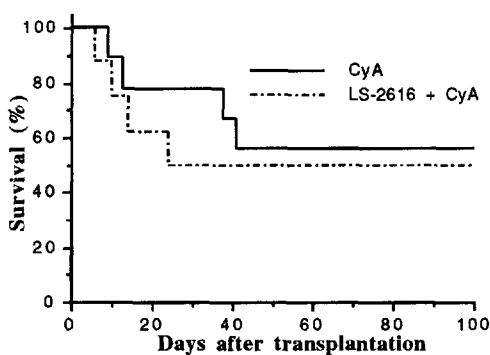


Figure 8. Survival after semi-syngeneic transplantation. Animals were either treated with CyA (15 mg/kg) days 0-20 or with CyA in combination with continuous LS-2616 (160 mg/kg).

LS-2616 administered from day 0 in combination with CyA (15 mg/kg) on days 0 - 20 resulted in 50% permanent survival (figure 8). This can be compared with the 56% permanent survival obtained after CyA-treatment alone. Starting the administration of CyA on day 5 with LS-2616 from day 0 gave a median survival time of 9 (6 - 20) days, whereas CyA from day 5 without LS-2616 resulted in the permanent survival of 3 out of 4 animals.

Syngeneically transplanted controls given LS-2616 continuously from the day of transplantation survived for more than 100 days with one exception; this animal developed ileus and was sacrificed on day 22.

The effects of LS-2616 were investigated also in the fully allogeneic Lewis to BN combination. Untreated animals or animals given LS-2616 orally showed signs of rejection - swollen abdomen and diarrhoea - on about day 6 post transplantation. The same was also true of the rats treated with LS-2616 locally in the transplanted intestine; however, in this group, unmistakable signs of GVHR appeared at the same time as the first clinical signs of rejection became visible. These animals displayed redness of the ears and occasionally the paws typical of GVHR. All animals were sacrificed on day 8, whereafter liver and ear biopsies were sectioned and stained with OX6 in order to detect class II antigen expression.

Furthermore, livers from semi-syngeneically transplanted animals sacrificed on days 3, 5 or 7 were stained for OX6. In this latter group liver sections from untreated rats taken on day 3 post transplantation showed a similar staining pattern as sections from untreated, non-transplanted animals - i.e. in the portal zones there were some positive cells with a dendritic morphology and in the parenchyma there were a few scattered positive cells (table 3). By days 5 and 7 there was a progressive increase in the number of cells staining positive for class II both in the parenchyma and in the portal zones. On day 3 sections from animals treated with LS-2616 showed positive dendritic cells in the portal zones and there were more positive cells in the parenchyma than in sections from untreated animals day 3. On day 5 there was an obvious difference in the number of class II positive cells when comparing the two groups: in untreated animals there was a moderate increase whereas in the LS-2616 treated animals the increase was pronounced. On day 7 there was no difference between the two groups investigated; in both, there were large numbers of class II positive cells throughout the sections.

Table 3. Class II antigen expression in the liver after semi-syngeneic small-bowel transplantation (Lewis to LxBN). Animals were either untreated or given LS-2616 orally, 160 mg/kg. The liver sections are graded arbitrarily for positive staining on a scale from 0 to ++++ where 0 represents a staining pattern similar to that seen in sections from untreated, non-transplanted animals.

<u>Treatment</u>	<u>Day 3</u>	<u>Day 5</u>	<u>Day 7</u>
None	0	+ / ++	+++
LS-2616 orally	+	+++	+++

Table 4. Class II antigen expression in the liver and the ear skin on day 8 after fully allogeneic small bowel transplantation (BN to Lewis). Animals were either untreated or given LS-2616 locally in the graft or orally, 160 mg/kg. The sections are graded arbitrarily on a scale from 0 to ++++ (liver biopsies) or 0 to ++ (ear biopsies) where 0 represents a staining pattern similar to that seen in sections from untreated, non-transplanted animals.

<u>Treatment</u>	<u>Liver</u>	<u>Skin</u>
None	+++	0
LS-2616 locally	++++	+
LS-2616 orally	+++	0

In liver sections from fully allogeneically transplanted animals, there were large numbers of positive cells in all groups investigated but, in the group treated with LS-2616 locally in the graft, the numbers of positive cells were even more pronounced than in the untreated group or the group given LS-2616 orally (table 4).

In ear skin biopsies from normal, non-transplanted animals, only the Langerhans cells stained positive for class II in the epidermis. The same staining pattern was observed in biopsies from untreated, fully allogeneically transplanted animals and from animals given LS-2616 orally. In the group given LS-2616 locally in the transplant, however, not only the Langerhans cells but also the epithelium of many hair follicles were class II positive (table 4).

Immunosuppressive therapy

The vitamin D analogue MC 1288 was evaluated for its possible immunosuppressive properties in vivo. The substance was first used in the heart rejection model to identify an optimal dose. In this PVG to Wistar/Kyoto combination untreated grafts are rejected on about day 8 (7 - 9). Treatment with MC 1288 at a daily dose of 0.05 µg/kg given intraperitoneally on days 0 - 9 slightly prolonged graft survival to 12 (8 - 22) days, while 0.1 µg/kg resulted in rejection after 22 (16 - 27) days. Higher doses did not have any further positive effects. MC 1288 0.1 µg/kg in combination with LS-2616, 160 mg/kg given orally starting on day -1 and administered daily for as long as the graft functioned, gave a median graft survival time of 11 (8 - 19) days.

To investigate the effects of MC 1288 on rejection after small-bowel transplantation, the Lewis x BN to Lewis combination was used and rejection was determined by measuring the HA content in the intestinal lumen on day 6 post transplantation. During the first 10 minutes of perfusion, the recovery of HA from untreated, rejecting grafts was 29.5±5.3 ng/min and cm (figure 9). After treatment

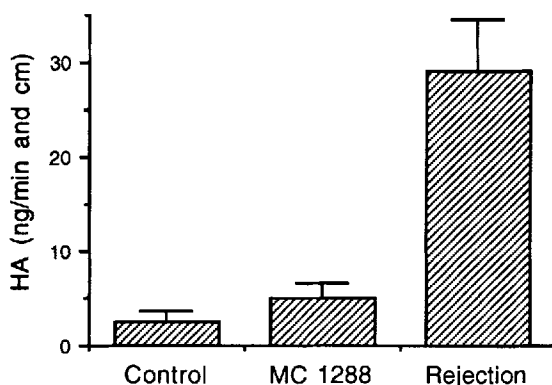


Figure 9. The recovery of hyaluronan (HA) during the first 10 minutes of perfusion of small bowel grafts following syngeneic transplantation (control), semi-allogeneic transplantation with treatment of the recipient with MC 1288 0.1 µg/kg (MC 1288) or semi-allogeneic transplantation with untreated recipients (rejection).

with MC 1288, 0.1 µg/kg, the levels of HA on day 6 were 5.0 ± 1.6 ng/min and cm and in syngeneic controls (Lewis to Lewis) 2.6 ± 1.0 .

The immunosuppressive properties of MC 1288 were also tested in the GVHR model (Lewis to Lewis x BN). Here a lethal GVHR developed at about the same time as in untreated animals. The median survival time, however, was somewhat shorter, viz. 9.5 (7 - 14) vs 14 (10 - 16) days, but it is uncertain whether the earlier deaths in the drug-treated group were fully due to GVHR.

DISCUSSION

Monitoring rejection

Since histological analysis of biopsies is an insufficient method of detecting early rejection after small-bowel transplantation, more reliable techniques have long been sought and evaluated experimentally. Histological routine staining has been developed into more refined immunohistochemical stainings and it is thus possible to detect increased numbers of class II-positive cells (134) or macrophages (140), and decreased amounts of brush border enzymes such as sucrase-isomaltase (140), during the development of rejection. However, these techniques suffer from the same drawbacks as the conventional histological analysis - rejection can appear patchy and there is a risk of missing affected areas when random biopsies are taken (46, 133).

There is also the risk of perforating the graft since full thickness biopsies are necessary (5, 109, 133).

In some studies, the monitoring of the uptake of glucose from the intestine after the local administration of ^{14}C -labelled glucose via the proximal stoma has been found to correlate well with rejection (18, 57, 116), but, in other studies with slightly different experimental settings, no such correlation could be observed (63). The absorption of other actively transported substances such as maltose (8) and short-chain fatty acids (152) has been observed to decrease during rejection. In all kinds of absorption tests, however, a high degree of mucosal damage has to be present before a determinable reduction in absorption capacity can be observed, and these techniques must thus be regarded as non-sensitive. In this context, it is more favourable to study the leakage of normally impermeable compounds, such as ^{51}Cr -EDTA (45, 48), from the transplanted bowel.

The serum markers so far investigated have not proven to be ideal (104, 148). Certainly the procoagulant activity in peripheral blood appears to rise early during the course of rejection, but the interindividual variation is striking (148) and increased levels of procoagulant activity may also be induced by septic complications.

Electrophysiological monitoring of the intestine for rejection has also been evaluated experimentally (95, 107). This technique appears to be cumbersome and also requires large amounts of fluid to be administered into the luminal segment studied.

As seen from the above, there is a need for easy, non-invasive, sensitive methods of monitoring rejection after small-bowel transplantation. Segmental perfusion of the graft and subsequent analysis of the amounts of HA in the perfusate appears to be a method at least as sensitive as conventional staining techniques. From day 2 to day 6, our studies have shown a 15-fold increase in the amounts of HA recovered from the intestinal lumen in rejecting animals. Although in the experimental setting the animals were anesthetized and a laparotomy was performed, this is not necessary in the clinical situation where a catheter with occluding balloons can be inserted into the intestine via the distal stoma (80, 81). The minor increase seen from day 2 to day 4 in the syngeneically transplanted group is probably due to ischemic injury, surgical trauma, and/or surgical damage to the lymph vessels leading to a slight accumulation of HA in the intestinal wall. This minor effect in the early post operative period has also been observed in the investigations of other rejection markers (45, 148).

The mechanisms behind the accumulation of HA in rejecting organs, which has been demonstrated in kidneys (53, 175), hearts (54), and small bowels (170), have not been ascertained, but it can be assumed that the local synthesis of HA by mesenchymal cells is stimulated by immunomodulators released from cells invading

the transplant during rejection. This hypothesis is supported by the in vitro observations that several inflammatory products can stimulate the synthesis of HA in fibroblasts (29, 56, 61, 177).

Since HA possesses strong water-binding properties (106), an accumulation of HA will also be reflected by water retention; this may be one factor that contributes to the development of transplantation oedema. Transplantation oedema is more pronounced in cardiac and renal grafts during rejection than in intestinal grafts, probably due to the varying HA elimination routes. HA should be cleared by lymphatic drainage and/or enzymatic degradation from the interstitial tissue of hearts and kidneys (30) whereas there also exists a luminal clearance from the small bowel, as was demonstrated.

The interstitial accumulation of HA is also seen during various inflammatory conditions, such as alveolitis (115) and myocardial infarction (167). Increased levels of HA should thus be regarded as a non-specific rejection marker; the same is also true for most other techniques that attempt to monitor rejection after small-bowel transplantation. However, the recovery of HA from the intestinal lumen was found to correlate well with rejection and, since the method is rapid and safe to perform, it could well function as a complement to the conventional and widely used biopsy technique.

Prevention of GVHR

The risk of GVHR after small-bowel transplantation has long been known and, ever since the one-way GVHR model in the rat was first described (110), extensive research work has been performed utilizing this model. Semi-syngeneic transplantation of the small bowel inevitably leads to GVHR, although it is not always lethal in all strain combinations (66). After fully allogeneic transplantation, however, lethal GVHR is uncommon but has occasionally been described after immunosuppressive treatment (13, 113). Thus, one has to be careful not to overemphasize the risk of GVHR when interpreting results obtained in the one-way model. Nonetheless, this model can provide excellent initial information when investigating new techniques for the prevention of GVHR or when studying the mechanisms involved in GVHR.

GVHR can be prevented experimentally by irradiation of the graft (93, 110) or by surgical removal of mesenteric lymph nodes (26, 122). These techniques are, however, not applicable in the clinical situation. Treatment of the donor rat with ALS (28, 142) or monoclonal antibodies (144) has also been successful in preventing GVHR, but the treatment had to be started 24 or 48 hours before procuring the intestine for transplantation, which, in a clinical situation, would be both inconvenient and unethical.

Administration of monoclonal antibodies *ex vivo* during organ procurement and preservation would be a simple and safe way of pretreating the graft. Such experiments have been performed with the aim of inactivating dendritic cells and thus reducing the antigenicity of the graft in order to avoid rejection. Infusion of a single dose of antibodies into the renal artery reduced the incidence of rejection after human kidney transplantation (12), while *ex vivo* normothermic hemoperfusion with monoclonal antibodies for 3 hours resulted in a slight prolongation of the survival of grafted rat pancreases (100). The same perfusion technique has also been used for the conditioning of small-bowel grafts and resulted in a good labelling of dendritic cells (154), but, in order to be able to transplant the intestines, several drugs had to be added to the perfusate (153).

In our initial experiments we found that the perfusion of the vascular system of the intestine with an OX8-containing solution for 20 - 60 minutes resulted in an almost total labelling of CD8-positive cells in the intestinal wall and the mesenteric lymph nodes. When performing the same experiments with R73, however, the transplanted animals developed GVHR at the same time as untreated animals did. To obtain prolonged survival, the vascular system of the graft had to be perfused for 15 minutes at the same time as the graft was incubated for 60 minutes with R73 in the intestinal lumen. Upon revascularization, the grafts were resected to a length of 14 cm. Without resection, the transplanted animals developed swollen abdomens and had to be sacrificed, probably due to an extensive hypersecretion from the transplant. These results indicate that incubation with antibodies in the intestinal lumen can be effective but, in order to obtain sufficient labelling of the T lymphocytes, such high pressure has to be applied that considerable hypersecretion develops. This theory is supported by the results from the animals where the transplanted bowel was resected to a final length of 14 cm; these rats either survived indefinitely or showed prolonged survival. This was in contrast to resected, untreated animals in which a typical, lethal GVHR developed. The phenomenon with swollen, dilated grafts may be, at least partly, due to the heterotopic transplantation technique used. It is known that in this model dilatation of the transplanted bowel can occur during rejection and, occasionally, also during the early postoperative period, probably due to disturbed motility and the lack of bile and pancreatic secretion (139).

When looking at the labelling of R73-positive cells in the mesenteric lymph nodes of the graft, there was a somewhat better labelling after intravenous than after *ex vivo* administration of the antibody. A possible explanation for these findings is that when the antibody is administered intravenously, it may reach the lymph nodes both via the vascular and the lymphatic systems, whereas only the vascular route is available during *ex vivo* perfusion. A similar technique of administering monoclonal antibodies *ex vivo* was tested by Smith et al. who used the antibody MRC OX-19

conjugated with the A chain of ricin and were able to obtain prolonged, but not indefinite, survival in the GVHR model (149). On day 7 post transplantation, the number of donor cells found in the lymphoid tissues of the recipient was significantly lower when the grafts had been treated with the antibody than if the intestines were not pretreated (67).

GVHR appears to be more difficult to control with immunosuppressive drugs than rejection (77, 158). In addition, GVHR may exert an immunosuppressive effect on the recipient by affecting both the humoral and cell-mediated immune responses (47). A fully developed GVHR may give rise to severe infectious complications. Moreover, a subclinical GVHR may, in combination with the high doses of immunosuppressive drugs given to the patient, lead to a state of over-immunosuppression, that plasma drug levels do not provide any information about. Severe infections are common in patients who have undergone small-bowel transplantation and, possibly, GVHR has in some cases been underdiagnosed (69).

During recent years, it has been debated whether one should try to prevent potential GVHR by pretreatment of the graft or not (68). However, as there are cases of lethal GVHR described in recent years and as the outcome after small-bowel transplantation has so far not been satisfactory, it would probably be safer to remove the potential GVHR-inducing cells during organ procurement, thereby reducing the risk of both subclinical and lethal GVHR.

GVHR and rejection - mirror images?

Based on Cohen's investigations with irradiated grafts in the canine model (19), a balance between GVHR and rejection was postulated. In the rat model, experiments have been performed both with irradiated donors (132) and with donors pretreated with ALS (14) where pretreatment has led to earlier rejection than that observed in untreated animals. These experiments support the theory that a mild or subclinical GVHR could be a favourable state which may make rejection less intense. However, the association between GVHR and rejection has been difficult to study because of lack of easy, standardized experimental models.

In order to learn more about GVHR and about the association between GVHR and rejection, we used the immunomodulatory drug LS-2616 both in the one-way GVHR model and in the fully allogeneic situation. In the semi-syngeneic model, treatment with LS-2616 was found to significantly enhance the onset of GVHR when compared with untreated animals. The effect was similar irrespective of whether the donor rats were pretreated with LS-2616 or not. In the group where the donors had previously received a heart graft, the onset of GVHR was slightly enhanced. As regards the outcome after combined treatment with LS-2616 and CyA, a similar percentage of animals survived indefinitely in this group as that treated with CyA-

treatment alone. When the administration of CyA was started on day 5 post transplantation, however, there were no permanent survivors in the group receiving both drugs, while in the group given CyA alone the majority survived indefinitely.

Compared with previous results in the heart rejection model, we observed two differences in the present study: (i) for the first time in a transplantation model we could show an effect of LS-2616 on its own, i.e. the immunological reaction was enhanced, and (ii) in this model LS-2616 does not inevitably extinguish the immunosuppressive effect of CyA as it does in the heart rejection model. The explanation for these findings could be either that GVHR and rejection are not mediated via exactly the same effector mechanisms, or that the findings reflect the quantitative difference between GVHR and rejection. The first theory could imply that the two reactions are unequally sensitive to certain cytokines, which directly or indirectly become induced by LS-2616. LS-2616 enhances the production of IL-2 (89), but the dose of CyA used is probably sufficient to also inhibit the effect of LS-2616 on IL-2 production. Our findings that CyA in the presence of LS-2616 can still protect the recipient from GVHR led us to speculate that the mechanisms involved in the development of GVHR include at least one step that is IL-2 dependent. In contrast, rejection can still occur in the presence of both LS-2616 and CyA, suggesting that rejection can also be mediated by a non-IL-2-dependent mechanism.

Rejection can be mediated by a larger number of cells (the recipient's total supply of immunocompetent cells) than GVHR (the T lymphocytes transferred with the grafted intestine) and this fact could also explain some of our findings. The enhanced onset of GVHR observed during treatment with LS-2616 from day 0 could thus be explained by means of a stimulation of T lymphocytes within the graft to a faster proliferation rate, leading to an earlier onset of GVHR. Rejection, on the other hand, is already such a rapid process that it is not possible to enhance it any further. Starting the treatment on day 5 leads to a similar GVHR pattern as that obtained in untreated animals, indicating that 5 days is enough time for the grafted cells to proliferate to such an extent that the GVHR cannot be further enhanced by drug manipulation.

The different results obtained in the rejection and the GVHR models could also be due to a combination of these theories; e.g. rejection and GVHR are unequally dependent on certain cytokines - probably IL-2 - but also the quantitative difference is of importance.

To confirm that the earlier deaths in the group given LS-2616 were really due to GVHR, the MHC class II antigen expression in the livers was studied immunohistochemically. As early as day 3 post transplantation, there was a slight difference between the sections taken from untreated animals and animals treated with LS-2616, with an increased number of cells staining positive for class II in the LS-2616 treated

group. On day 5, the difference was even more pronounced. Class II antigen expression in the liver has previously been shown to correlate with the development of GVHR (169).

After local LS-2616 treatment of the grafted intestine in the fully allogeneic BN to Lewis combination, a typical GVHR developed at the same time as the first signs of rejection became visible. Neither untreated animals nor animals treated orally with LS-2616 showed any clinical signs of GVHR. At laparotomy on day 8, all animals - regardless of treatment - suffered from severe rejection. In both ear and liver biopsies a much more intense class II antigen expression was seen in sections from animals that showed clinical signs of GVHR than in those that did not.

As described previously, a balance between GVHR and rejection has been suggested. In our experiments, however, no such balance could be observed. The animals exhibited clinical signs of GVHR and rejection simultaneously and rejection occurred at the same time as in untreated animals and was, thus, not delayed, despite the ongoing GVHR process. Experiments with vigorously manipulated donors and recipients have been performed by Langrehr et al., who injected recipient splenocytes into the donors on days -21 and -14 and pretreated the recipients with CyA and ALS; in addition, the recipients were also splenectomized. The purpose with this complicated system was to simulate a situation where the donor has been unsuspectedly sensitized to the recipient and where the recipient has a reduced immunocompetence. Four out of 9 treated animals developed clinical signs of GVHR. The survival times in this group were, however, significantly shorter than those in the group treated without donor sensitization, in which no GVHR was observed (85). These findings also argue against a balance between rejection and GVHR.

To our knowledge, no group has previously reported on such a standardized incidence of GVHR after fully allogeneic small-bowel transplantation as we obtained in our experiments. Eighty-three percent (5 out of 6) of the rats treated with LS-2616 locally in the grafted intestine showed clinical manifestations of GVHR simultaneously with rejection. Our findings indicate that even if there, under normal circumstances, exists some kind of balance between GVHR and rejection, this balance can under certain conditions be circumvented.

GVHR and rejection have also been said to be almost mutually exclusive (34), which was here not shown to be the case. Furthermore, our findings contradict one of Billingham's original requirements for GVHR, namely, that for GVHR to occur, the host must be incapable of reacting immunologically against the graft. As we have shown, this is not the case because a normal, lethal rejection developed simultaneously with the GVHR.

The mechanisms behind GVHR, as well as the association between GVHR and rejection, are poorly understood. It has been argued that the risk of GVHR in small-

bowel transplantation is a more or less artificial problem since it is so rarely observed after fully allogeneic transplantation. A reason for this is probably that rejection is such an early event that an eventual GVHR in untreated animals has not had time to develop before the animals suffer from end-stage rejection. During immunosuppression, both temporary (28, 113) and lethal (13, 113) GVHRs have been described. From studies of bone-marrow transplantation, it is known that the risk of chronic GVHR increases if the patient has one or more incidents of acute GVHR (31, 147); thus, a mild or subclinical GVHR might predispose for the later development of chronic GVHR.

In our experiments, we have been able to enhance the rate of the GVHR pharmacologically and we have thus been able to demonstrate the simultaneous occurrence of GVHR and rejection. Taken together, our results indicate that GVHR and rejection are not complete mirror images. As long as the mechanisms behind GVHR and the association between GVHR and rejection are not ascertained and since there are recent clinical cases of GVHR described after small-bowel transplantation, we do not think that one can assume that GVHR is merely theoretical in this context.

Immunosuppressive therapy

During recent years, several new substances with immunosuppressive effects have been developed and tested experimentally and in clinical trials for their ability to prevent and treat rejection and GVHR. The immunosuppressive drugs used today are certainly potent but, at the same time, they have severe side-effects. Furthermore, they are non-specific, i.e. they affect the whole immune system of the recipient.

To prevent rejection after small-bowel transplantation, extremely high doses of immunosuppressants are needed and, as mentioned before, the therapeutic window for immunosuppression appears to be more narrow for the small bowel than for other transplanted organs.

In the quest for new immunosuppressive substances, vitamin D has become an interesting candidate. This substance has long been known to possess immunoregulatory properties but, because of the effects exerted on calcium homeostasis, it is impossible to use it systemically in the treatment of immunological disorders. Large series of vitamin D analogues with less calcemic effects have recently been synthesized, of which MC 1288 is one. In the heart rejection model, MC 1288 was found to have immunosuppressive qualities with an optimal effect when administered at a dose of 0.1 µg/kg. With this treatment, the median graft survival time was similar to that obtained after treatment with an optimal dose of CyA. The same dose, 0.1 µg/kg, was also effective in suppressing rejection after small-bowel transplantation, as measured by analysing the amounts of intestinal, intraluminal HA on day 6 post

transplantation. In the one-way GVHR model, however, no positive effects of MC 1288 could be observed.

Rejection after transplantation of the small bowel is normally more difficult to control than rejection occurring in the heart transplantation model. Thus, higher doses of immunosuppressants are usually needed by small-bowel graft recipients. MC 1288 was, however, found to be effective in preventing the development of intestinal rejection at the same dose as that found optimal in the heart model.

GVHR is often even more difficult to control by immunosuppressive drugs than rejection (77, 158) and, in the one-way GVHR model, we were not able to observe any positive effects of MC 1288. This lack of efficacy is difficult to explain since most immunosuppressive substances investigated can, at least to some degree, postpone GVHR. One reason could be that the dose used is insufficient, another that if GVHR and rejection are mediated by slightly different mechanisms, MC 1288 might be able to control rejection but not GVHR.

The promising in vitro effects of MC 1288 (10) were confirmed in vivo by the findings that MC 1288 was effective in preventing rejection. In the future, immunosuppressive therapy may consist of a combination of drugs with different mechanisms of action and/or different adverse effect profiles. Our results suggest the possibility that it may be worthwhile to further explore MC 1288, or other vitamin D analogues, for this purpose.

Future perspectives

In 1959 when Lillehei presented his initial experimental work with small-bowel transplantation, Dr. O H Wangenstein uttered the words "I believe it would be fair to say it is an adventure in the exploration of adversity". Thirty-five years have now passed and the sentence has proven to be true - small-bowel transplantation is still an experimental activity.

In recent years, there has been considerable progress in this field and great efforts have been made by many investigators to answer some of all the questions still existing. The first large series of clinical small-bowel transplantations has been presented, showing that small-bowel transplantation really can be feasible. However, when looking at the overall experience in organ transplantation, our knowledge about the small bowel is far behind that of other organs. The main obstacle after human small-bowel transplantation has so far been rejection, although infections are also a severe threat to these patients. For small-bowel transplantation to become a realistic therapy in the future, deeper knowledge is required about several aspects, the most important probably being the specific immunology of the intestine. It is of importance to know whether the transplant should be manipulated during organ procurement in order to remove the potential GVHR-inducing cells and/or reduce the

antigenicity of the organ to reduce the risk of rejection. One further point of interest is the association between rejection and GVHR. Is there a balance between these immunological reactions and, if so, can this balance under certain circumstances be disturbed? The development of new drugs, immunosuppressive and antibiotic, is also of vital importance and would probably contribute to improving the situation for transplanted patients substantially. Therefore, further investigations are required before small-bowel transplantation can become standard treatment in intestinal failure as kidney transplantation has become in renal failure.

CONCLUSIONS

The amount of HA secreted into the intestinal lumen was found to correlate well with the development of rejection and could thus function as a rejection marker.

Ex vivo administration of monoclonal antibodies during organ procurement resulted in good labelling of target cells in the intestinal wall and mesenteric lymph nodes. GVHR was totally prevented or postponed when these pretreated intestines were transplanted.

An immunomodulatory substance (LS-2616) was found to enhance the onset of GVHR after semi-syngeneic transplantation. In contrast to the findings in the heart rejection model, LS-2616 did not extinguish the immunosuppressive effect of CyA, suggesting that GVHR and rejection are not mediated via exactly the same effector mechanisms.

It was possible, under certain conditions, to observe rejection and GVHR simultaneously after fully allogeneic transplantation.

The novel vitamin D analogue MC 1288 was found to have immunosuppressive effects on both small-bowel and heart grafts.

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