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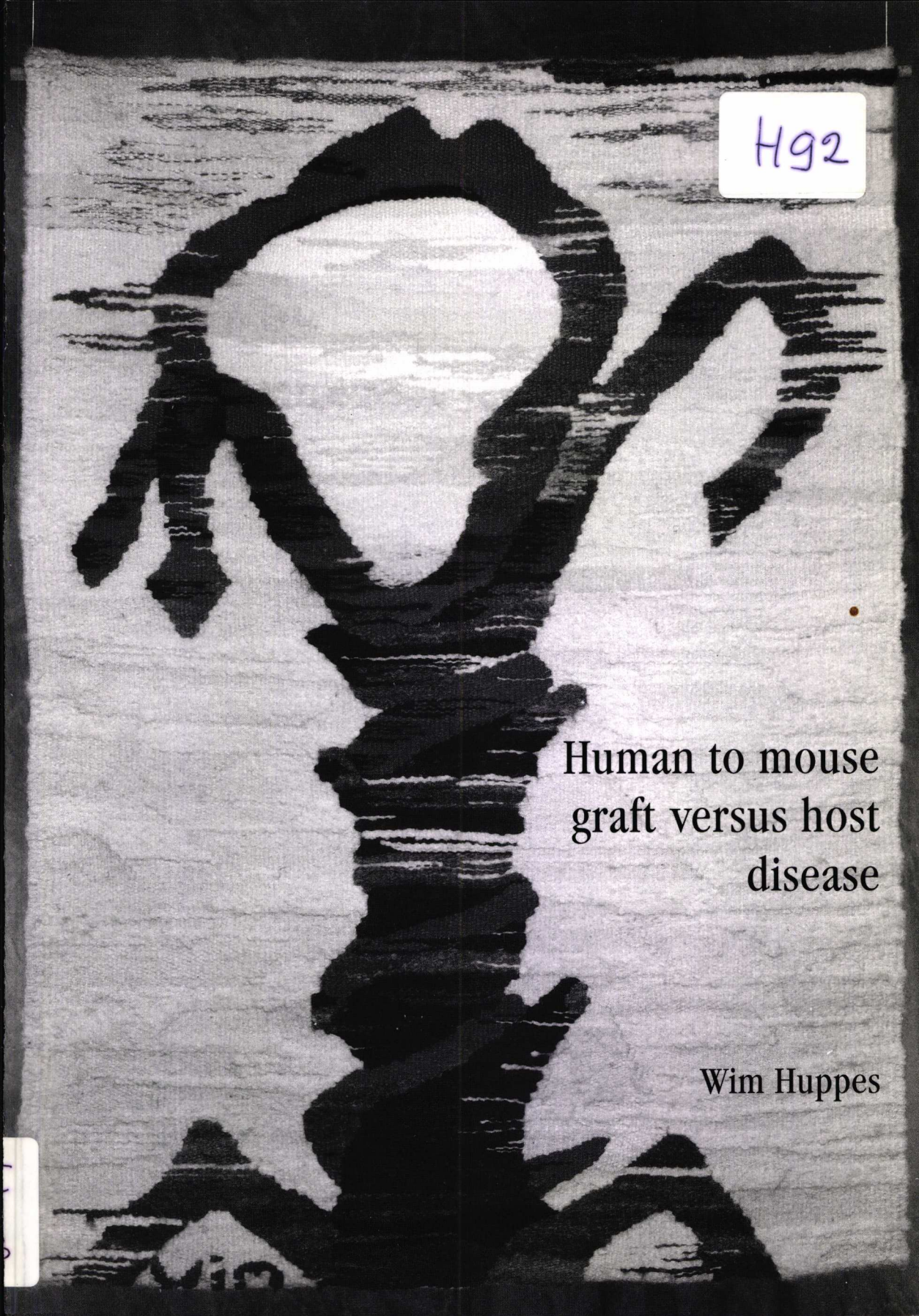

Human to mouse graft versus host disease

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HUMAN TO MOUSE GRAFT VERSUS HOST DISEASE

A new model for AIDS research

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HUMAN TO MOUSE GRAFT VERSUS HOST DISEASE

A new model for AIDS research

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Willem Huppes
geboren te Aruba

Promotor: prof.dr. P.I.J.M. Wuisman

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To my parents

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Chapter 1

General Introduction

General introduction

Arguments for a new murine model for AIDS research

Onset of the acquired immune deficiency syndrome (AIDS) epidemic in the early 1980s and identification of the etiologic agent as a human retrovirus stimulated interest in models for medical research[1].

It appeared that non-human primates like the chimpanzee are not an ideal AIDS model, mostly so because replication of macrophage tropic human immuno-deficiency virus-1 (HIV-1) in these animals is poor. This probably causes their apparent resistance to AIDS[2,3], as a comparable AIDS disease has not been observed in chimpanzees after experimental infection with HIV. In addition this model is limited by the fact that world-wide only a few hundred chimpanzees are available as laboratory animals.

In-vitro culture systems appear of limited value too, because they do not allow complex interactions with, for instance, the immune system. These interactions are characteristic of biological systems. In addition, it has appeared that not all pseudo-strains of HIV-1 can be propagated in tissue culture. Cultures also rapidly select for viral variants[4]. Thirdly, the phenotype of HIV-1 accessory mutants appears to be more obvious in the *in vivo* models than in tissue culture[5,6]. This is particularly true for *nef*-deletion mutants, which are associated with appreciable delay in infection and CD4 T cell depletion[6].

Most earlier work on retroviruses had focused on the oncogenic properties of RNA tumor viruses, and immuno-suppressive properties of murine and feline leukemia viruses were largely overlooked. Moreover, most oncogenic retroviruses were type-C viruses with simple genomes, whereas human immuno-deficiency virus genome is more complex[7]. Instead the model-virus should be HIV-1 or a similar lentivirus with the same regulatory genes as HIV-1. Simian immuno-deficiency virus-1[8] (SIV-1) of macaques is a close second choice and feline immuno-deficiency virus[9] (FIV-1) of cats a more distant third choice[17]. Both HIV-1 and SIV-1 use CD4 to infect T cells, whereas FIV principally infects macrophages in a CD4-independent fusion event. However, even SIV gene-regulation appears to be different, as pathogenic SIV isolates induce relatively rapid CD4 T cell depletion and simian AIDS, compared with HIV-1 infection of humans, but the clinical course is relatively protracted, with the exception of rare, highly pathogenic isolates[10,11]. With these highly cyto-pathogenic isolates disease progression in macaques is rather quick, with lethal disease occurring within a few weeks. However, whatever SIV-isolate is used, the viral genomes of HIV and SIV are too divergent to allow direct conclusions from experiments. Moreover, experiments with non-human primates like macaques are ethically demanding and expensive. Alternative experimental strategies have to be developed.

Therefore it is important to develop small animal models of AIDS, which also would allow for efficiency of scale. This relative advantage as compared to chimpanzees, monkeys and cats is quite obvious in terms of costs and time efficiency. An additional advantage is the wealth of prior information on the immunology and virology of the mouse. Consequently, one type-C retrovirus infection of mice that has been found to reproduce several of the clinical features of AIDS could be considered a model for some aspects of AIDS. It has been termed the murine AIDS (MAIDS) model[12,13]. However, although the model can be used for certain areas of research, e.g., into prevention of opportunistic infection or lymph-adenopathy with secondary leukemia, this murine leukemia virus (MuLV) is very different from HIV-1. The regulatory genes are different and infection is not through CD4. Therefore, the MAIDS model is far from being ideal.

In 1983 a severe combined immuno-deficiency (SCID) mutation in the mouse was reported[14]. Mice homozygous for this mutation are severely deficient in functional B and T lymphocytes. Consequently, these mice do not reject allogeneic and xenogeneic (e.g., human) tissue grafts. This was a break-through, because it became theoretically possible to study intrinsic human disease in a murine model.

In 1988 two different models for the generation of human to mouse chimeras were published using this SCID mutant mouse[15,16]. Since then, it has been shown that these models allow significant *in vivo* replication of HIV. The produced chimeras are susceptible to all clinical isolates of HIV-1 and HIV-2 and show relatively rapid induction of $CD4^+$ T cell depletion with maintenance of diversity of pseudo-strains[17].

The first model, developed by the group of Mosier, consists of human peripheral blood leukocytes (PBL) intra-peritoneally (ip) injected into non-conditioned mice with severe combined immune-deficiency (SCID). The PBL graft was given in such a dose that the recipient mice had no signs of graft versus host disease (GvHD)[18].

The disadvantage of the model is that the human T cells in the graft become mostly activated $CD8^+$ cells that are anergic to T cell receptor activation[19,20]. In addition, the mice contain few human cells. Lastly, clear evidence of HIV-1 infection in this type of chimera was in the range of 40-75%[18,21,22,23,24,25,26]. (Occasionally, small groups of mice have shown a 100% rate of infection[23].)

The advantage of the model is that every HIV-1 isolate that was tested (including laboratory pseudo-strains) could be replicated[22,27,28] and that genotypic diversity of HIV-1 primary isolates, the so-called quasi-species of viral genomes[29], is maintained[30]. Infection with most HIV-1 isolates leads to rapid induction of CD4 T cell depletion (within weeks)[28,31] and increased human immuno-globulin production[18,22] Lastly, the phenotype of HIV-1 accessory gene mutants, particularly of nef-deletion mutants, is more obvious in the model than in tissue culture[5,6].

The second model, developed by the group of McCune, consists of small segments of human fetal thymus grafts in the subcapsular space of the kidney of non-conditioned SCID mice[32]. When fragments of human fetal liver were co-implanted adjacent to the fetal thymus graft, the two organs are vascularized and fuse to form a conjoint thymus/liver hemopoietic organ (Thy/Liv)[33]. For periods as long as 6-12 months, these are capable of proliferating and differentiating through all the normal

stages of thymocyte maturation, including the predominant $CD3_{low}CD4^{+}CD8^{+}$ cortical thymocyte population, and the more mature medullar population expressing either CD4 or CD8. Each of these sub-populations exists in a relative proportion that is similar to that found in normal human thymus[34].

Approximately 50-80% of SCID mice with Thy/Liv grafts show evidence of circulating human T cells in the peripheral blood, depending on the detection methods such as flow cytometry or PCR analysis. These cells display phenotypic and functional characteristics similar to those found in humans. The CD4/CD8 ratio is normal, there is no evidence of T cell activation as measured by expression of CD69 and CD25[36]. Even though the human cells are in the circulation for relatively long periods of time, there is no observation of graft-versus host disease, which suggests that the cells are tolerant of the $H\text{-}2^{d}$ backgrond of the SCID mouse of the laboratory[34]. These observation show that the Thy/Liv organ xenograft provides stable conditions for a stromal microenvironment requisite for maintenance and differentiation of human progenitor cells to T cell lineages[35].

A disadvantage of this model is that it is demanding to make the chimeras. First of all, because one has to be able to influence standard operation techniques of suction abortion in humans, because it would lead to pieces of fetal thymus which are too small to be of any use. Secondly, one has to be able to perform fast histological analysis to check selected tissues. Thirdly, one has to be able to perform micro-surgery. Lastly, the produced chimeras do not have human immunological function, because the fetal thymus grafts do not have that and they do not populate the murine tissues significantly[36].

In contrast, the advantages of the model are that HIV-1 primary isolates were able to infect the graft lymphocytes and histiocytes successfully. In addition these primary isolates remained polyclonal and retained their quasi-species composition after primary HIV-1 infection[37,38,39]. The infection proceeds with anticipated pattern of tropism and cytophatic effects consistent with those observed in HIV-1infected humans. Both $CD4^{+}$ T cells and myeloid cells are infected and support active viral replication[40,41]. In addition to inversion of the CD4/CD8 ratio in the medulla of the thymus and within the human T cell pool in the peripheral circulation, thymocyte depletion is associated with massive destruction of the $CD4^{+}CD8^{+}$ cortical thymocyte subpopulation and eventual depletion of the $CD3^{-}CD4^{+}CD8^{-}$ intra-thymic progenitor cell population[42].

In summary it can be concluded that the human/mouse chimeras as developed by the groups of Mosier and McCune are promising models for medical research. They are now in the phase of being used to screen antiviral compounds in the drug development pathway, as an adjunct to prioritise ones that should move forward towards the clinic. In addition, their contribution to fundamental AIDS research is beyond doubt.; e.g., a key finding has been that HIV-1 virus and not secondary immune responses cause patho-genicity, by depleting T cell progenitors and $CD4^{+}$ T cells[17,35].

However, the final conclusion is that the practical use of both these models is significantly hampered by their disadvantages, i.e., that the grafts show defective immunological capacities and that few donor-type cells can be recovered from the recipient mice.

Need to examine xenograft transplantation barrier

The models of the group of McCune and Mosier showed for the first time that discordant cellular grafts (human peripheral blood leukocytes) could be grafted into a murine host without being hyper-acutely rejected.

Human to mouse chimeras had been made containing solid tissue grafts in athymic nude mice[43]. However, a murine model for human disease has to include hemopoietic cells in order to allow the study of AIDS. During the past 30 years, many attempts to transplant human hemopoietic cells in severely immuno-suppressed rodents, including athymic nude mice, did not result in consistent takes, except for malignant cells of lympho-hemopoietic origin in newborn rats[44].

The break-through with the models of the groups of Mosier and McCune in 1988, with the use of the SCID mutant mouse[18,32] was therefore encouraging. As the only difference between heavily conditioned normal mice and SCID mice is presence of plasma antibodies, it was reasonable to suppose that these caused rejection of discordant xenogeneic grafts. Due to the fact that the hosts were naive, it could be conjectured that the hyperacute rejection of the non-SCID hosts had to be due to the presence of natural antibodies (NAb).

NAb are an important component of the normal B cell repertoir[45]. They are present in sera of normal mammals and in lower phylogenetic species. They are thought to arise from immune activation by microbial agents and food antigens. NAb are poly-reactive, appear early in ontogony, and have sequences close to germ-line configuration[46].

One major line of research in this thesis is the investigation whether these NAb are indeed the major barrier for xenografts, because further research could then be directed at interventions of this transplantation barrier. This is important because it could lead to the use of rodents for medical research that would be easier to breed then severe-combined immuno-deficient (SCID) mice. Breeding of SCID mice is demanding for three reasons: (1) SCID mice have B and T cell deficiency and are therefore prone to opportunistic infection. (2) SCID mice recover from immuno-deficiency in about a year, a phenomenon which is called "leakiness" [47,48] and this is passed on to next generation (unpublished results B. DeGeus, CDI, Leliestad, The Netherlands and W. Huppes, Hilversum, The Netherlands). (3) Breeding of SCID mice is demanding, because females have iron deficiency anaemia (personal communication with J. Paulonis, Oncogene Science Inc., New York, USA).

An additional advantage of having strategies to overcome the natural antibody transplantation barrier would be that discordant xenogeneic donor transplantations would become more achievable. This is important to medicine, because some human diseases can principally be cured with discordant xenogeneic organ grafts. A method to circumvent the Nab barrier was investigated in Chapter 3.

The *in-vitro* relation between pre-existing antibodies, i.e., natural antibodies (NAb) and the human ABO (H) blood-groups was identified in 1911[49] with the discovery of the Forssman Ab: rabbit sera exhibited anti-human blood-group A, B and O (H) activity. Only the anti-A agglutinins also lysed sheep erythrocytes and were defined as Forssman Ab[50]. The glycolipid Forssman Ag appears to be ubiquitous and its reactive serum Ab is supposedly induced in mammals by the bacterium Escherichia freundii[51]. NAb reacting with cells of other species have been described in the serum of many animals[52,53]. For chickens the reactivity of the NAb with human blood-group B has been demonstrated[54]. Cross species NAb assumably play a pivotal role in the hyperacute rejection of vascularized organ grafts, but their influence on the transplantability of human cells into mice had not been studied so far.

At first sight it is difficult to understand why mouse anti-human ABO NAb would lead to the rejection of human cells, because ABO (H) antagonism is not an obstacle for allogeneic bone marrow nor for allogeneic vascularised organ grafts. However, in contrast to the ABO (H) antibodies, xenogeneic serum is toxic and causes lysis of nucleated cells[55]. In the present study the occurrence of murine serum NAb against human blood cells (Chapter 2) is examined in relation to the success of engraftment of human blood cells. More specifically the relation with the ABO (H) blood-group antigens (Chapter 3) is examined. It was also investigated whether normal adult mice could be constructed devoid of natural antibodies (Chapter 4).

Need to get donor-type environment for discordant xenograft

As discussed, the human xenograft murine models that were developed by the groups of Mosier and McCune have human grafts that are immunologically non-functional. This is a significant drawback of these models, because integration of human immunology is a prerequisite for many research areas, including that of AIDS[17]. Consequently the work in this thesis concentrated on solving that difficulty. The goal was to generate human xenografts in a small animal that would generate a functional human immune-system, with abundance of functional human immune-cells through *in vivo* proliferation.

The evolutionary gap between humans and mice is 70×10^6 years[56]. This large gap is thought to explain the non-functionality of the grafted human peripheral blood leukocytes in the murine host. It causes lack of appropriate environment, most importantly of human cytokines like interleukin-2 (Il-2), Il-4 and γ interferon. Therefore, it was conjectured that large numbers of human $CD4^+$ T cells would overcome the lack of these cytokines by auto-production of these proteins, thereby reaching the threshold levels needed for proliferation. Consequently, the work concentrated on whether xenogeneic graft versus host disease (GvHD) could be induced with transplantation of large numbers of human peripheral blood cells (PBL).

It was envisioned that successful functional engraftment of human PBL would lead to graft versus host disease (GvHD). This form of GvHD, i.e., discordant xenogeneic, had not been described yet.

The *allogeneic* GvHD had been known since 1956 as secondary disease[57]. It is the characteristic complication of non-syngeneic transplantation of grafts containing sufficient numbers of mature lymphocytes[58]. The reacting cells in allogeneic GvHD represent T cells[59]. Primate and human bone marrow and blood contain large numbers of these immuno-competent cells and transplantation usually leads to the rapid, that is within 5-10 days, development of violent GvHD, which is lethal within 15 days after transplantation[60,61]. This acute GvH reaction is the result of a cell-mediated immune response against the host tissues by lymphocytes which were already present in the graft [58,61,62]. The cause of death during acute GvHD is attributed to severe damage to organs and tissues involved by donor type lymphocytes.

When xenogeneic GvHD could be induced with large numbers of human leukocytes (Chapter 2), it was envisioned that subsequently it would be investigated how the number of human PBL needed to induce GvHD could be decreased. This was done with the treatment of chimeras with donor-type cytokine interleukin-2 (Chapter 5). Subsequently, it was investigated, which cell type was causing this xenogeneic GvHD (Chapter 2, 5, 6). In addition, histological documentation (Chapter 2, 6) and functional tests of the graft (Chapter 6) were done to test whether the model would be useful for human immunological research. Lastly, it was investigated whether the grafted human immune system allowed human virus replication and immunological interaction (Chapter 7).

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Chapter 2

Acute Human Versus Mouse Graft Versus Host Disease in Normal and Immunodeficient Mice

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Acute human *vs.* mouse graft *vs.* host disease in normal and immunodeficient mice

Recent reports of persistent engraftment of human lymphocytes and myeloid cells in hereditary immunodeficient severe combined immunodeficient mice (SCID) and beige athymic nude X-linked immunodeficiency (Bg/Nu/XID) mice have raised the question of why attempts to graft human cells into artificially immunosuppressed normal mice have failed so far. In the present study we provide evidence that this difference is due to the absence of natural antibodies in the mutant mice. We demonstrate that human PBL can be grafted in normal mice immunosuppressed by heavy doses of total body irradiation, provided the transplant is performed when the recipients lack natural antibodies in their serum, *e.g.* as in newborn normal mice, in mice treated with anti-mouse IgM antibody from birth, and in 3-week-old B cell-deficient CBA/N mice. In all cases, large numbers of human PBL were required.

Under these conditions an acute and fatal graft *vs.* host disease (GVHD) developed in the recipients, regardless of whether these were artificially immunosuppressed or hereditary immunodeficient. The clinical manifestations and the histopathology of this xenogeneic acute GVHD are quite different from those of allogeneic GVHD. The former is primarily confined to the hematolymphoid tissues and locations close to accumulations of proliferating lymphoblasts, such as the peritoneal cavity in case of i.p. transplantation. The discordant xenogeneic GVHD is induced by human T lymphocytes and can be abrogated by treatment with anti-human T cell serum.

1 Introduction

Engraftment of mismatched allogeneic or of concordant xenogeneic donor T lymphocytes causes GVH disease (GVHD) in all donor-recipient combinations studied so far [1]. Unexpectedly, in recent reports of the successful transplantation of human T lymphocytes in immunodeficient mice [2–4] and in normal lethally irradiated mice grafted simultaneously with severe combined immunodeficient (SCID) BM and human cells, there was no mentioning of the development of acute GVHD [5]. Only in one study a GVH reaction that was limited to the portal triad of the liver was observed in three out of six mice that had been grafted i.p. with human PBL 8 weeks previously. The reaction consisted of small infiltrates of human T cells and plasma cells around the bile ducts [6].

The characteristic pathology of acute allogeneic GVHD, i.e. necrosis of parenchyma cells and bile ducts in the liver, dermatitis and colitis, is similar in mice and humans. Its absence in the human-mouse chimeras can, therefore, not be due to this particular recipient-donor combination. However, it is possible that the species specificity of certain cytokines, like IL 2, IL 4 and IFN-γ, prevents the development of acute GVHD in this discordant xenogeneic combination. Another explanation might be sought in specific defects in antigen expression or presentation associated with SCID and beige-athymic nude X-linked (Bg/Nu/XID) immunodeficiency.

We attempted to induce GVHD in normal immunosuppressed mice by the injection of large numbers of human PBL, hoping that this would facilitate the subsequent engraftment of hemopoietic and lymphoid tissue and would eventually result in more complete chimerism than is currently achieved in the various immunodeficient recipients. Our initial attempts using normal adult mice conditioned with a lethal dose of total body irradiation (TBI) all failed, in that the human cells rapidly disappeared from the circulation and from the hemopoietic tissues. In view of the assumed role of natural antibodies (NAb) in preventing the take of discordant xenogeneic organ grafts [7] we developed the hypothesis that the failure to graft human hemopoietic cells in immunosuppressed mice of normal strains was due to the presence of NAb reacting with human cells. In a survey of preformed antibodies (agglutinins) between combinations of 50 species in 5 zoological orders (primates, carnivores, even-toed ungulates, equines and rodents) the highest titers were found in rodent sera against human erythrocytes [8]. Cytotoxic activity against lymphocytes of other species was demonstrated in the serum of all common laboratory animals, including the mouse [9]. Furthermore, it seemed plausible to assume an absence of NAb in SCID and Bg/Nu/XID mice, which would explain the reported engraftment of human cells in these mutant strains.

In the present study, evidence of proliferation of grafted human PBL in lethally irradiated recipient mice that lacked anti-human NAb, is presented. These were newborn mice, 3-week-old CBA/N mice, which have a hereditary B cell deficiency and 6-week-old BCBA mice, which had been

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Abbreviations: **NAb:** Natural antibodies **GVHD:** GVH disease **TBI:** Total body irradiation

injected with anti-mouse IgM antibodies since birth to prevent the development of NAb. In all these recipients, a fatal acute atypical GVHD developed. We were also able to induce a similar clinical syndrome in cyclophosphamide-treated SCID mice and in irradiated Bg/Nu/XID mice, by grafting large numbers of human lymphocytes. The disease that develops in these mice differs from the classical acute GVHD of allogeneic and concordant xenogeneic host-donor combinations. In the following we describe the pathological changes in the tissues of these animals as well as the conditions required to induce discordant xenogeneic GVHD.

2 Materials and methods

2.1 Mice

Inbred BALB/cRij, BALB/c-SCID, CBA/Rij, CBA/N, C3H/Rij, BCBA [C57BL × (CBA)F_1] and random bred CBA/N (XID) and Bg/Nu/XID (gift of Dr. A. Sebesteny, Imperial Cancer Research Fund, London, GB) mice were bred and kept under specific pathogen-free (SPF) [10] conditions in our own breeding colony. Fetal liver cells for support of lethally irradiated CBA/N mice were obtained from timed pregnancies of CBA/N mice, that were randomly bred on BALB/c and C57BL background, because the number of offspring of inbred CBA/N was insufficient. Mice in experiments were kept in horizontal laminar flow cabinets and were given the following additives per liter distilled drinking water: 100 mg polymyxin B sulfate (Pfizer, New York, NY), 100 mg ciprofloxacin (Ciproxin, Bayer, Leverkusen, FRG) 100 mg natamycin (Delvovid, Gist-Brocades, Delft, The Netherlands) and 50 g saccharose. Groups of BALB/c, CBA and C3H mice were bred and kept under germ-free (GF) conditions as well [11]. All mice were fed autoclaved pellets (AM2; Hope Farm, Woerden, The Netherlands) and acidified water *ad libitum*.

2.2 NAb

FCM was used for determining the presence of NAb against human PBL in decomplemented mouse sera. Mouse serum was diluted 1 : 10 and 1 : 100 in Hepes-buffered HBSS and incubated with 10^6 human PBL for 60 min. After two washes in HBSS, the cells were resuspended in 50 µl of 1 : 100 diluted rat anti-mouse IgM (RaM IgM-FITC; Janssen Pharmaceutica B.V., Beerse, Belgium) in HBSS with 5 µl human serum of a healthy blood donor. After a 30 min incubation, cells were washed in HBSS and analyzed on a FCM (Becton Dickinson, San Jose, CA). To detect (unexpected) IgG NAb the sera were checked with RaM-IgG-FITC as well. Anti-HLe-1-FITC (anti-human-CD45-FITC; Becton Dickinson) served as positive control and human serum as negative control. Some of the sera of normal mice showed no signal with RaM-IgM-FITC at the 1 : 10 dilution (due to the pro-zone effect) and a clear signal at the 1 : 100 dilution, indicating high titers of NAb in these animals. Therefore both dilutions were always used. Background staining was about 1% and samples showing 2.5% of cells or more stained with RaM-IgM-FITC were considered as positive. To prevent the development of NAb [12], newborn BCBA mice were treated with goat anti-mouse IgM antiserum [13]. Treatment was started with a dose of 50 µg between 150 and 240 min after birth and continued with a dose of 100 µl three times a week for 6 weeks.

2.3 Conditioning of mice for transplantation

TBI was performed with a ^{137}Cesium source, type Gamma Cell 20 (Atomic Energy of Canada, Ottawa, Canada), at a dose rate of 0.87 Gy/min. Since SCID mice are abnormally sensitive to irradiation [14], we decided to condition them instead with CY (Endoxan, Asta, Frankfurt am Main, FRG). CY was dissolved in distilled water and injected i.p. The doses used were 200, 400 and 800 mg/kg. The bladder epithelium was protected with mesna (Uromitexan, Asta-Werke, Degussa Pharma Gruppe, Bielefeld, FRG) 1 : 10 diluted in HBSS at a dose of 80 mg/kg 1 h before, and 8 and 16 h after CY treatment.

2.4 Transplantation procedures

All supralethally irradiated mice were given a small dose of mouse hemopoietic cells as supportive care to prevent mortality from BM aplasia. Initially in experiments with CBA/N recipients, we employed fetal liver (FL) cells from day-14 timed pregnancies to minimize the presence of NAb-producing cells in the graft. These cells are allogeneic to the recipient inbred CBA/N strain (see Sect. 2.1). Later, we observed that the use of syngeneic BM cells from NAb-positive animals (12-week-old BCBA mice) did not influence the results. Therefore, in subsequent experiments, inbred mice received supportive care with syngeneic mouse BM, which was obtained by flushing femurs with HBSS. Non-inbred mice received autologous BM as supportive care to exclude allogeneic GVHD. Autologous BM was obtained by transcutaneous aspiration of the BM of one femur, using ether anesthesia.

FL cell suspensions were prepared by trypsin digestion of parenchymal cells, as reported by Löwenberg et al. [15]. Mononuclear BM suspensions were prepared by filtration through four layers of 100-µm nylon gauze.

Human PBL were isolated from fresh buffy coat cells of healthy blood donors. T cells were isolated by rosetting with SRBC, as described by Saxon et al. [16]. In some experiments, SRBC-selected human T cells were depleted of remaining MΦ by culturing 10^8 cells/50 ml RPMI 1640 with 10% (v/v) heat-inactivated FCS in 162-cm^2 cell culture flasks for 24 h. FCM analysis of nonadherent cells with anti-LeuM3-FITC (anti-human-CD14-FITC; Becton Dickinson; detecting human MΦ), anti-κ Ig L chain-FITC (anti-κ-FITC) and anti-λ Ig L chain-FITC (anti-λ-FITC) both detecting human B cells (Nordic Immunology, Tilburg, The Netherlands) showed $< 0.1\%$ contamination of T cell suspensions with MΦ or B cells.

Cells were counted after staining with trypan blue 0.4% solution prior to transplantation. Transplantation of the various cells was performed within 5 h of irradiation and 50 h of conditioning with CY. Human PBL were injected i.p. Mouse cells were injected i.v. except for the newborn mice which received their grafts i.p.

2.5 Analysis of chimerism and GVHD

Cell suspensions of mouse thymus, livers, BM, spleen, LN and blood were filtrated through four layers of 100-μm nylon gauze. Lymphocytes of blood and liver were separated from remaining cells using lymphocyte separation medium [16]. FCM analysis of cells was performed after incubation with each of the following fluorochrome-labeled human leukocyte specific-Ab: anti-HLe-1-FITC (anti-human-CD45-FITC), anti-Leu2A-PE (anti-human-CD8-PE), anti-Leu3A-PE (anti-human-CD4-PE), anti-human CD14-FITC (Becton Dickinson), anti-human-κ-FITC and anti-human-λ-FITC.

Karyotyping was performed according to Haeyry et al. [17]. Cells were obtained by filtering mashed spleen through four layers of 100-μm nylon gauze. After culturing the cells for 3 days in the presence of PHA (Sigma, St Louis, MO), colchicine mitotic arrest and preparation of slides was carried out.

Enumeration of thrombocytes was performed with a Platelet Analyzer (Baker Diagnostics, Bethlehem, PA) and under light microscope with May-Grünwald (Merck, Darmstadt, FRG) and Giemsa (Gurr, Poole, GB) stained peripheral blood smear slides.

2.6 Histopathology

Complete gross necropsy was performed on mice from the various experiments. Tissue samples were fixed in phosphate-buffered 4% formaldehyde, routinely processed to 3-μm histological sections and stained with hematoxylin, phloxine and saffron (HPS). Skeletal tissues were decalcified using formic acid solution. Immunofluorescence microscopy was conducted on the liver of human PBL-transplanted CBA/N mice. After killing these animals, the liver was snapfrozen at − 70 °C. Sections of 6 μm, prepared on a cryostat, were incubated with the following anti-human Ab: anti-Leu4-FITC (anti-human-CD3-FITC; Becton Dickinson), anti-human CD14-FITC, anti-human κ-FITC and anti-human-λ-FITC. To exclude cross-reactions, all reagents were tested on sections of human tonsil. No cross-reactions were observed.

3 Results

3.1 NAb

A survey for the presence of NAb was made in the sera of normal mouse strains, bred and kept under either GF or SPF conditions, using RaM-IgM-FITC as a reagent for FCM analysis (Table 1). All sera showed NAb reacting with human peripheral blood cells: erythrocytes, granulocytes, lymphocytes and MΦ. The test showed no signal when human serum instead of mouse serum was used. Also, none of these mouse sera showed reactivity against human cells when RaM-IgG-FITC was used as a reagent.

From 24 h after birth on, NAb were detected in BCBA mice. In contrast, no NAb could be detected in 6-week-old BCBA mice treated with goat anti-mouse IgM serum from birth. NAb were absent in sera of CBA/N mice, until the age of 21 days. In sera of 6-week-old SCID and Bg/Nu/XID mice no NAb were found. It is evident from the data in Table 1 that GVHD, indicative of engraftment of human PBL, only developed in mice without NAb.

Table 1. Relation between presence of NAb and occurence of xenogeneic GVHD following administration of human PBL

Strain	Flora	Age[a)]	NA[b)]	GVHD[b)]
BCBA	SPF	Newborn	− (4)	+ (20)
BCBA	SPF	2 d	+ (4)	ND
BCBA	SPF	3 d	+ (4)	ND
BCBA	SPF	12 w	+ (4)	− (2)
BCBA[c)]	SPF	6 w	− (4)	+ (2)
SCID	SPF	6 w	− (4)	+ (2)
Nude	SPF	Newborn	− (4)	+ (6)
Nude	SPF	12 w	+ (4)	− (2)
Bg/Nu/XID	SPF	6 w	− (4)	+ (2)
CBA/N	SPF	3 w	− (4)	+ (20)
CBA/N	SPF	4 w	± (4)	ND
CBA/N	SPF	5 w	+ (4)	ND
CBA	GF	12 w	+ (4)	− (6)
CBA	SPF	12 w	+ (4)	− (6)
C3H	GF	12 w	+ (4)	− (6)
C3H	SPF	12 w	+ (4)	− (6)
BALB/c	GF	12 w	+ (4)	ND
BALB/c	SPF	12 w	+ (4)	ND

a) w = weeks; d = days.
b) Between brackets number of animals.
c) Daily treatment with anti-mouse-IgM, from day of birth.

3.2 Conditioning for transplantation

Initially, we tried to construct human to mouse chimeras using NAb-positive normal mice. BCBA (6 and 12 weeks old), C3H and CBA mice (12 weeks old; both SPF and GF raised) received a graft of 3×10^8–4×10^8 human PBL, after conditioning with 9 Gy TBI and 5×10^5 syngeneic BM cells. None of the animals developed GVHD (Table 1), a finding which underlines the importance of NAb.

Consequently, high-dose radiation regimens were investigated for NAb-negative mice: newborn BCBA, 3-week-old CBA/N and 6-week-old Bg/Nu/XID and SCID mice. Of these strains, groups of four mice were subjected to graded doses of TBI. Supportive care with mouse hematopoietic BM cells was provided following irradiation doses of 9 Gy and more. Mortality data after a 3-month period are presented in Table 2. The highest dose tolerated by newborn BCBA mice given 5×10^5 syngeneic BM (of 12-week-old donors) was 9 Gy. CBA/N mice at the age of 3 weeks tolerated 9 Gy with 10^6 day-14 allogeneic FL or 5×10^5 syngeneic BM. Six-week-old Bg/Nu/XID mice tolerated 9 Gy irradiation with 10^6 autologous BM well. As reported by Biedermann et al. [14], SCID mouse cells are abnormally sensitive to γ irradiation. In our experiments a moderate dose of 4 Gy TBI was lethal for SCID mice. The mice showed wasting with diarrhea, suggesting intestinal damage as the cause of death. On the other hand, high-dose CY up to 400 mg/kg was very well tolerated. For supportive care, these mice received 5×10^5 syngeneic BM of 6-week-old SCID mice i.v. 50 h after CY administration.

Table 2. Results of conditioning with graded doses of TBI in the various mouse strains

Mousestrain	TBI dose (Gy)	No. of hemopoietic cells $\times 10^{-5}$	Deaths/ total	Cause of death
Newborn	7	–	0/4	–
BCBA[a)]	8	–	0/4	–
	9	–	4/4	BM aplasia
	9	1	3/4	BM aplasia
	9	5	0/4	–
	10	5	3/4	Neurological syndrome
	11	5	4/4	Intestinal syndrome
3-week-old	8	–	0/4	–
CBA/N[b)]	9	–	4/4	BM aplasia
	9	1	3/4	BM aplasia
	9	10	0/4	–
	10	10	3/4	Intestinal syndrome
	11	10	2/4	Intestinal syndrome
3-week-old CBA/N[a)]	9	5	0/4	–
6-week-old	7	–	0/4	–
Bg/Nu/XID[c)]	8	–	0/4	–
	9	–	4/4	BM aplasia
	9	10	0/4	–
	10	10	2/4	Intestinal syndrome
6-week-old	3	–	0/4	–
SCID	4	–	1/4	Intestinal syndrome
	5	–	3/4	Intestinal syndrome
	6	–	4/4	Intestinal syndrome

a) Supportive care with syngeneic BM.
b) Supportive care with allogeneic FL cells.
c) Supportive care with autologous BM.

3.3 Occurence of xenogeneic GVHD and chimerism

Newborn BCBA mice, which were conditioned with 9 Gy TBI and supported with 5×10^5 syngeneic BM cells, were injected i.p. with 2×10^7 human PBL. All animals died between day 6 and day 11 after transplantation ($n = 10$). FCM analysis of spleen and BM of these animals showed the presence of between 5–60% of human CD45$^+$ cells (results not shown). Histological examination of the tissues of four animals revealed a pattern of lesions that we now consider to be specific for discordant xenogeneic GVHD. In contrast, newborn mice ($n = 10$) treated the same way but without a graft of human cells, survived. Reactivity with human-specific anti-CD45 Ab in these mice was $< 0.1\%$. Newborn BCBA given only 2×10^6 human PBL also survived the conditioning regimen (Fig. 1) and showed no signs of GVHD. After 30 days, DNA was isolated from spleen and BM of these mice and spotted on a nylon membrane (NY13N; Schleicher and Schuell, Dassel, FRG). Hybridization with ^{32}P-labeled pBLUR8, a plasmid which contains the human Alu-sequence, revealed that approximately 0.01% of the DNA was of human origin (results not shown).

Induction of acute xenogeneic GVHD was further explored by using lower doses of TBI for conditioning. With a dose of 8 Gy only two out of four animals grafted with 2×10^7 human PBL developed GVHD and with 7 Gy none out of four (results not shown).

In contrast to normal young adult BCBA mice, those treated with goat anti-mouse IgM from birth until 6 weeks of age ($n = 2$), subjected to 9 Gy TBI and supported with syngeneic BM cells (5×10^5) developed fatal GVHD following i.p. injection of 3×10^8 human PBL. FCM analysis showed large numbers of human CD45$^+$ cells in the spleen, the BM and the ascites fluid. BCBA mice ($n = 2$) treated in exactly the same way, but not grafted with human cells, survived. FCM analysis showed an absence of human CD45$^+$ cells.

CBA/N mice were used at 3 weeks of age because in mice of that strain aged 5 weeks and older, NAb were detected (Table 1). The highest TBI dose tolerated in combination with supportive care (allogeneic FL) was 9 Gy. They were grafted with 2×10^8 human PBL i.p. These CBA/N mice ($n = 10$) died with acute GVHD between day 9 and 17 after transplantation (Fig. 2). FCM analysis at time of death revealed between 5% and 60% of human CD45$^+$ cells in their BM, spleen and ascites fluid. In contrast, very few positive cells, usually $< 1\%$, were detected in the spleen 3 days after grafting of 2×10^8 human PBL. When, instead of 2×10^8, only 2×10^7 human PBL were grafted, acute GVHD did not develop, and the animals survived. DNA isolated from the spleen and the BM of these mice 30 days after transplantation was spotted and hybridized against ^{32}P-labeled pBLUR8. Again, only approximately 0.01% of the DNA was of human origin (results not shown).

Karyograms of spleen cells were made to confirm the presence of human cells as found with FCM analysis in similar experiments. Cultured spleen cells of a CBA/N mouse, which died 11 days after grafting of 2×10^8 human

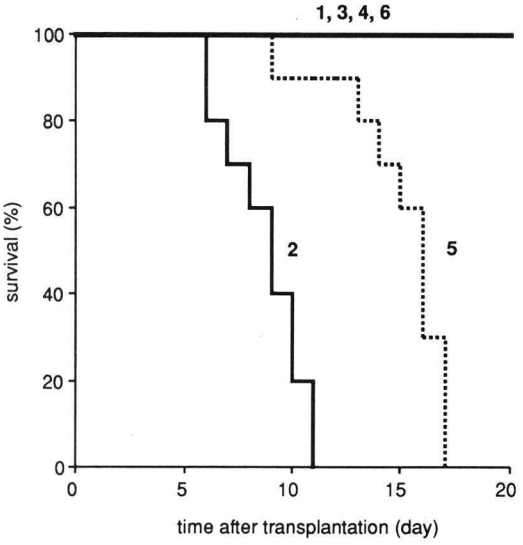


Figure 1. Survival curves of newborn BCBA mice and 3-week-old CBA/N mice. 1: Newborn BCBA mice (n = 10); 9 Gy TBI and i.p. transplantation of 5×10^5 syngeneic BM cells. 2: Newborn BCBA mice (n = 10); 9 Gy TBI, i.p. transplantation of 2×10^7 human PBL and 5×10^5 syngeneic BM cells i.v. 3: Newborn BCBA mice (n = 10); treatment as group 2, but 2×10^6 human PBL. 4: Three-week-old CBA/N mice (n = 10); 9 Gy TBI and i.v. transplantation of 10^6 allogeneic FL cells. 5: Three-week-old CBA/N mice (n = 10); 9 Gy TBI, i.p. transplantation of 2×10^8 human PBL and i.v. transplantation of 10^6 allogeneic FL cells. 6: Three-week-old CBA/N mice (n = 10); treatment as group 5, but 2×10^7 human PBL.

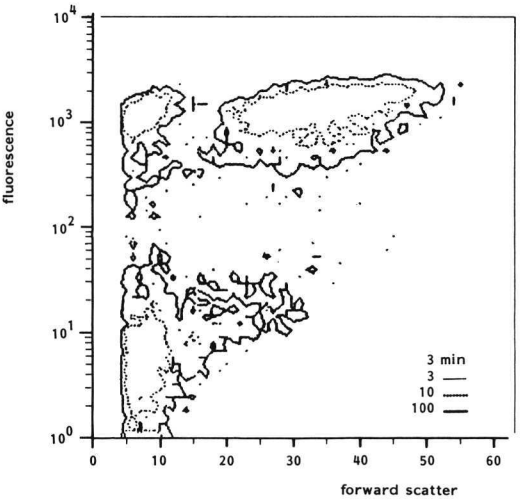


Figure 2. Size distribution of CD45$^+$ cells in ascites of a Bg/Nu/XID mouse, 6 days after i.p. inoculation of 3×10^8 human PBL. Conditioning: 9 Gy TBI. Anti-human CD45-FITC was used as a probe.

PBL, showed, among numerous metaphase plates, roughly 60% with the human karyotype.

The highest dose of radiation tolerated by SCID mice was 4 Gy. After higher doses of TBI these mice died within 8 days with heavy diarrhea, which made further experimentation using hemopoietic support unattractive. Surprisingly, the i.p. injection of up to 3×10^8 human PBL after TBI with a dose of 4 Gy did not result in GVHD. The results of grafting graded numbers of human PBL following conditioning with 400 mg/kg of CY are listed in Table 3. Under those conditions, the high cell number of 3×10^8 PBL caused GVHD and this was again accompanied by high percentages of human lymphoblasts in the spleen.

In an initial experiment Bg/Nu/XID (n = 2) mice were conditioned with 4 Gy TBI, the same dose as used by Kamel-Reid et al. [2]. These mice received 3×10^8 human PBL i.p. and did not develop GVHD. FCM analysis of Bg/Nu/XID spleen cells 1 month after treatment showed 5% and 3% human CD45$^+$, respectively in two mice. The effect of conditioning Bg/Nu/XID mice with various doses of TBI was investigated in more detail (Table 4). Of each group of four animals, two received 3×10^8 human PBL, which were injected i.p. and the other two served as controls. Following TBI doses of 7 Gy or more, grafting with human PBL resulted in GVHD. Just like the BCBA and CBA/N mice with GVHD, these Bg/Nu/XID mice showed between 5 and 60% human of CD45$^+$ cells in BM, spleen and ascites. As in the other strains, two subpopulations of human cells resembling medium sized and large lymphoblastic cells could be seen on scatter diagrams (Fig. 2).

3.4 Grafting of selected cell populations of human PBL

To investigate the cell type capable of inducing xenogeneic GVHD, selected cells of human PBL were transplanted into

Table 3. Human PBL titrated in CY-conditioned SCID mice[a)]

No. of mice	Human PBL i.p.	Days post-transplantation	Physical condition	GVHD	Percentage of human CD45$^+$ cells in spleen
2	3×10^8	11	Moribund	+, +	73, 24
2	3×10^7	30	Healthy	−, −	5, 1.0
2	3×10^6	30	Healthy	−, −	1.0, 0.1
4	–	30	Healthy	−, −, −, −	0.2, 0.1, 0.1, 0.0

a) CY: 400 mg/kg, supportive care: 5×10^5 syngeneic BM cells.

Table 4. Effect of TBI conditioning dose on outcome of transplantation of 3×10^8 human PBL i.p. in Bg/Nu/XID mice

γ-irradiation dose (Gy)	Human PBL	Day of death[a]	Acute GVHD	Percentage of human CD45$^+$ cells in spleen
4	+		−	0.4, 0.4
4	−		−	0.0, 0.0
5	+		−	0.5, 0.5
5	−		−	0.0, 0.0
6	+		−	0.8, 0.7
6	−		−	0.0, 0.0
7	+	6, 12	+	27, 42
7	−		−	0.2, 0.1
8	+	8, 11	+	53, 8
8	−		−	0.2, 0.0
9[b]	+	6, 10	+	22, 60
9[b]	−		−	0.1, 0.0

a) When not specified, mice were killed 3 months after transplantation.
b) Mice received supportive care with 10^6 autologous BM cells i.v.

Table 5. Occurence of GVHD after transplantation of T and B lymphocyte and MΦ selections of human PBL in CBA/N mice

Transplanted human cells[a]	Day of death	GVHD/total
Unseparated PBL[b]	7, 8	2/2
B + MΦ[b]	−, −	0/2
T + MΦ	10, 11	2/2
T	10, 12, 16, 17	4/4
None	−, −, −, −	0/4

a) All mice received supportive care with 10^6 allogeneic FL cells.
b) Including 5%–10% granulocytes.

groups of 3-week-old CBA/N mice conditioned with 9 Gy TBI and given 10^6 allogeneic CBA/N day-14 FL cells as hematological support. The selection of human T cells was performed by SRBC-rosetting and 24-h culture. In that way $< 0.1\%$ human MΦ and B cells remained in the graft. Only the purified T cells proved to be able to induce GVHD in these CBA/N mice (Table 5), within a period of 17 days, while equal numbers of B cells or MΦ were without effect.

3.5 Clinical manifestations of GVHD

The immediate cause of death in mice suffering from severe GVHD appeared to be multifocal hemorrhages. Platelet counts of mice with terminal disease were $< 40 \times 10^6$/ml. Control CBA/N mice grafted with allogeneic CBA/N FL cells only ($n = 4$), had 800×10^6–1600×10^6/ml on the same day after grafting. At the time GVHD was fully developed (day 12) FCM showed that the proportion of CD45$^+$ cells in the BM, spleen, thymus, LN, blood and liver was between 12% (thymus) and 100% (blood) in one completely analyzed CBA/N mouse. The ratio of CD4 CD8 cells was between 4.0 and 9.0 compared to 3.5 for the grafted peripheral blood cell suspension.

One human mouse chimera suffering from GVHD, with 24% CD3$^+$ human T cells in the spleen, was analyzed by FCM for human B cells and MΦ. Cell suspensions of liver, spleen and ascites cell showed about 1.5% anti-human κ-FITC$^+$ B cells and 0.75% anti-human λ-FITC$^+$ B cells (non-xenografted control: $< 0.1\%$). No human MΦ were detected with anti-human CD14-FITC in the chimera.

In an effort to prevent mortality from GVHD, two CBA/N mice suffering from GVHD at 11 days after transplantation were injected i.p. with 300 μg of anti-human thymocyte globulin (ATG, a gift of Dr. J. Kreeftenberg, R.I.V.M., Bilthoven, The Netherlands). The treatment was successfull, as all animals survived. These mice were killed at the age of 3 months. FCM analysis at that time, of the spleen with anti-human CD45-FITC revealed 5% and 4% human cells, respectively (control = CBA/N mice grafted with FL cells only: 0.1%).

3.6 Histopathology

Pathology data were derived from moribund BCBA, CBA/N and SCID mice transplanted i.p. or i.v. with human PBL as described earlier and from CBA/N mice killed between 6 and 14 days after i.v. transplantation of 2×10^8 human PBL and supportive care with 5×10^5 syngeneic BM cells. The latter study was performed to investigate the time course of pathological changes. Mice which were killed when moribund were severely dehydrated and at necropsy showed multifocal hemorrhages. Diarrhea and skin disease were notably absent.

Microscopic examination of mice killed moribund and of mice killed on schedule revealed the following sequence of events. Following an early regeneration of normal mouse BM constituents on day 6, with erythroid and myeloid colonies appearing first, the still depleted T cell areas of the spleen were repopulated by highly proliferative lymphoblastic cells. These areas expanded into the red pulp of the spleen replacing the normal mouse hemopoietic tissues on day 12 and thereafter (Fig. 3 C). These lymphoblastic cells were then also observed in the portal triads and sinusoids of the liver often growing into the walls of portal and central veins. They were also observed in LN, thymus, BM (Fig. 3 D) and in the perivascular tissues of lungs, salivary glands and meninges. Many chimeric animals, which suffered from GVHD, showed large numbers of lymphoblastic cells in the lumen of blood vessels. Occlusions of blood vessels with local proliferation of lymphoblastic cells was seen in various organs, especially the liver. Hemorrhages were seen at many sites among them the brain. In the mice which received i.p. grafts of human PBL, lymphoblastic infiltrates were observed in all intraperitoneal tissues and sometimes retroperitoneally as well.

In late stages, these infiltrates had the appearance of a malignant process consisting of rather monomorphic large blastic cells. Interdispersed between these blastic cells were medium-sized lymphoid cells. Their presence in spleen, LN and other tissues was associated with invasion into sur-

rounding tissues and destruction of vascular structures with associated hemorrhage, fibrinous exsudate and edema.

When comparing light microscopy with immunofluorescence microscopy it was observed that the infiltrations consisted of human cells. The liver of a CBA/N mouse after xenoengraftment revealed large groups of human $CD3^+$ cells, often around the portal triad. Among these, approximately 2% human B cells (anti-human κ-FITC- and anti-human λ-FITC-positive cells) were seen. No human MΦ were observed with anti-human CD14-FITC as a probe. A CBA/N mouse, which had been grafted with allogeneic CBA/FL cells, served as control and showed no positive cells at all.

In addition to the replacement of normal mouse hemopoietic tissue by human lymphoblasts, there were signs of active destruction (Fig. 3 E, F, G). Next to areas with normally repopulated mouse BM, areas were observed devoid of hemopoietic tissue (Fig. 3 G). This was replaced by edematous tissue containing a few blastic cells, local hemorrhage, fibrinoid necrosis, and early fibroblastic proliferation. This constitutes a very characteristic histological picture in discordant xenogenic GVHD.

Pathological changes characteristic of allogeneic GVHD were absent. Notably, lesions in the coecum, one of the prime targets in mouse GVHD, were never encountered. In the skin, a slight increase of dyskeratotic cells was occasionally observed. The liver lesions were dominated by lymphoblastic proliferation and coagulative necrosis, while bile duct destruction was absent. Lymphoid tissues were enlarged in the early stages and later became atrophic.

The cause of death in xenogenic GVHD seems to be mainly related to the destruction of the mouse hemopoietic tissue and to vascular damage by infiltrating lymphoblasts resulting in multifocal hemorrhages.

4 Discussion

4.1 Conditions allowing engraftment of discordant xenogeneic lymphocytes

4.1.1 Absence of NAb

So far transplantations of human PBL have only succeeded in the heriditary immunodeficient mouse strains SCID and Bg/Nu/XID [2–4, 6]. As it is unlikely that the level of immunosuppression in these mice is much different from that induced in heavily irradiated normal mice, the observed rejections in the latter have to be attributed to other factors that are not inactivated by high dose TBI. NK cells are not a likely candidate since these cells are absent in Bg/Nu/XID and present in SCID mice, while both mutant strains are equally receptive for grafts of human cells.

The most obvious other factor capable of rejecting hemopoietic cells is of course circulating antibodies. The early literature on experimental BM transplantation contains several reports demonstrating that actively induced antibodies directed against donor tissues prevent grafting of allogeneic BM or leukemic cells [18–22] even when these antibodies were passively transferred to the recipients prior to grafting. Recently, antibodies that bind to BM cells of the rat were demonstrated in the serum from normal mice by Aksentijevich et al. [23] who suggest that this might explain the large numbers of rat BM cells required for engraftment in lethally irradiated mice.

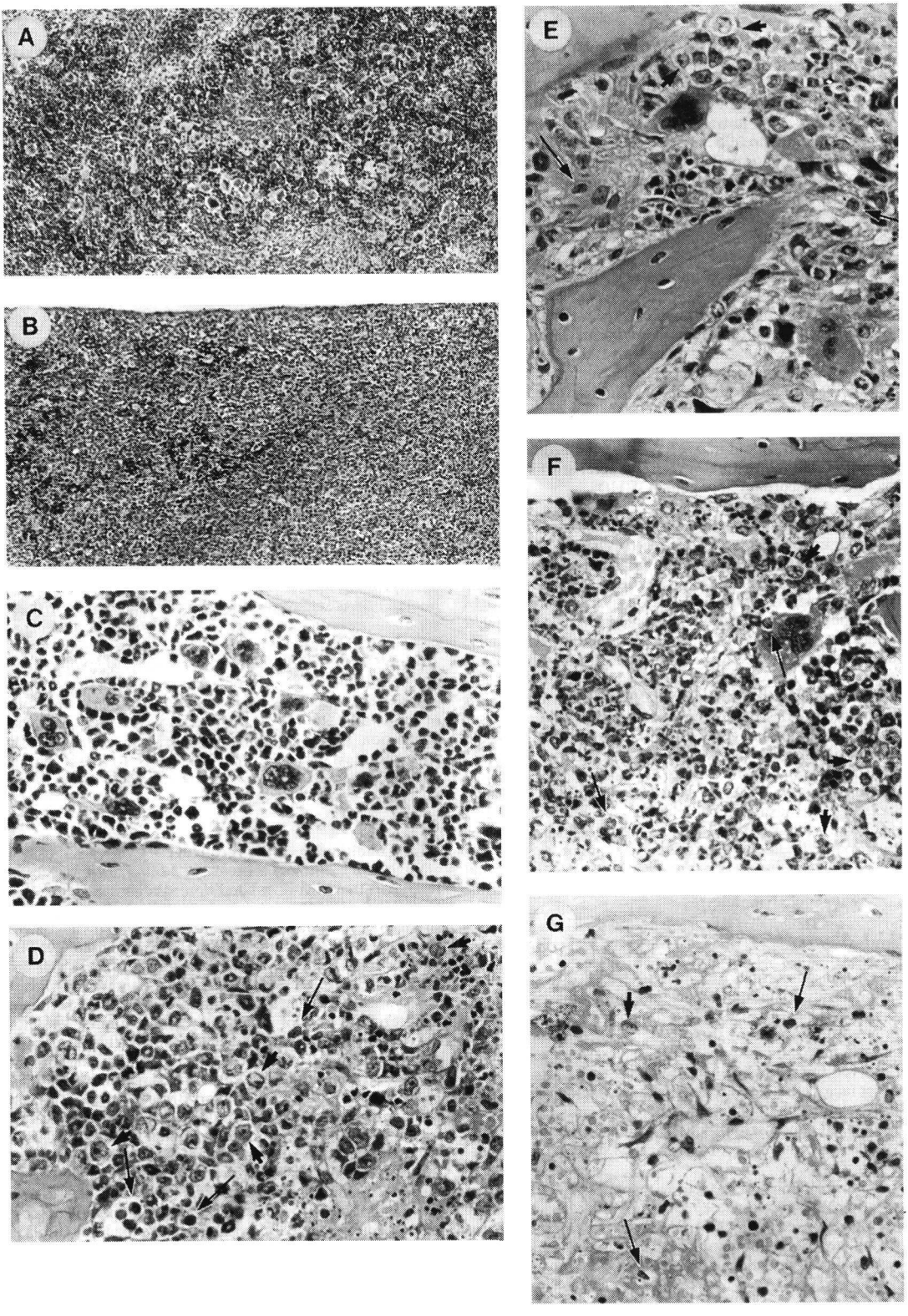
NAb are postulated to arise in all mammalian species in response to microbial antigens, some of them cross-reacting with erythrocytes and other cells of different species. As expected we could not demonstrate NAb in SCID and Bg/Nu/XID mouse sera. In the B cell-deficient CBA/N mouse, NAb were absent untill the age of weaning at 5 weeks and in newborn mice of normal strains NAb were not detected untill 24 h after birth. This latter finding would explain the take and proliferation of human lymphoma cells in newborn rats, reported by Southam et al. [24].

We detected normal titers of NAb reacting with human lymphocytes in our normal mouse strains that were bred and maintained under GF conditions, which is in contrast with the absence of NAb in Minnesota miniature swine reared under GF conditions [25]. However, the swine were raised on an antigen-free diet (Mullsoy, Syntex Lab., Palo Alto, CA), which is not the case with our mice, that receive sterile food containing bacterial antigens.

Our present results show that proliferation of grafted human PBL is achieved in all recipients that lack NAb, be it spontaneously due to heriditary factors, or induced as in the normal BCBA mice that were treated with anti-mouse IgM Ab from birth. All this seems to provide rather compelling evidence in favor of NAb being a major barrier in human to mouse transplantation of lymphatic cells. The recent results of Lubin et al. [6] may appear to be inconsistent with this conclusion. However, their mice were supported with SCID BM and it is possible that this leads to the eventual disappearance of Nab.

4.1.2 Conditioning of the recipients

It appears that high-dose conditioning regimens are needed to induce discordant xenogeneic GVHD in all mouse strains, because moderate conditioning of SCID mice with up to 4 Gy TBI, of Bg/Nu/XID with up to 6 Gy TBI (Table 4) and of newborn BCBA with up to 7 Gy TBI did not result in GVHD. However, Table 4 shows that lower doses of TBI, which fail to induce GVHD, do result in long-term survival of small numbers of human lymphocytes. Similarly, Kamel-Reid et al. reported the presence of a few percent of human cells in the spleen and BM of Bg/Nu/XID mice that were conditioned with a dose of 4 Gy TBI and grafted with 10^7 human BM cells without developing GVHD. It seems therefore that lower doses of TBI permit a limited engraftment of human cells, but it is not clear whether the requirement of higher conditioning doses for the extensive proliferation of human lymphocytes and the development of GVHD, has to be ascribed to the provision of more space in the lymphohemopoietic tissues, or to the more complete eradication of some unidentified immunocompetent cell population capable of rejecting xenogeneic cells.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4.1.3 Grafts containing high cell numbers

Human and mouse MHC are separated by an evolutionary gap of about 70×10^6 years [26]. Nevertheless the murine immune system recognizes human MHC Ag, as is obvious from the non H-2-restricted xenogeneic lymphocytotoxic mouse to human HLA reactivity [27]. Conversely, the human T cell anti-mouse H-2 response has been demonstrated to occur in the absence of human APC provided that human cytokines are added [28], implicating that human T cells can recognize mouse MHC, but require certain human-specific lymphokines for maximal reactivity. Accordingly, our present finding that xenogeneic GVHD can only be induced with large numbers of human T lymphocytes can be explained by assuming that under these conditions sufficient amounts of human cytokines are produced. Our results show that at least 2×10^7 human PBL per gram bodyweight are needed to induce GVHD in irradiated NAb-negative mice.

The finding that these numbers are similar in all different types of recipients argues against the assumption that the large numbers of PBL are required to suppress residual recipient barrier. Such a mechanism is also unlikely in view of the sustained engraftment of smaller numbers of human PBL or BM cells in the absence of symptoms of GVHD, as has been reported by others [2–4, 6] and in this study in newborn BCBA and weaned CBA/N mice, conditioned with high-dose TBI. We have demonstrated that T lymphocytes are the effector cells for inducing xenogeneic GVHD, and that MΦ and B cells do not possess this capacity. In addition we have been able to mitigate the xenogeneic GVHD by treatment of the affected mice with a polyclonal anti-human T cell globulin, which resulted in long-term survival, interestingly with small numbers of human lymphocytes persisting in the tissues. In this respect, xenogeneic GVHD is not different from allogeneic GVHD.

4.2 Symptoms and histopathology of discordant xenogeneic GVHD

The GVHD which developed in our immunodeficient and normal mouse strains following the grafting of human PBL, bears little resemblance to the acute GVHD as commonly observed following transplantation of allogeneic T cells and of rat BM into mice. The highly specific pathological changes observed in our human-mouse chimeras were also recorded in the tissues of mouse grafted with large numbers of rhesus monkey PBL or BM, not described in this report. We therefore propose the term discordant xenogeneic GVHD.

Clinically, wasting is the most predominant feature and the general condition of the animals only deteriorates 1 or 2 days before death. Diarrhea does not occur. Necrosis of the intestinal crypts and dermatitis characteristic for allogeneic GVHD have not been observed. During the first week after transplantation, the lymphohemopoietic tissues show a limited number of human lymphoblasts; about 10–12 days after transplantation these tissues are massively invaded by proliferating human lymphoblasts and in the BM aplastic areas have appeared. Our histopathological survey gives the impression that the mouse BM cells are killed in areas around the lymphoblasts and that the lymphoblastic cells themselves die when there are no target cells left. Proliferation and infiltration of these tissues and others like the liver and the meninges with lymphoblasts is at times so abundant that it resembles lymphoblastic leukemia. In this stage, many hemorrhages occur in various tissues, among them the brain and the heart, and these are probably the cause of death. It is likely that vascular damage induced by the human lymphoblasts is causing the hemorrhages, which are probably aggravated by the severe thrombopenia.

In the animals grafted i.p. with human PBL, lymphoblastic cells are also abundant in the peritoneal cavity, with infiltration of the abdominal organs and most strikingly in the omentum and abdominal fat. Ascites fluid, sometimes hemorrhagic, accumulates in the peritoneal cavity. A very similar pathology has been described by Gorer and Boyse [19] in F_1 hybrid mice given large numbers of lymphocytes from parental strain donors that had been immunized with cells of the other parental strain. However, these descriptions are not conclusive as regards the identity of the two syndromes. On the other hand our findings leave no doubt that discordant xenogeneic GVHD is quite different from concordant xenogeneic GVHD as described for instance in mice grafted with rat BM and spleen cells [1]. The latter chimeras develop all the characteristic lesions of allogeneic GVHD and the same pattern of clinical manifestation and mortality. We expect the difference with discordant GVHD to be caused by the lack of immunosurveillance function of the donor lymphocytes, due on the one hand to abundance of xenoantigens in the recipients' reticuloendothelial system which keep the incoming human lymphocytes from migrating, and on the other hand to species differences in lymphocyte homing receptors, like ICAM and LFA.

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◀ *Figure 3.* Depletion of mouse lymphohemopoietic tissues by the human xenograft. Photomicrographs of HPS-stained tissue sections of control CBA/N mice, transplanted with 5×10^5 syngeneic BM cells (A and C) and CBA/N mice with terminal xenogeneic GVHD (B, D–I) at 14 days post transplantation. Normal recovery of mouse hemopoiesis in red pulp of spleen (A, × 100) and BM (C, × 250). Spleen still shows partly depleted white pulp areas (A, left upper corner). (B) Spleen (× 100). Normal architecture lost and completely replaced by tumor-like proliferation of lymphoblastic cells. Only a few small foci of normal erythropoiesis remain. (D) BM (× 250). Foci of large lymphoblastic cells (short arrows) and medium-sized lymphoblastic cells (long arrows) between remaining mouse BM constituents with focal apoptotic cell death and fibrinoid stromal changes (right lower corner). (E, F, G) BM (× 250). Showing progressive stages of destruction of mouse BM cells and associated stromal changes. These sections were obtained from a single mouse. Arrows point to lymphoblastic cells as indicated under (D).

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Chapter 3

The Role of Natural Antibodies and ABO (H) Blood Groups in Transplantation of Human Lymphoid Cells into Mice

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The role of natural antibodies and ABO (H) blood groups in transplantation of human lymphoid cells into mice*

Recently, evidence was presented that natural antibodies (NAb) are a crucial barrier to human cellular engraftment in severely immunosuppressed normal mice (*Eur. J. Immunol.* 1992. *22:* 197.). In this report we show that normal mouse serum contains low titers of NAb against human cells of blood groups type O (H) and B and high titers against human cells of blood group A. Accordingly, human peripheral blood leukocytes (PBL) of group O (H) and B donors could be grafted successfully into normal BCBA mice (H-$2^{b/k}$) following irradiation with high dose total body irradiation (TBI). PBL of blood group type A donors did not engraft in normal mice but could be transplanted without difficulty in B cell-deficient CBA/N mice which lack NAb, after conditioning with high dose TBI.

Treatment of lethally irradiated normal BCBA mice with cobra venom factor (COF), which eliminates the third factor of complement, and liposomes containing dichloromethylene diphosphonate (Cl_2DMP), which eliminates macrophages, resulted in engraftment of human blood group type A PBL. This implies that the NAb barrier for discordant xenogeneic cell transplantation can be abrogated.

A method utilizing directly labeled probes and flow cytometry is described for the quantitation in mouse serum of NAb, reacting with human cells. Using sera of H-$2^{b/k}$ mice we show that murine NAb react with human stem cells, granulocytes, lymphocytes and monocytes of blood group A and only weakly with similiar cells from blood group O (H) and B donors. Sera of H-2^b, H-2^d and H-2^k mice of different ages and microflora possess NAb against human erythrocytes of blood group type A and occasionally demonstrate weak titers against erythrocytes of blood groups B and O (H) and the Rhesus factor.

1 Introduction

During the past 30 years, many attempts to transplant human hemopoietic cells in severely immunosuppressed rodents has not resulted in consistent takes, except for malignant cells of lymphohemopoietic origin in newborn rats [1]. In recent years, engraftment of human hemopoietic and lymphatic cells has been reported in severe combined immunodeficient (SCID) [2–3] and beige/nude/X-linked immunodeficient (Bg/Nu/XID) [4] mice.

We have recently shown that natural antibodies (NAb) against human PBL present a barrier to the xenotransplantation of human lymphocytes [5]. In XID mice, newborn mice and mice treated with anti-mouse IgM from birth, these NAb are absent. After lethal irradiation, these mice accept grafts of human PBL equally well as the SCID or Bg/Nu/XID mice. The titers of NAb in normal mouse serum reacting with human cells varied widely from one experiment to another. Subsequently, these variations were found to be donor associated and dependent on the ABO (H) group of the donor.

The *in vitro* relationship between NAb and the human ABO (H) blood groups has been identified in 1911 [6] with the discovery of the Forssman Ab: rabbit sera exhibited anti-human blood group A, B and O (H) activity. Only the anti-A agglutinins, which also lysed SRBC, are defined as Forssman Ab. The glycolipid Forssman Ag appears to be ubiquitous and the Ab is supposedly induced by the bacterium *Escherichia freundii* [7]. NAb reacting with cells of other species have been described in the serum of many animals [8, 9]. The reactivity of chicken NAb with human blood group B has been demonstrated [10]. Cross-species NAb assumably play a pivotal role in the hyperacute rejection of vascularized organ grafts, but their influence on the transplantability of human cell grafts in mice so far has not been studied.

It is difficult to understand why mouse anti-human ABO (H) erythrocyte NAb would lead to the rejection of human cells, since ABO (H) antagonism is not an obstacle for either allogeneic bone marrow or allogeneic vascularized organ grafts. However, in contrast to the human ABO (H) antibodies, xenogeneic serum is toxic and causes lysis of nucleated cells [11]. In the present study the occurrence of

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Abbreviations: **NAb:** Natural antibodies **GVHD:** Graft-*vs.*-host disease **TBI:** Total body irradiation **COF:** Cobra venom factor **Cl_2MDP:** Dichloromethylene diphosphonate-containing liposomes **SCID:** Severe combined immunodeficiency **Bg/Nu/XID:** Beige/nude/X-linked immunodeficiency

Key words: Natural antibodies / ABO(H) blood groups / Transplantation

murine serum NAb against human E, granulocytes, lymphocytes, monocytes and stem cells in relation to the ABO (H) blood group is examined. These Ab are demonstrated in mice with different microfloras and of various ages. We explored if the engraftment of human PBL is dependent on the titer of NAb in the recipient mice. In these experiments engraftment is assesed by the occurrence of fatal acute graft-*vs.*-host disease (GVHD) [5].

Furthermore, attempts to abrogate the NAb barrier were made. The mechanism by which NAb lead to elimination of xenogeneic cells or tissues is attributed to three host effector mechanisms: Ab-dependent phagocytosis, C-dependent cell lysis and NK cell cytotoxicity. The conditioning with a heavy dose of total body irradiation (TBI) presumably reduces NK cells to very low levels [12–13]. We studied the effect of abrogating the other two mechanisms on the engraftment of human PBL in lethally irradiated mice with normal NAb levels. The first method was the depletion of the monocyte and macrophage system with liposomes containing dichloromethylene diphosphonate (Cl_2MDP), as has been shown to be effective in rats by Van Rooijen et al. [14]. The second method was the utilization of cobra venom factor (COF), which has been demonstrated by White et al. [15] to eliminate the third factor of C. In these experiments we also assessed engraftment by the occurrence of acute fatal human to mouse GVHD.

2 Materials and methods

2.1 Mice

Inbred BALB/cRij, BALB/c-SCID, (C57BL × CBA)F_1 (BCBA), CBA/Rij, CBA/N, Beige/athymic Nude/X-linked Immunodeficient [5], C3H/Rij, C57BL/Rij, C57BL/KaRij and DBA/2Rij mice were kept and bred under specific pathogen-free (SPF) [16], "conventional" or "Houston" [17] conditions. The term "conventional" pertains to an unrestricted environment, exposed to human and airborne microbial contact. The SPF flora consists of undefined anaerobic bacteria and *Streptococcus faecalis, Staphylococcus aureus* and *epidermidis* and *Escherichia colii* (5144572, biotype API 20 E). The "Houston" flora is a conventional but defined flora, which was imported from the M.D. Anderson Cancer Institute, Houston, TX. It contains many more different gram-negative bacteria than the conventional flora in our institute (data not published), among which are strains of *Proteus* and *Pasteurella* bacteria. This flora, in contrast to the SPF and the conventional flora, renders rodents susceptible to GVHD after allogeneic bone marrow transplantation [17].

All mice were fed autoclaved pellets (AM2; Hope Farm, Woerden, The Netherlands) and provided with acidified water *ad libitum*. In the experiment with non-immunodeficient mice, only females were used. Both sexes were used of the immunodeficient strains.

2.2 Hemagglutination assay for NAb

Individual decomplemented mouse sera were tested against individual human blood groups. The human blood was collected in heparinized tubes, washed four times in a large volume of cold PBS to remove the plasma, and the E were suspended in Alsever's solution at a concentration of 0.5 %. The mice were anesthetized with ether and bled from the orbital plexus. The blood stood for 2 h at 4 °C to allow coagulation and was decomplemented by heating in a water bath for 20 min at 56 °C. These decomplemented sera were then used immediately or stored at −20 °C. Serial dilutions were made in 1 % gelatin/PBS solution. Ten microliters of each dilution of mouse serum was then tested in U-bottom 96-well plates (Greiner, Alphen a/d Rijn, The Netherlands) against 10 µl/well of the human E suspension. The incubation was overnight at 4 °C on a shaker at 60 cycles/min with an amplitude of 1 cm. As a negative control, decomplemented autologous human serum was used. As a positive control, pooled serum of conventionally housed 2-year-old C57BL/Ka mice was employed. A positive agglutination reaction was scored when spiked agglutinates were visible at the boundary of the cell pellet.

2.3 FCM NAb assay

The preparation of the human blood cells and of the mouse sera was identical with that for the hemagglutination assay. Serial dilutions of the mouse sera were incubated overnight with 10^6 human blood cells tube. The next morning the cells were washed twice with PBS and incubated with directly labeled probes for 30 min at 4 °C, either with 3 µl of FITC-rat anti-mouse IgM (Janssen Pharmaceutica B.V., Beerse, Belgium) or with 50 µl FITC-goat anti-mouse Ig (Nordic Immunology, Tilburg, The Netherlands) absorbed with human E. After incubation the cells were washed twice with 2 ml of cold PBS and suspended in 0.5 ml of PBS, followed by FCM (Becton Dickinson, San Jose, CA). Goat anti-mouse absorption was performed in Eppendorf tubes. Goat anti-mouse serum was diluted 1:50 in PBS, after which one-tenth of the volume of human E was added and the mixture was incubated at 4 °C for 30 min. The supernatant was separated from the cells in a centrifuge, by spinning for 6 min at 1400 rpm. The supernatant was used for staining.

The FCM test was performed in the same way against human PBL [5]. The test was also used for double staining of human BM cells with CD34, a marker for lymphohemopoietic precursor cells. After the standard procedure for NAb staining the cells were incubated for 30 min with biotin-CD34 (biotin-mouse anti-human-CD34; Nordic Immunology). Subsequently the cells were washed twice and incubated with PE-streptavidin (Becton Dickinson) for 20 min. After washing twice the fluorescence intensities were measured with the FCM. As a negative control for the CD34 marker on the BM cells served a human B cell line (BSM) immortalized by EBV.

2.4 Treatment of mice with LPS and CFA

The SCID mice of the first shipment into our colony proved to be infected with *Mycoplasma pneumoniae*. These animals were "leaky", as they showed NAb against human blood group type A PBL and E (other blood groups were not tested). A later shipment was not infected with pathogens. SPF offspring of the latter were obtained via

cesarian section and foster parents. To reproduce the "dirty" animal effect, two SCID mice of 3, 6 and 12 months of age were injected with either the immunostimulants LPS (Wellcome Pharmaceuticals B.V., Utrecht, The Netherlands) or CFA (Difco Laboratories, Detroit, MI). Fifteen micrograms LPS was administrated twice i.p. with an interval of 1 week. CFA (0.5 ml) was injected s.c. into the footpad of the right hindleg. One month later the mice were tested for the presence of NAb.

2.5 Transplantation procedure

Xenogeneic transplantation of human PBL was conducted in supralethally irradiated BCBA and CBA/N mice just after weaning. The BCBA mice were weaned at day 17, when a low body mass of 6.5 g permits a smaller number of human cells to induce a GVH reaction. Also the NAb titers against the different human ABO (H) blood groups appeared the most discriminate in young animals. The mice were housed five per cage to preserve the nesting situation and to prevent hypothermia. The CBA/N mice were weaned at day 21 after birth, with an average weight of 10 g. This is the earliest age at which these animals tolerate high-dose TBI. A dose of 9 Gy TBI was administrated with a ^{137}Cesium source, type Gamma Cell 20 (Atomic Energy of Canada, Ottawa, Canada). Hematological support was provided with 5×10^5 syngeneic bone marrow cells via the intravenous route.

Human PBL were isolated from fresh buffy coat cells of healthy blood donors. The cells were separated on a Lymphocyte Separation Medium density gradient [5] and were injected i.p. The various cells were transplantated within 5 h post irradiation.

In an effort to eliminate the NAb barrier, mice were treated i.p. with 10 ml/kg body weight Cl2MDP in liposomes [14] (gift from Boehringer Mannheim, Mannheim, FRG) 1 day before irradiation and from that time on also with i.p. injections on alternate days of COF (a kind gift of D. White [15]) at a dose of 0.5 mg/kg recipient body weight for the duration of the experiment.

3 Results

3.1 Hemagglutination *vs.* FCM NAb assay

The expression of red blood cell Ag is routinely determined by agglutination assays. This procedure yields low titers of NAb [8]. A recent report describes quantitative FCM of human Ab against ABO (H) RBC, which proves to be superior as it could, in contrast to hemagglutination assays, detect ABO (H) heterozygotes [18]. Table 1 shows the difference in sensitivity between the traditional E agglutination assay and FCM, for different human blood groups. The same sensitivity ratio of the two methods is consistently seen with sera of BCBA mice of various ages when compared against blood group A erythrocytes of a single individual (Fig. 1). The titers obtained with FCM are easy to determine (Fig. 2), as is the agglutination test, when characteristic "spiking" of the E pellet is taken as criterion for a positive test. Further dilutions of the positive sera shown in the hemagglutination test tightly packed E,

Table 1. Comparison of the sensitivity between FCM and hemagglutination assay for NAb in mouse serum

Human blood group[a)]	FCM titer	Agglutination titer
O^- (JP)	1:16	0
O^- (KG)	1:16	0
O^+ (WH)	1:16	0
A^+ (DB)	1:256	1:4
A^+ (MG)	1:512	1:4
B^+ (EG)	1:128	1:2
AB^+ (PH)	1:128	ND

a) End point dilutions of serum from a 14-week-old BCBA mouse against E of different human blood groups.

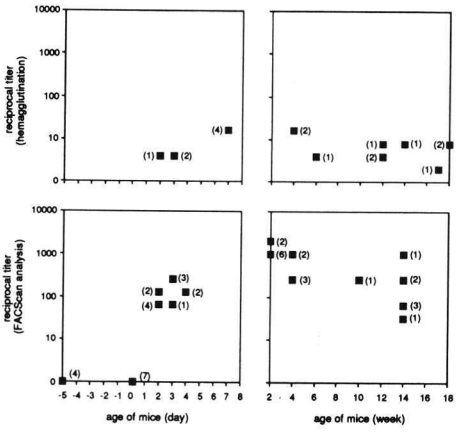

Figure 1. The occurrence of NAb with age in BCBA mice against human blood group type A. The data of the FCM and the hemagglutination assay are compared. Between brackets the number of mice.

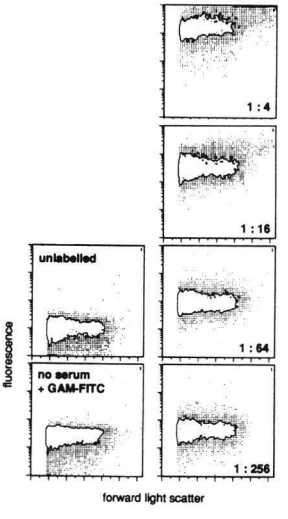

Figure 2. FCM of titration of serum of a 17-day-old BCBA mouse against E of human blood group type A. FITC goat anti-mouse was employed as label.

resembling a honeycomb. If this pattern is used as an end point instead of the traditional spiking of the cell pellet, the sensitivity appears similar to that of the FCM. However, we were not able to score the titer of the sera unambiguously when utilizing the "honeycombing" effect, because it did not show a sharp cut-off point.

3.2 NAb levels in normal mouse strains

NAb are absent at birth and during the first 4 h thereafter [5], a fact which is confirmed in this study utilizing BCBA mice (Fig. 1). We have confirmed the sustained absence of NAb in BCBA mice treated from birth with goat anti-mouse IgM [5]. The high titers of NAb in the control mice treated with normal goat serum is probably due to aspecific immune stimulation (Table 2).

As previously reported, the germ-free mice in our colony contain NAb, much like the SPF ones [5]. We speculated that this fact was due to the presence of bacterial remnants in the food. In the present study we measure the titer of NAb in mice, which are fed in the same way, but have progressively more aerobic bacterial colonizations: the SPF, the "conventional" and the "Houston" flora. Compared are sera from 17-day-old SPF BALB/c, C3H, C57BL and BCBA mice, a few 17-day-old "conventional" BALB/c and BCBA mouse sera and a few 17-day-old "Houston" CBA and BALB/c mouse sera. We consistently found high levels of NAb against human blood group type A, rhesus factor pos (A^+) and absence or low levels against type B^+ and O^+ blood groups, as demonstrated in Fig. 3. The same pattern is observed in 14-week-old SPF BALB/c, C3H, C57BL and BCBA mice (Fig. 4). In these latter cases, we also analyzed the reactivity against blood group type O^- and found the same results as against blood group O^+. According to Stern and Davidsohn [19] the sera of conventionally housed mice

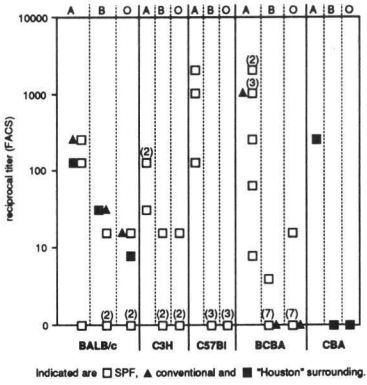

Figure 3. NAb titer of sera from 17-day-old mice of different strains against E of human donors of different ABO (H) blood groups. Titers were determined by FCM using FITC-goat anti-mouse. Open squares: SPF, black triangles: conventional, black squares: Houston flora

contain a Forssman-like Ab reactive with human blood group type A erythrocytes and with SRBC.

However, we found that 17-day-old BCBA sera ($n = 3$) were not reacting with SRBC ($n = 2$) neither with FCM, nor the hemagglutination assay. Furthermore, the serum of a human with blood group B, did not block the reaction between mouse serum and human blood group A erythrocyte. This indicates that the E epitopes, to which mouse NAb reacts are not identical to those that are recognized by human serum Ab. There is no relationship between the H-2 type of mouse and the reactivity of the murine serum against the human cells of different blood groups (Figs. 3 and 4).

Table 2. Inhibition of development of NAb reactivity with human E and of IgM^+ cells in the spleen of newborn mice by treatment with anti-mouse IgM serum

Treatment	BCBA mouse-n°	Number of cells ($\times 10^7$) per spleen	Fluorescence of spleen cells with FITC-rat anti-mouse-IgM[a]	NAb titer against human type O erythrocytes
None	1	5.0	+	1:16
	2	3.7	+	1:4
	3	3.4	+	1:16
	4	2.9	+	1:16
		3.8		
	mean	3.8		
Goat anti-mouse IgM serum[b]	1	3.8	−	0
	2	2.5	−	0
	3	1.8	−	0
	4	2.0	−	0
	mean	2.5		
Normal goat serum	1	5.5	+	1:256
	2	6.0	+	1:64
	3	5.6	+	1:64
	mean	5.7		

a) Detection limit was 2%. b) Treatment was according to [5].

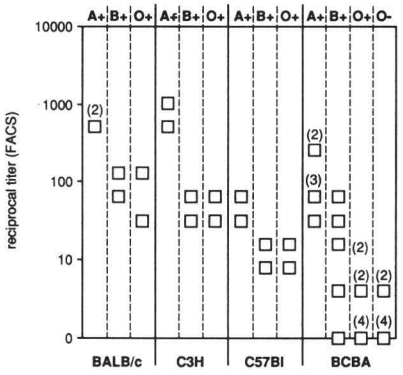

Figure 4. NAb titer of 14-week-old mice of different strains against E of human donors of different ABO (H) blood groups. Methods and symbols as in Fig. 3.

We previously reported that NAb of most mouse strains react with human E, granulocytes, monocytes and lymphocytes [5]. In the present report, titration with FCM shows identical reactivity of BCBA mice sera ($n = 3$) against human blood group type A erythrocytes as well as granulocytes, lymphocytes and monocytes from a single human donor. All showed titers between 1:1024 and 1:2048 and absence of reactivity with similar cells from a type O (H) donor. The different populations of the PBL were defined by scatter patterns. The titer of the reactivity of BCBA mouse serum against $CD34^+$ cells from two individuals with blood group type A is 1:1024, whereas against cells from two individuals with blood group type O, it is negative.

3.3 NAb levels in different immunodeficient mouse strains

Sera from 6-, 9- and 12-month-old Bg/Nu/XID mice ($n = 2$), shows only one positive (1:64) sample against human blood group type A^+; this serum is from a 12-month-old mouse. In Fig. 5 the occurrence of NAb in the sera of individual CBA/N mice against human blood group type A^+ is presented. NAb is detectable beginning at the age of 7 weeks. Sera from 6-, 9- and 12-month-old BALB/c-SCID mice ($n = 2$) do not contain NAb against human blood group type A^+ and O^+. SCID mice of the same age were challenged with LPS or FCA. This treatment did not induce positive NAb responses in the mice. In contrast, two 14-week-old BCBA mice with pretreatment levels of anti-A of 1/64 and 1/128 and negative anti-B and anti-O levels developed anti-A titer of 1/2048 and simultaneously retained negative anti-B and anti-O titers after being stimulated with LPS.

3.4 GVHD in NAb-negative mice

Fatal xenogeneic GVHD characterized by the presence of lymphatic proliferation and ascites, with human $CD3^+$ cells in the mouse lymphohemopoietic organs and ascites fluid [5] was established as criterion for successful engraftment. Graded numbers of human PBL of blood group type A^+, B^+, O^+ and O^- were transplanted into 17-day-old BCBA and 21-day-old CBA/N mice after conditioning with 9 Gy gamma TBI. Table 3 shows that 1.3×10^8 PBL resulted in GVHD in all combinations except with human blood group type A^+ in BCBA mice. However, if the mice were given additional treatment with Cl_2MDP and COF they developed fatal GVHD after transplantation of human PBL blood group type A^+ (Table 4). Neither additional treatment with the Cl2MDP-liposomes alone, or with COF alone were effective. Mice treated with high dose TBI, syngeneic bone marrow transplantation, COF, Cl_2MDP and no human cells survived ($n = 5$), indicating that the regime of COF and Cl_2MDP treatment was not toxic to the incoming graft (data not shown).

Table 3. The occurrence of fatal GVHD after transplantation of graded numbers of human PBl of different blood groups

Mouse strain[a)]	Number of cells per g bodyweight	Human blood group A-pos	B-pos	O-pos	O-neg
CBA/N	6×10^6	−(5)[b)]	−(5)	−(5)	−(5)
CBA/N	1.3×10^7	+(3)	+(3)	+(3)	+(3)
BCBA	6×10^6	−(5)	−(5)	−(5)	−(5)
BCBA	1.3×10^7	−(3)	+(3)	+(3)	+(3)

a) Twenty-one-day-old CBA/N mice (body weight 10 g) and 17-day-old BCBA mice (body weight 6.5.g) were conditioned with 9 Gy gamma irradiation, transplanted with 5×10^5 syngeneic BM cells i.v. and with indicated numbers of human PBL i.p.
b) +: fatal GVHD; −: no GVHD; numbers between brackets indicate number of mice per group.

Table 4. Effect of treatment of recipients with cobra venom factor (COF) and liposomes containing diphosphonate (Cl_2MDP) on occurrence of fatal GVHD after transplantation of PBL of different blood groups

Treatment[a)]	Human blood group A	B	O
None	0/1[b)]	2/2	2/2
COF	1/5	5/5	5/5
Cl_2MDP	1/5	5/5	5/5
COF + Cl_2MDP	8/8	8/8	5/5

a) Seventeen-day-old BCA mice, irradiated with a dose of 9 Gy and supported with 5×10^5 syngeneic BM cells.
b) Human PBL (1.5×10^7 body weight) were grafted i.p.; number of dead mice from GVHD *vs.* total number of mice grafted.

4 Discussion

4.1 Natural antibodies

Germ-free swine raised on a soya bean protein-base hypoallergic milk substitute diet (Mullsoy), develop IgM Ab but remain free of NAb for up to 1 year [20]. In germ-free mice raised on an Ag-free diet, also nearly normal levels of IgM have been found [21], but reactivity of the sera of these mice with discordant xenogeneic cells has

not been determined. In the present study, no difference in serum NAb reactivity with erythrocytes of ABO (H) blood groups is observed among SPF-, "conventional" – and "Houston" microflora-carrying mice. In a previous study we reported normal levels of NAb in the serum of germ-free mice [5]. This is attributed to the fact that our germ-free diet is not Ag free.

In contrast with the report of Stern and Davidsohn [19], the NAb found in the 17-day-old SPF BCBA mice in our colony does not seem to be a Forssman Ab, as it did not lyse SRBC. We speculate that this difference is due to the fact that our murine colonies' bacterial surroundings contain a decreased exogeneous load of Ag.

All sera from normal mice show high IgM Ab titers against human blood group type A^+, whereas no or weak reactivity against blood group type B^+, O^+ and O^- (Figs. 2–5) exists. In this context, it is of interest that upon immunization, E of human blood group A elicit a vigorous Ab response in mice, whereas blood group B Ag is poorly immunogenic [22]. Our observation that stimulation with LPS results in a 32-fold elevation of the Nab titer against blood group A erythrocytes, while the same treatment does not elicit reactivity against blood group B cells, seems to substantiate these findings.

The xenogeneic NAb of the mice in our colony appear to be unlike human allogeneic ABO (H) NAb, as the anti-human blood group A murine NAb were not blocked by serum from a human blood group B donor. Terasaki et al. [11] reported that the reactivity of NAb is not only directed against E, but also against lymphocytes. We extended these findings by showing that murine NAb react with human E, granulocytes, lymphocytes, monocytes and $CD34^+$ stem cells.

B cell-deficient CBA/N mice have a delayed maturation of IgM-producing $CD5^+$ cells [23]. We showed the rapid postnatal development of NAb in non-immunodeficient BCBA mice (Fig. 1) and absence of NAb up to the age of 7 weeks in CBA/N mice (Fig. 5).

Reimann et al. induced in SCID mice "leakiness", *i.e.* SCID-derived IgM-producing B cells, by transplanting

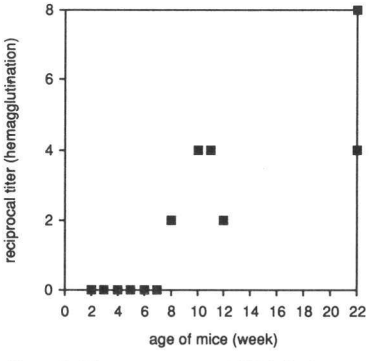

Figure 5. The occurrence of NAb in hemagglutination assay of CBA/N mice of different ages against human blood group type A.

cloned self-reactive T cells from normal cogenic donor mice [24]. We observed "leakiness", *i.e.* NAb to human blood group type A erythrocytes, in three out of four SCID mice raised in a "dirty" surrounding and infected with *Mycoplasma pneumoniae*. As NAb seem to be encoded by primitive genes [25], it would be attractive to have a technique that allows the induction of NAb in these SCID mice. However, we were not able to reproduce the "leaky" effect in SCID mice of our colony with the immunostimulants LPS and CFA.

4.2 Xenogeneic transplantation

The relationship between absence of NAb and engraftment of human cells has been demonstrated in newborn normal mice [5]. Newborn rats also show absence of NAb against E from a rhesus monkey and several individual human beings with the hemagglutination assay (results not shown), which may explain the successful transplantation of non-Hodgkin lymphomas into these animals [1]. The FCM data of Figs. 2–5 show that most mouse strains do not develop significant titers of NAb against human cells of B and O (H) blood groups before weaning age and that PBL of those human donors cause fatal GVHD following transplantation into heavily irradiated BCBA mice. In contrast, transplantation of PBL from human blood group type A, against which significant levels of NAb are shown to exist in these 17-day-old BCBA mice, did not result in fatal GVHD. This implies that NAb are an important barrier against cell grafts in certain xenogeneic combinations. We performed these experiments in 17-day-old suckling mice, because some older mice had developed low titers of NAb against human blood group B and O (H) (Fig. 3 *vs.* Fig. 4) after weaning.

For many purposes, *e.g.* AIDS and cancer research, it is attractive to be able to employ normal mice for growing human lymphohemopoietic tissues *in vivo*. In a recent report Lubin et al. [26] described the successful transplantation of human bone marrow in 16 of 20 adult BALB/c mice. These animals were conditioned with 10 Gy gamma irradiation and had hematological support with i.v. transplanted SCID mouse bone marrow cells. These interesting results may be explained by the fact that the human bone marrow was only in four cases derived from blood group type A donors (Y. Reissner; personal communication). It seems obvious that the success of future transplantation experiments of human cells in rodents will be facilitated by the matching of the donor cells ABO (H) blood group with the recipient mouse serum.

In all our experiments, the most severe murine barrier against human cells was provided by NAb against blood group A xenografts. We showed that this strong resistance can be abrogated by pretreatment of the recipient mice with COF and Cl_2MDP. Interestingly, Thierfelder et al. found that COF blocked the elimination of T cells by low-affinity anti-Thy-1 Ab but not by high-affinity Ab. They suggested that the latter failure may be due to synthesis of C1q by the macrophage [27]. This assumption is supported by our finding that treatment with Cl_2MDP of the recipient mice in conjunction with COF overcomes resistance against blood group A human PBL.

In view of the continuing scarcity of human organ grafts, it seems worth while to investigate whether the use of COF and CL_2MDP will result in the abrogation of the xenograft barrier for discordant pancreatic islet and vascularized organs grafts.

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Chapter 4

Thymectomy and SCID Bone Marrow Transplantation Eradicates Natural Antibody of Mice to Human Blood Cells

THYMECTOMY AND SEVERE COMBINED IMMUNE-DEFICIENT BONE MARROW TRANSPLANTATION ERADICATES NATURAL ANTIBODY OF MICE TO HUMAN BLOOD CELLS

In spite of numerous attempts, transplantation of cellular grafts of normal hemopoietic or lymphopoietic human cells into artificially immune-suppressed rodents has failed until recently. However, a limited engraftment of human leukemic cultured cell lines has been reported in newborn rats (*1*) and in nude mice (*2*). After the discovery of the hereditary immune-deficient severe combined immune-deficient (SCID)* mouse and the generation of the Beige-athymic nude-X-linked immune-deficient (Bg/Nu/XID) mice, these mice were found to sustain the proliferation of human cells of hemopoietic origin for prolonged periods of time (*3*). In general, however, the numbers of human cells recovered from the tissues of the mutant mice are small.

Recently, Lubin et al. (*4*) reported the engraftment of human bone marrow in normal mice that had been conditioned with a supralethal dose of irradiation and allogeneic bone marrow from SCID mice. These results were difficult to reconcile with our finding that natural antibodies (NAb) in the serum of normal mice react with all human hemopoietic cells (*5*), including $CD34^+$ progenitor cells, and thereby prevent engraftment (*6*). All mouse sera tested had high antibody titers against human cells of blood group A and low or negligible titers against cells of human blood group B and O (H). Lubin et al. grafted the human bone marrow 24 hr after the total body irradiation (TBI). It is unlikely that the NAb would have disappeared by that time. Therefore, Lubin's result can only be explained if the majority of the bone marrow donors had been of blood group B and O (H). Unfortunately, the blood group of these human donors was not reported. Alternatively, it could be that a proportion of the human hemopoietic stem cells survive in the presence of NAb and subsequently successfully produce progeny at a time when the pre-existing circulating NAb have disappeared. If repopulation is by SCID cells, NAb levels will not be restored, because the SCID mutation represents a defect in gene rearrangement, resulting in the absence of immune-competent T and B lymphocytes and of NAb.

Therefore, it remains to be established whether the stratagem of Lubin et al. (i.e., the transplantation of murine SCID bone marrow) is an absolute requirement for the subsequent take of the human bone marrow. Accordingly, we investigated the immune status of normal mice after transplantation with bone marrow from SCID donors. To our surprise, we found that at 4 weeks after transplantation, these mice had numbers of T and B lymphocytes as well as NAb titers similar to control mice that had been grafted with syngeneic marrow. The interval of 4 weeks after transplantation was selected because by then, repopulation of the lymphatic tissues is complete in syngeneic chimeras. Apparently, the few surviving host stem cells had by that time repopulated the lymphatic tissues. We had previously observed (*6*) in Bg/Nu/XID mice that the period of absence of NAb is 1 year as compared to 8 weeks in CBA/N (XID) mice, indicating that in the absence of the thymus, NAb production is inhibited. In addition, the group of Wortis (*7*) had observed that thymus grafts restore B cell function in nude XID mice. Therefore, the experiment was repeated with thymectomized BCBA mice, and here we found the expected deficiencies in T and B cells as well as the absence of NAb.

Twelve-week-old BCBA mice were subjected to 9.25 Gy of gamma irradiation from a 137 Cs source, type Gamma Cell 20, dose rate 0.87 Gy/min (Atomic Energy of Canada, Ottawa, Canada), and after 4 hr transplanted i.v. with 1.5×10^7 bone marrow cells of, respectively, BCBA and BALB/c-SCID mice. One group of recipient mice was subjected to thymectomy at the age of 4 weeks. These animals were anesthetized with ether inhalation. Thymectomy was performed through a medial skin incision and upper sternal cleavage, followed by removal of the gland by suction. The wound was closed by sternal cerclage and a skin suture.

Four weeks after transplantation, at the age of 16 weeks, the BCBA mice were bled via the orbital plexus. The sera were decomplemented and analyzed by FACScan for the presence of NAb (*5, 6*) using goat antimouse-Ig-FITC (Nordic Immunology, Tilburg, The Netherlands). The blood cells were separated on Lymphocyte Separation Medium (*5*). T cells were enumerated by incubation for 30 min at 5×10^5 blood cells with 0.1 μg of rat antimouse Lyt 2, 0.1 μg of rat antimouse L3/T4 epitope P, and 0.1 μg of rat antimouse epitope Q (*8*) in 50 μl of 0.01 mM PBS (pH 7.4) containing 4% BSA (Sigma, St. Louis, MO). Next, the cells were washed twice in the same buffer and subsequently incubated for 30 min with the conjugated goat antirat-Ig-FITC (Tago, Inc., Burlingame, CA). Subsequently, the cells were washed twice and analyzed on the FACScan machine. Detection of B cells was done by incubation for 30 min of 5×10^5 blood cells with 5 μl of goat antimouse-Ig-FITC in 50 μl of PBS containing 4% BSA.

The results in Table 1 show that 1 month after transplantation, all intact BCBA mice, conditioned with 9.25 Gy of TBI and engrafted either with BCBA bone marrow cells or with SCID bone marrow cells, had the same titers of NAb and numbers of blood T and B cells, whereas SCID mice and thymectomized BCBA mice engrafted with SCID bone marrow did not have detectable NAb titers nor mature T and B cells. This indicates that cells of recipient origin had repopulated the euthymic mice after transplantation with SCID bone marrow. In fact, based on recent calculations, the number of surviving long-term repopulating stem cells may be substantial after irradiation with 9.25 Gy of TBI of BCBA mice, being the maximally tolerated radiation dose not causing gastrointestinal irradiation mortality (*9*). The difference between the radiation dose used in our experiments and that used by Lubin et al. (i.e., 10 Gy) is negligible considering variations in dosimetry and irradiation conditions. In any case, it does not warrant the conclusion that in Lubin et al.'s experiments, some stem cells of the host did not survive.

The idea of Lubin et al. to use SCID bone marrow for hematological support was based on the assumption that this graft would not produce any significant numbers of B and T lymphocytes as a result of the genetic defect. Our results demonstrate that under similar conditions, repopulation occurs with recipient type cells and, subsequently, complete recovery of T and B cells. To prevent such repopulation, preirradiation thymectomy is required. We speculate that successful engraftment of human lymphohemopoietic tissues of all human blood groups may be attained in any heavily irradiated thymecto-

* Abbreviations: Bg/Nu/XID, Beige-athymic nude X-linked immune-deficient; NAb, natural antibody; SCID, severe combined immune-deficient; TBI, total body irradiation.

TABLE 1. Occurrence of T cells, B cells, and NAb in 16-week-old BCBA mice[a]

	BCBA	BCBA→BCBA	SCID→BCBA	SCID	SCID→ BCBA-TMX (thymectomy)
Lyt-2/L3T4 cells (%)	39, 44, 49, 51	27, 29, 36	22, 27, 30, 44	<2 (n=3)	<2 (n=8)
Ig-bearing cells (%)	28, 30, 34, 37	15, 22, 22, 32	18, 19, 19, 25	<2 (n=3)	<2 (n=8)
Reciprocal titer of NAb against human A-positive erythrocytes	64, 256, 256, 1024	64, 256, 1024, 1024, 2048	16, 256, 1024, 1024, 1024	<4 (n=8)	<4 (n=13)

[a] Thymectomy of BCBA mice was at 4 weeks and bone marrow transplantation at 12 weeks after conditioning with 9.25 Gy. The transplant contained 1.5×10^7 bone marrow cells.

mized normal mouse using supportive bone marrow grafts of SCID mice. However, such complex preparations are not required, as we have demonstrated that X-linked immune-deficient CBA/N mice do not have NAb against peripheral blood cells of any human blood group (5). The deficiency of these mice is subtle and defined as lack of the antibody response to pneumococcal polysaccharide capsule, and they are just as easily kept and bred as any normal mouse strain. Therefore, they seem to be the recipients of choice for xenografts of human hemopoietic cells.

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Chapter 5

Cytokine Dependence of Human to Mouse Graft versus Host Disease

Cytokine Dependence of Human to Mouse Graft-versus-Host Disease

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Human peripheral blood leucocytes (PBL) induce chronic graft versus-host disease (GvHD) in non-conditioned severe combined immunodeficient mice. Chronic GvHD was observed in such animals after transplantation of 6×10^7 human PBL per g body weight. However, acute xenogeneic GvHD results from grafting at least 2×10^7 human PBL per g body weight to heavily conditioned murine hosts. The large numbers of human PBL were thought to be required to produce above threshold amounts of certain cytokines. We show that treatment of the recipient mice with human interleukin 2 reduces the number of cells to inflict acute GvHD by a factor of ten. Human T cells and not B cells or macrophages, were previously shown to generate acute xenogeneic GvHD, when selected cell types from peripheral blood were grafted. Most of the infiltrating cells had the $CD4^+$ phenotype. We demonstrate that $CD4^+$ T cells are the main mediator, as the disease is abrogated by treating the mice with cytotoxic CD4 antibodies, but not with CD8 antibodies. A survival pattern, similar to that seen in GvHD, was induced by transplantation of a Herpesvirus saimiri transformed human $CD4^+$ clonal T cell line in conjunction with daily interleukin 2 injections. Herpesvirus saimiri transformed human T cells allow easily reproducible graft properties in chimeric mouse models for human diseases.

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INTRODUCTION

Chimeric mouse models are an essential tool for the study of human disease. Human T lymphocytes have been successfully grafted to severe combined immunodeficient (SCID) mice, mainly following two principal protocols. McCune *et al.* transplanted small segments of human fetal thymus of about 14 weeks of gestation (about 10^7 T cells) into the dorsal subcapsular space of the kidney of non-conditioned SCID mice [1]. The thymus grafts remained functional, sustaining follicles for up to a year, but the human lymphocytes failed to populate the mouse organs significantly, although these cells were functionally immuno-competent *ex vivo* [2]. Mosier *et al.* also used non-conditioned SCID mice recipients, which were injected i.p. with about 10^7 human peripheral blood leucocytes (PBL). The cells survived for up to 1 year, but without effecting signs for GvHD [3]. These human T cells were activated in the mouse environment, but proved anergic to TCR-activation [4]. However, they acquired normal T-cell functions after a 1 month culture in the presence of IL-2 [5].

It can be concluded from these investigations, that as many as 10^7 discordant xenogeneic T cells (about 3.5×10^5 cells/g recipient body weight) are unable to induce graft versus-host disease (GvHD) in non-conditioned hosts. The present report deals with the following three issues.

(1) Recently, we described the conditions which allow the induction of acute GvHD in 100% of recipient mice, i.e. the transplantation of 2×10^7 human PBL per gram body weight of heavily conditioned recipient mice, which have low or absent levels of natural antibody [6–8]. Conditioning was done with a large dose of total body irradiation or with a large dose of cytostatic drugs. It was assumed that only large numbers of human $CD4^+$ cells are able to produce enough cytokines like IL-2, IL-4 and interferons in the murine environment to reach the threshold needed for proliferation [6]. This threshold hypothesis was tested by injecting the chimeric mice with human IL-2.

(2) Elucidation of the major cell type causing the acute

Abbreviations: GvHD, graft-vs.-host disease; TBI, total body irradiation; PBL, peripheral blood leucocyte; SCID, severe combined immunodeficient (mice); LCA, leucocyte common antigen (CD45); FCM, flowcytometry; BM, bone marrow (cells); IP, intra peritoneal; IV, intra venous; Ab antibody.

GvHD would increase the application possibilities of this model. Previously, we had shown that sheep erythrocyte rosetting human T cells, and not B cells or macrophages were able to induce acute GvHD [6]. In this study, the contribution to acute GvHD of NK cells [9] and of T-cell subpopulations was investigated.

(3) Most of the infiltrating human T cells in mice with acute GvHD were previously shown to be $CD4^+$. Using a Herpesvirus saimiri $CD4^+$ monoclonal [10] human T cell line, we analysed whether the polyconality of PBL was essential to this model.

In this report, we demonstrate that IL-2 dependent $CD4^+$ cells are the main mediator of acute (within 2 weeks) xenogeneic GvHD in mice.

MATERIAL AND METHODS

Conditioning of mice. Inbred BALB/c-SCID/Rij, C57BL/Rij, CBA/Rij, CBA/N/Rij mice and outbred XID mice on C57BL/Rij, BALB/c/Rij and CBA/N background were kept and bred under specified pathogen-free (SPF) conditions in our own breeding colony [11]. All mice were fed autoclaved AM2 pellets. SCID mice received SRM-A pellets instead, which contain more iron (Hope Farm, Woerden, The Netherlands), because the females were not fertile on AM2 (results not shown).

Total body irradiation (TBI) was done with a ^{137}Caesium source, type gamma-Cell 20 (Atomic Energy of Canada, Ottawa, Canada), at a dose rate of 0.87 Gy/min. The dose of 9 Gy for CBA/N mice was previously published to be optimal. Conditioned mice had supportive care with 5×10^5 syngeneic bone marrow (BM) cells i.v. [6]. The CBA/N mice used for experiments were 4 weeks old. Mice in experiments were kept in horizontal laminar flow cabinets and were given the following additives per l distilled drinking water: 100 mg polymyxin B sulfate (Pfizer, New York, NY, USA), 100 mg ciprofloxacin (Bayer, Leverkusen, Germany), 100 mg natamycin (Gist-Brocades, Delft, The Netherlands) and 50 g saccharose.

Transplantation procedure. Graded numbers of the cytokine independent human monomyeloid cell line HL60 [12] were transplanted into groups of four non-conditioned 12-week old SCID mice, to define the minimal cell numbers needed for a discordant xenograft.

To study the interaction of allo- and xenogeneic GvHD, 14 CBA/N mice were conditioned with 9 Gy TBI and transplanted with 10^6 pooled fetal liver cells (FL) instead of syngeneic BM cells. In order to induce limited chronic GvHD of gut and liver, FL were taken from day-16 timed pregnancies of outbred XID mice (unpublished observations). FL cells were prepared by 30-min incubation with Puck's trypsine, as reported by Löwenberg *et al.* [13]. Six mice also received i.v. grafts of 2×10^7 human PBL/g, the other eight served as controls.

In another experiment it was investigated whether chronic GvHD can be efficiently induced in non-conditioned mice. Therefore 10^9 human PBL were i.p. grafted into each of 23 1 month-old SCID mice (17 g: 6×10^7 PBL/g body weight). Every mouse had cells from a different human donor.

Human PBL, before xenografting, were isolated from fresh buffy coat cells of healthy blood donors using Lymphocyte Separation Medium (LSM, Organon Teknica B.V., Boxtel, The Netherlands) [6]. The clonal IL-2 dependent $CD4^+$ CB15 cell line was obtained by transformation with Herpesvirus saimiri of human cord blood cells [10]. Cells were counted after staining with Trypan Blue 0.4% solution before transplantation. Within 5 h of total body irradiation, the transplantation of cultured PBL or cell lines were performed.

When the graft was given i.p., the number of 4×10^7 was chosen instead of the previously published 2×10^7 human PBL/g recipient body weight [6], after realizing that the i.p. 2×10^7 PBL/g grafts did not elicit acute GvHD in rare exemptions (results not shown).

Interleukin-2 treatment of mice. To investigate activation of PBL *in vivo*, CBA/N mice transplanted with human PBL received daily i.p. injections with 10^4 IU recombinant human IL-2 (a kind gift from Eurocetus Benelux B.V., Amsterdam, The Netherlands). As had been found for LAK cell therapy in humans, the treatment of these mice with elevated doses of IL-2 was lethal in the third and fourth week after transplantation when repopulation is complete (results not shown). Therefore, directly post TBI, these animals also received 250 μl ascitic fluid with anti-mouse CD3 Ab [14]. With this treatment the mice receiving IL-2 survived the observation period of 4 weeks. Histology of lymphoid tissues of these animals revealed severe lymphoid depletion (results not shown). The ascitic fluid was produced in Bg/Nu/XID mice, which were conditioned 1 week before inoculation of the Ab-producing cell line [14] with 200 μl 2,6,10,14-tetramethylpentadecane (Pristane, Aldrich-Chemie, Steinheim, Germany).

Culture of GvHD-inducing cells. Before transplantation, HL60 was cultured in RPMI-1640 (Gibco, Paisley, UK) with 10% decomplemented bovine calf serum (Hyclone, Logan, UT, USA), referred as culture medium.

Herpesvirus saimiri transformed lymphoblasts were cultured in the culture medium with addition of 100 IU IL-2/ml.

To investigate the effects of activation before transplantation in heavily conditioned CBA/N mice, PBL were incubated for 1 h with either 10 μg/ml anti-human CD3 (OKT3, American Type Culture Collection, Rockville, MD, USA) (5 CBA/N mice), or with 10 μg/ml PHA (Wellcome, Weesp, The Netherlands) and 100 IU IL-2/ml (7 CBA/N mice) in RPMI with 10% bovine serum. The sham procedure was incubation of PBL in medium (5 CBA/N mice). Transplantation was i.p. with 4×10^7/g human PBL.

To investigate whether NK cells contribute to the acute GvHD, they were enriched *in vitro* for 4 weeks according to the culture method described by Van De Griend *et al.* [15]. This culture method is routinely used by us and at least doubles the relative number of NK cells in such a period (results not shown). Ascitic fluid cells (2×10^7) of a 6 weeks old CBA/N mouse suffering from acute GvHD were used. This mouse had been conditioned with 9 Gy TBI and transplanted i.p. with 4×10^7 human PBL/g at the age of 4 weeks.

Phenotype of GvHD-inducing cells. To evaluate the contribution to the GvHD of the CD4 and CD8 bearing lymphocytes separately, mice were given cytotoxic Ab to either CD4 (OKT 4A a kind gift of Coulter Immunology, FL, USA, RIV-6 a kind gift of RIVM, Bilthoven, The Netherlands) or CD8 protein (OKT 8F a kind gift of Coulter Immunology) i.p. in a dose of 10 μg per g body weight 1 h of the transplantation of the human PBL.

Interleukin-6. Levels of serum IL-6 in allogeneic and xenogeneic GvHD were determined by stimulation of the murine B6 cell line. Its proliferation was measured by thymidine incorporation [16].

Flow cytometry of human cells. Cell suspensions of mouse tissues were generated by pressing the organs through four layers of 100-μm nylon gauze. FCM of cells was done after incubation with anti-human CD4, CD8 and CD19 Ab (Becton and Dickinson, San Jose, CA, USA) [6]. FCM of the cultured cells was done after labelling them with the anti-human Ab anti-TCR α/β (BMA 0.31, a kind gift

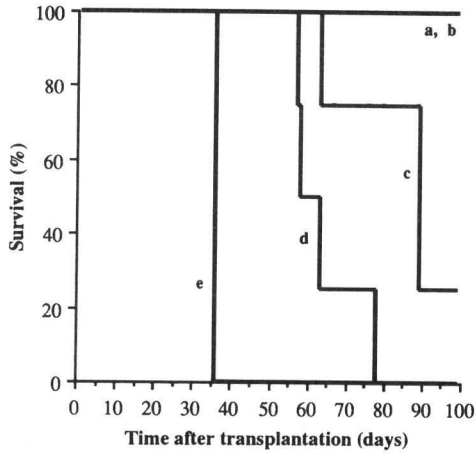


Fig. 1. Survival curves of groups of four non-conditioned SCID mice, after the i.p. transplantation of graded numbers of the human monomyeloid cell line HL60. The number of grafted cells was: a, 10^4; b, 10^5; c, 10^6; d, 10^7; e 10^8.

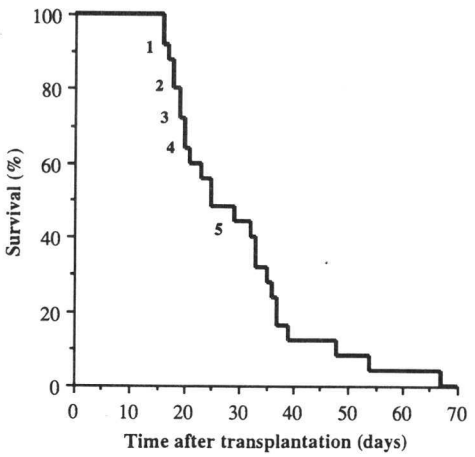


Fig. 2. Survival percentage of 23 non-conditioned SCID mice after the i.p. transplantation of 10^9 human PBL. The CD4/8 ratio and the percentage of $CD20^+$ cells of the spleen cells of the indicated affected mice was determined: 1: CD4/8 = 1.4, CD20 = 0.5%; 2: CD4/8 = 1.0, CD20 = 0.0%; 3: CD4/8 = 0.8, CD20 = 1%; 4: CD4/8 = 0.4, CD20 = 0.4%; 5: CD4/8 = 0.4, CD20 = 0.1%.

from Behring Pharma N.V., Amsterdam, The Netherlands), anti-TCR γ/δ (TCR-γ/δ-1), anti-CD3, anti-CD11c or anti-CD16 (Becton and Dickinson, San Jose, CA, USA) and goat anti-mouse-Ig-FITC (Nordic Immunology, Tilburg, The Netherlands). FCM was performed at the Dr. Daniël den Hoed Cancer Hospital, Rotterdam, The Netherlands.

Histopathology. Complete gross necropsy was done on mice from the various experiments. The tissue samples were fixed in phosphate-buffered saline with 4% formaldehyde, routinely paraffin imbedded, processed to 3 μm sections and stained with haematoxilin, phloxine and saffron (HPS) [6].

RESULTS

Grafts of cytokine-independent HL60

The minimal cell number of a xenogeneic cytokine-independent cell line was estimated, which is required for tumour development. Therefore graded numbers of the human HL60 monomyeloid cell line [12] were i.p. grafted in non-conditioned 12-week-old SCID mice. Subsequently, the occurrence of leukaemia was observed. The results are shown in Fig. 1. As few as 10^6 cells were sufficient to cause the lethal monomyeloid tumour in recipient adult mice (3.6×10^4 cells/g).

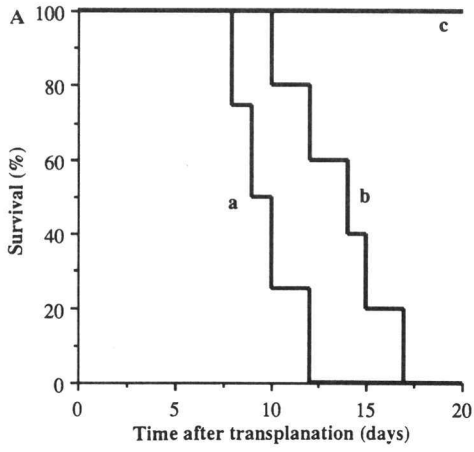
Chronic GvHD

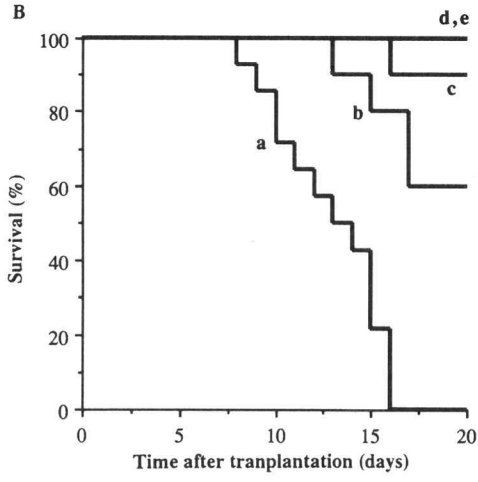
In order to induce chronic GvHD, almost all white blood cells from 450 ml human blood (10^9 human PBL) were grafted i.p. in non-conditioned SCID mice (6×10^7 cell/g). The results are depicted in a survival curve (Fig. 2) and show that 23/23 of the mice expired within 3 months, with signs of chronic GvHD. Starting a week before their death, the mice had weight loss, a ruffled skin, a rounded back and tachypnoe. At necropsy the lungs had yellowish discoloration. Some animals had enlarged spleens, which were less than double the normal size. There were some adhesions in the abdomen and the omentum appeared thickened and white. FCM of the spleen revealed CD4/8 ratios and CD20 absolute number consistent with chronic GvHD [17–19] and not with B cell lymphoma [20] (Fig. 2).

IL-2 dependence of acute GvHD

Large numbers of PBL are necessary to cause lethal GvHD. We investigated whether a reduction of the cell number could be compensated by supplementation of IL-2 by i.p. injection. The dose estimation studies of human to mouse chimeras for human IL-2 supplementation are shown in Fig. 3A and 3B. In comparison to the daily dose of 10^3 IU, or the $3 \times$ /week dose of 10^4 IU IL-2, the daily dose of 10^4 IU was optimal and allowed a reduction of the cell number to one tenth.

We analysed further if *in-vitro* activation of the human T cells prior to transplantation could lead to prolonged spontaneous cytokine production *in vivo*, allowing growth of the graft without exogenous IL-2. However, the *in vivo* IL-2 injections could not be replaced by pretreatment *in vitro* either with 10 μg/ml anti-human CD3 or with 10 μg/ml PHA and 100 IU IL-2. It appeared that neither of these latter treatments caused earlier GvHD in heavily conditioned CBA/N mice (12 per group). The mean survival was for sham treated PBL 14 days, for pretreated with PHA and human IL-2 13 days and for pre-





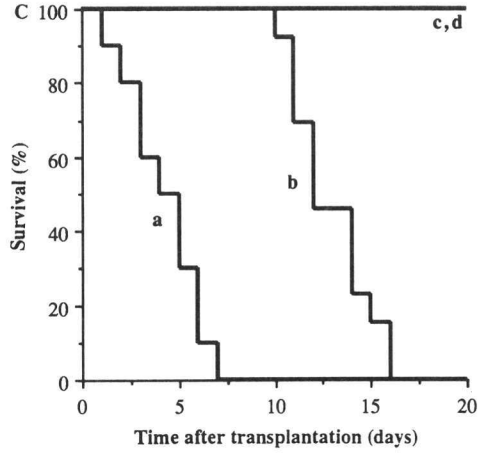


Fig. 3. Effect of injection of human IL-2 on the survival curves of heavily conditioned CBA/N mice grafted i.p. with human PBL, or the lymphoid cell line CB15. (A) 4×10^7 human PBL/g and daily 10^4 IU IL-2 ($n = 4$; line a), 4×10^7 human PBL/g ($n = 5$; line b) and 4×10^6 human PBL/g ($n = 10$; line c). (B) 4×10^6 human PBL/g and daily 10^4 IU IL-2 ($n = 14$; line a), 4×10^6 human PBL/g and 3×/week 10^4 IU IL-2 ($n = 10$; line b), 4×10^6 human PBL/g and daily 10^3 IU ($n = 10$; line c), 4×10^6 human PBL/g ($n = 10$; line d) and 10^6 human PBL/g and daily 10^4 IU IL-2 ($n = 5$; line e). (C) 5×10^6 CB15 $CD4^+$ cells/g and daily 10^4 IU IL-2 ($n = 10$; line a), 1.3×10^6 CB15 $CD4^+$ cells/g and daily 10^4 IU IL-2 ($n = 13$; line b), 5×10^5 CB15 $CD4^+$ cells/g recipient bodyweight and daily 10^4 IU IL-2 ($n = 5$; line c) and 1.3×10^6 CB15 $CD4^+$ cells/g recipient bodyweight ($n = 5$; line d).

incubation with anti-human CD3 14 days. This is in contrast to the results of Murphy *et al.*, who showed that anti-human CD3 pretreatment facilitated i.p. engrafment of 5×10^7 human PBL in SCID mice [21]. Table 1 shows the results of FCM analysis of human $CD4^+$, $CD8^+$ and $CD19^+$ cells in three groups of 5-week old CBA/N mice, conditioned with 9 Gy TBI, supported with 5×10^5 syngeneic BM cells and transplanted i.p. with human PBL. The first group received the usual dose of 4×10^7 PBL/g. This group had GvHD at the day of analysis with the typical predominance of human $CD4^+$ cells in the lymphohemopoietic organs. The second group received 4×10^6 cells/g. No GvHD was detected and few human cells with a relative predominance of $CD8^+$ cells were observed. The graft of the third group was identical with the second, but these mice received daily injections of 10^4 IU human IL-2. The results of the third group are identical with the first, i.e., both show an increase of the CD4/8 ratio which is usual for acute GvHD.

At least 100 CBA/N mice were examined clinically and post mortem. These had been heavily conditioned, grafted i.p. with 4×10^6/g human PBL and daily i.p. injected with 10^4 IU of human IL-2. They showed identical pictures as observed after the i.p. transplantation of 4×10^7 human PBL/g without injections with IL-2, as was described previously [6]. The mice had rapidly progressive cachexia with ruffled hair and had rapidly progressive dyspnoea and tachypnoea whereby they sat on top of each other and had a rounded back. They also showed severe anaemia and had hemorrhages.

Of three mice, which had received i.v. 4×10^6/g human PBL grafts and had been injected daily with 10^4 IU of human IL-2 the spleens were examined histopathologically with HPS-stained sections. The spleens were characterized by an enlarged white pulpa with infiltrating human cells. The findings were identical to those in the mice which had received i.v. 2×10^7 human PBL/g without IL-2 injections, as described previously [6].

In summary, small cell number grafts of human PBL supplemented with i.p. IL-2 injections are as efficient in eliciting acute GvHD as 10-fold PBL cell numbers without exogenous IL-2.

Table 1. FCM analysis of chimeric CBA/N mice*

	Ascites				Liver				Spleen				Thymus				PBL			
hu donor/hu marker	CD4[†]	CD8	4/8	CD19	CD4	CD8	4/8	CD19	CD4	CD8	4/8	CD19	CD4	CD8	4/8	CD19	CD4	CD8	4/8	CD19
neg. contr. 1	–	–	–	–	0.0	0.1	–	0.0	0.1	0.0	–	0.1	0.0	0.0	–	0.0	0.0	0.0	–	0.0
neg. contr. 2	–	–	–	–	0.0	0.0	–	0.1	0.1	0.0	–	0.1	0.0	0.1	–	0.0	0.0	0.0	–	0.0
neg contr. 3	–	–	–	–	ND	ND	–	ND	0.0	0.0	–	0.2	ND	ND	–	ND	0.1	0.0	–	0.0
hu 1; 4×10^7/g	66.5	18.6	3.6	0.1	46.5	16.9	2.8	0.1	12.8	4.1	3.1	0.0	2.6	5.2	2.0	0.0	0.0	0.0	–	0.0
hu 2; 4×10^7/g	59.5	18.8	3.2	0.2	45.5	14.3	3.2	0.1	3.3	1.0	3.3	0.0	6.8	5.9	1.2	0.0	0.0	0.0	–	0.0
hu 3; 4×10^7/g	53.2	17.1	3.1	1.4	33.0	19.9	1.7	0.1	22.7	10.0	2.3	0.3	6.4	7.9	0.8	0.0	0.2	0.1	2.0	0.0
hu 4; 4×10^7/g	40.9	22.5	1.8	0.6	30.0	30.7	1.0	0.3	19.7	17.5	1.1	0.1	7.1	5.8	1.2	0.0	0.2	0.1	2.0	0.0
hu 1; 4×10^6/g ($n = 8$)	0.4	0.4	1.0[‡]	<0.1	<0.1	<0.1	–	<0.1	<0.1	<0.1	–	<0.1	<0.1	<0.1	–	<0.1	<0.1	<0.1	–	<0.1
hu 3; 4×10^6/g + IL2[§]	61.1	20.7	3.0	1.2	24.8	13.7	1.8	0.1	20.8	9.5	2.2	0.5	9.8	5.8	1.7	0.0	0.4	0.2	2.0	0.0
hu 5; 4×10^6/g + IL2[§]	48.2	12.0	4.0	0.3	ND	ND	–	ND	6.5	6.3	1.0	0.1	ND	ND	–	ND	10.7	5.6	1.9	0.6

*All mice received 9 Gy TBI and were grafted i.p. with different numbers of human PBL from different donors, 4 weeks after birth. Control mice did not receive a xenograft. Mouse tissues were analysed by FCM 12 days after transplantation.

[‡]Standard deviation 0.1; the mean of the CD4 and CD8 percentage is given.

[†]Percentage of total number of nucleated cells.

[§]Daily i.p. treatment with 10^4 IU IL2.

NK cell analysis

Human NK cells were previously shown to contribute to xenogeneic GvHD [9]. Cells from the ascitic fluid from a chimeric mouse suffering from GvHD, induced by the i.p. injection of 4×10^7/g human PBL, were cultured for 4 weeks according to Van De Griend *et al.* [15] and subsequently analysed with FCM. Fresh CBA/N mouse spleen cells served for sham analysis. No fluorescent cells were observed after staining with anti-CD11c and anti-CD16. With anti-CD3 100%, with anti-TCR α/β 98% and with anti-TCR γ/δ 2% of the cells were reactive. Background staining was below 0.1%. Lymphocytes showed 5–28% staining with anti-CD11c or with anti-CD16. The normal distribution of the $CD3^+$ cells is 98% TCR α/β^+ and 2% TCR γ/δ^+. We were not able to demonstrate NK cells participating in GvH reaction.

Survival after CB15 graft

Herpesvirus saimiri transformed human T cell lines [10] have the phenotype of mature activated T lymphocytes [22, 23] and are permissive to T lymphocytotropic viruses such as human immunodeficiency virus [24]. As an alternative to PBL, the viral transformed $CD4^+$ human clonal T-cell line CB15 was engrafted i.p. into conditioned CBA/N mice. The survival curve is depicted in Fig. 3C. In conjunction with daily IL-2 injections, the virus immortalized T cells induced xenograft related death similarly to human PBL.

The deceased CBA/N mice ($n = 13$), which had been transplanted with 1.3×10^6 CB15 $CD4^+$ cells/g and had been injected daily 10^4 IU IL-2, had ascitic fluid, just like after human PBL grafts, containing cells in the range of 5×10^6–4×10^7, with a mean of 10^7 cells, which were determined by FCM, >90% human $CD45^+$.

Abrogation of GvHD by anti-CD4 Ab

Previously we demonstrated that anti-thymocyte globulin abrogates the GvHD induced by 2×10^7 human PBL/g [6]. In this study, 1 h after the i.p. 4×10^7 PBL/g injection, chimeric CBA/N mice were treated with cytotoxic monoclonal antibodies. Both Ab to CD4, but not the Ab to CD8, were able to abrogate the GvHD (Fig. 4). Therefore $CD4^+$ T cells can be considered the major mediator of acute xenogeneic GvHD.

IL-6 production at the time of GvHD

To compare cytokine levels of GvHD, induced by allogeneic and xenogeic lymphocytes, species non-restricted relative IL-6 concentrations were determined at the time of GvHD, using B6 cells [16]. It was shown that the transplantation procedure with heavy conditioning and syngeneic BM cells transplantation of the mice did not induce elevated levels of IL-6 (Fig. 5). Xenogeneic GvHD was induced with the i.v. injection of 2×10^7 human PBL/g into 9 Gy TBI conditioned CBA/N mice. Allogeneic GvHD was induced with the i.v. injection of 4×10^4 CBA mouse spleen cells/g into 12-week-old C57BL/RijxCBA/Rij F1 hybrids, which were conditioned with 9.25 Gy TBI. Figure 5 demonstrates that the IL-6 levels in allo- and zenogeneic GvHD were in similar range.

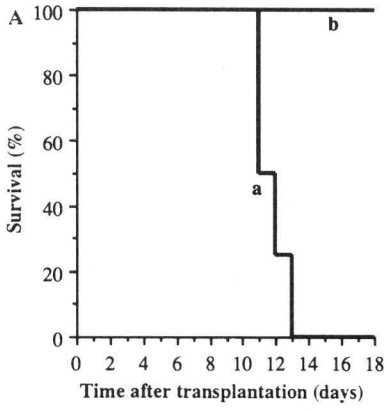
Allo- and xenogeneic GvHD

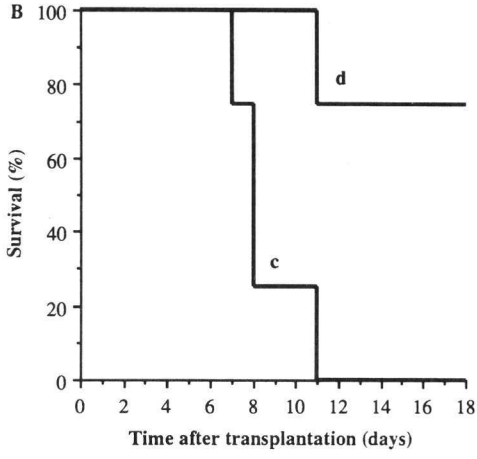
Human PBL grafts had been shown to grow preferentially in the murine lymphohemopoietic organ [6]. To study the interaction of lymphatic tissues of both species, low grade allogeneic GvHD was induced in conjunction with acute xenogeneic GvHD in heavily conditioned CBA/N mice. Typical xenogeneic GvHD was observed in the spleen, the mesenterial lymph nodes and the lung. Typical allogeneic low grade (I/IV [25]) GvHD was observed in the rectum. In the portal triads dramatic enhancement of the GvHD (Fig. 6) was seen (grade IV), resembling the vanishing bile duct syndrome, that is sometimes seen in human allogeneic liver rejection. The control animals ($n = 8$) with allogeneic GvHD had very small infiltrates in the portal triangles (grade I) (see Fig. 6), and few invading lymphocytes in the crypts of the rectum (grade I). The control animals with xenogeneic GvHD had small infiltrates in the portal triangles (grade I), without bile duct infiltration. In some of the triangles, parenchymal liver cell destruction was observed (see Fig. 6). We demonstrate enhancement of allo- and xenogeneic GvHD in the liver, which is affected in either disease separately.

DISCUSSION

Chronic GvHD

Murphy *et al.* [17] and Hoffmann-Fezer *et al.* [18, 19] demonstrated that the engraftment of 10^8 human PBL leads to a prolonged activation of human T cells in about 50% of little- or non-conditioned mice. From 6 weeks after transplantation, these mice suffer from chronic GvHD as characterized by generalized perivasculitis and infiltrates in the murine lymphohaemopoietic tissues both with a predominance of human $CD8^+$ lymphocytes [18, 19]. Murphy *et al.* grafted 19 SCID mice i.p. with 10^8 human PBL (about 4×10^6 cells/g) and treated nine of these with an antiserum against both human and mouse NK cells (anti-asialo GM1). Six weeks after PBL transfer, a chronic GvH reaction was observed in 10% of the non-conditioned and 50% of the antiserum conditioned mice [17]. Similarly, Hoffmann-Fezer *et al.* i.p. grafted 44 SCID mice with 10^8 human PBL. In the second and third month after transplantation, 60% developed lethal chronic GvHD [18, 19; personal communication]. Some human donors will have larger numbers of reactive T cells against murine cells than others, because the interindividual variance [26] in reactive cells is a factor 100. In this paper we show, that chronic GvHD is induced in 100% of recipients, when very large grafts of 10^9 human PBL are used,





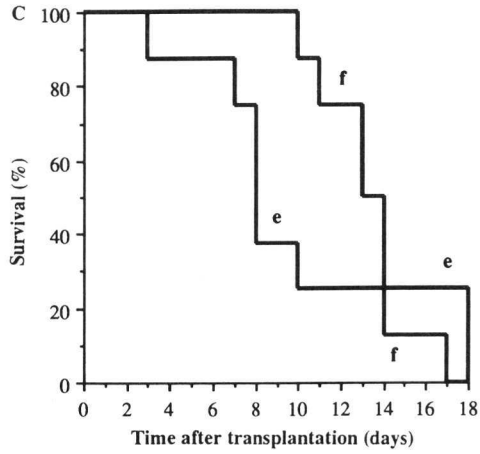


Fig. 4. Survival curves of heavily conditioned CBA/N mice grafted i.p. with 4×10^7/g human PBL and treated i.p. with 100 μg anti-human cytotoxic antibody. (A) with lines a and b, (B) with lines c and d and, (C) with lines e and f represent three different experiments. Mice treated with PBS ($n = 4$; line a), with α-CD4 Ab OKT 4A ($n = 4$; line b), with PBS ($n = 4$; line c), with α-CD4 Ab RIV-6 ($n = 4$; line d), with PBS ($n = 8$, line e) and with α-CD8 Ab OKT 8F ($n = 8$; line f).

corresponding to the number of white blood cells from 450 ml human blood (Fig. 2). Considering the long time period of 3 months until all the mice had died of chronic GvHD, we assume that lower numbers of PBL than 6×10^7 PBL/g might not induce GvHD in 100% of the recipients. This number of cells is higher than the 4×10^7 PBL/g needed to induce acute GvHD, via the i.p. route, in heavily conditioned mice. We have previously shown that non-conditioned SCID mice have a transplantation barrier for human PBL grafts [6]. It can be speculated that the larger number of cells needed to induce chronic GvHD, compared to that of acute GvHD, is caused by the fact that a certain critical mass of donor immune cells must overgrow the host resistance. Just like Murphy *et al.* [17] and Hoffmann-Fezer [18, 19] had demonstrated, this process appears to be relatively more advantageous for the cytotoxic $CD8^+$ lymphocytes, than for the helper $CD4^+$ lymphocytes or the B lymphocytes (Fig. 2).

Mosier *et al.* showed that 16–100% of non-conditioned SCID mice developed human B cell lymphomas in 8–18 weeks after the grafting of 5×10^7 PBL of an Epstein-Barr virus seropositive (> 90% of the adults) human blood donor ($n = 10$) [27,28]. Lower cell numbers of human PBL induced B cell lymphomas less frequently, appearing later after transplantation [29]. The cell numbers used by Mosier *et al.* were explicitly chosen below the number needed to induce GvHD (personal communication). We did not observe human B cell lymphomas after grafting as many as 10^9 human PBL, as lymphoma formation is probably a later event than the development of chronic GvHD.

GvHD inducing PBL grafts are cytokine dependent

When analysing the murine H_2-locus, Matzinger *et al.* demonstrated that the frequency of MHC responding T cells was not dependent on the individual MHC molecule, as long as it was not identical to the responder MHC. This frequency ranged from 1 to 0.01% of total T lymphocytes [26]. The frequencies of MHC reactive T cells in allogeneic and xenogeneic mixed lymphocyte reactions are widely assumed to be in a similar range. However, to inflict acute GvHD in heavily conditioned mice, as few as 5×10^4 allogeneic T cells per g recipient bodyweight [30] and as many as 2×10^7 discordant human PBL are needed as an i.v. graft [6], indicating that there is a pronounced difference between the threshold cell doses at which the grafted cells start producing pathogenic cytokines. When fewer discordant

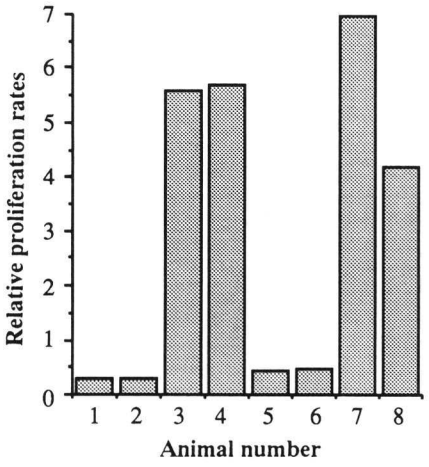


Fig. 5. Serum level of IL-6 (of relative proliferation) 2 weeks after transplantation. 1 : normal BCBA mouse; 2 : 9.25 Gy TBI conditioned BCBA mouse (F1 : C57BL × CBA) transplanted i.v. with 10^6 syngeneic BM cells; 3 and 4 : 9.25 Gy TBI conditioned BCBA mice transplanted i.v. with 10^6 BM and 10^6 spleen cells of a CBA mouse; 5 and 6 : 9 Gy TBI conditioned CBA/N mice transplanted i.v. with 5×10^5 syngeneic BM cells; 7 and 8 : 9 Gy TBI conditioned CBA/N mice transplanted i.v. with 5×10^5 syngeneic BM cells and 2×10^7/g human PBL.

xenogeneic T cells are used, peripheral tolerance appears to result in a lack of GvHD. Such has been demonstrated for Mosier's model, where 10^7 human PBL are transplanted into non-conditioned SCID mice [4, 5]. Supposedly, this peripheral tolerance occurred via primary immunization, secondary litigation of the human CD28 receptor would be excluded. Proteins like murine B7 and LFA-3 are species specific and therefore unable to deliver costimulatory signals of antigen presenting cells. It is generally accepted that a secondary signal is necessary to induce full IL-2 secretion, allowing efficient T-cell proliferation [31]. As high affinity IL-2 receptors are expressed after stimulation of T cells, exogenous human IL-2 might be able to induce proliferation of grafted human lymphocytes within the murine environment. In contrast to preactivation *in vitro* prior to transplantation, daily injections of human IL-2 *in vivo* into chimeric CBA/N mice allowed a 10-fold reduction of the cell number needed to inflict GvHD, from 4×10^7 to 4×10^6 human PBL (i.p.) per g body weight of the recipient (Fig. 3).

The reduced number of 4×10^6 xenogeneic human PBL under IL-2 supplementation is still substantially higher than the 5×10^4 spleen cells/g needed for allogeneic GvHD [30]. This amount of 5×10^4 cells/g also proved to be able to induce xenotransplanted monomyeloid leukaemia of the human cytokine-independent tumour cell line HL60 [12] in non-conditioned SCID mice (Fig. 1). Besides IL-2, other human-specific cytokines might be lacking in the xenogeneic situation. Further studies will show whether supplementation of additional factors facilitates the growth of the PBL graft.

Whereas species-specific activities of cytokines like IL-2 are limiting for xenogeneic GvHD, cytokines with species-unrestricted activity like IL-6 are not expected to be critical for limitation of initiation and progression of the disease. IL-6 levels were actually found identical in both allo- and xenogeneic GvHD (Fig. 5). The fact that non-restricted cytokine levels are mutually thriving is illustrated by the observation that allogeneic and xenogeneic GvHD show local enhancement in the liver triangles (Fig. 6).

Human $CD4^+$ cells induce GvHD

Human NK cells had been reported to induce acute xenogeneic GvHD [9]. In our hands, cultivation procedures failed to provide hints for involvement of NK cells in the described model.

At low cell numbers, species-specific cytokines like human IL-2 are limiting xenogeneic GvHD. Among the grafted PBL, the cytokine producing helper $CD4^+$ T lymphocytes have clear selection advantage in the murine environment. $CD4^+$ T cells had been previously shown to be main infiltrating cell type in xenogeneic GvHD. In this report, we demonstrate, that the disease was abrogated by treatment of the grafted mice with cytotoxic anti-human CD4 monoclonal Ab. Anti-Cd8 Ab only delayed marginally the onset of disease (Fig. 4). The mentioned selective advantage for cytokine producing human T cells might also be the reason why the infiltrates appear as tumours with little notice of cytotoxicity [6].

Chimeras with herpesvirus saimiri transformed lymphocytes

After transformation by Herpesvirus saimiri, human T-cell lines show the phenotype of mature activated lymphocytes [10]. Immortalized cell clones grow in the presence of IL-2, but without need for antigen or mitogen stimulation. Many features of the parental cultures are retained after transformation. The karyotypes were normal [32]. Biochemical signal transduction patterns and immunological functions like antigen specificity were found identical to untransformed counterparts [22, 23, 33, 34]. The cell line CB15 has been analysed in detail [10, 22, 23, 33, 34]. CB15 and other Herpesvirus saimiri transformed T cell lines had been grafted without exogenous IL-2 to nude and SCID mice in conventional tumour test experiments, similarly to the presented HL60 assay. As early as 1 week after inoculation no surviving human T cells were detected. Such animals survived for 3 months without any disturbance and did not show any alteration upon autopsy (H. Fickenscher *et al.*, unpublished observations). From these experiments the conclusion was drawn that Herpesvirus saimiri transformed human T cells are not tumourigenic in conventional assays without exogenous human IL-2.

In this report, Herpesvirus saimiri transformed human

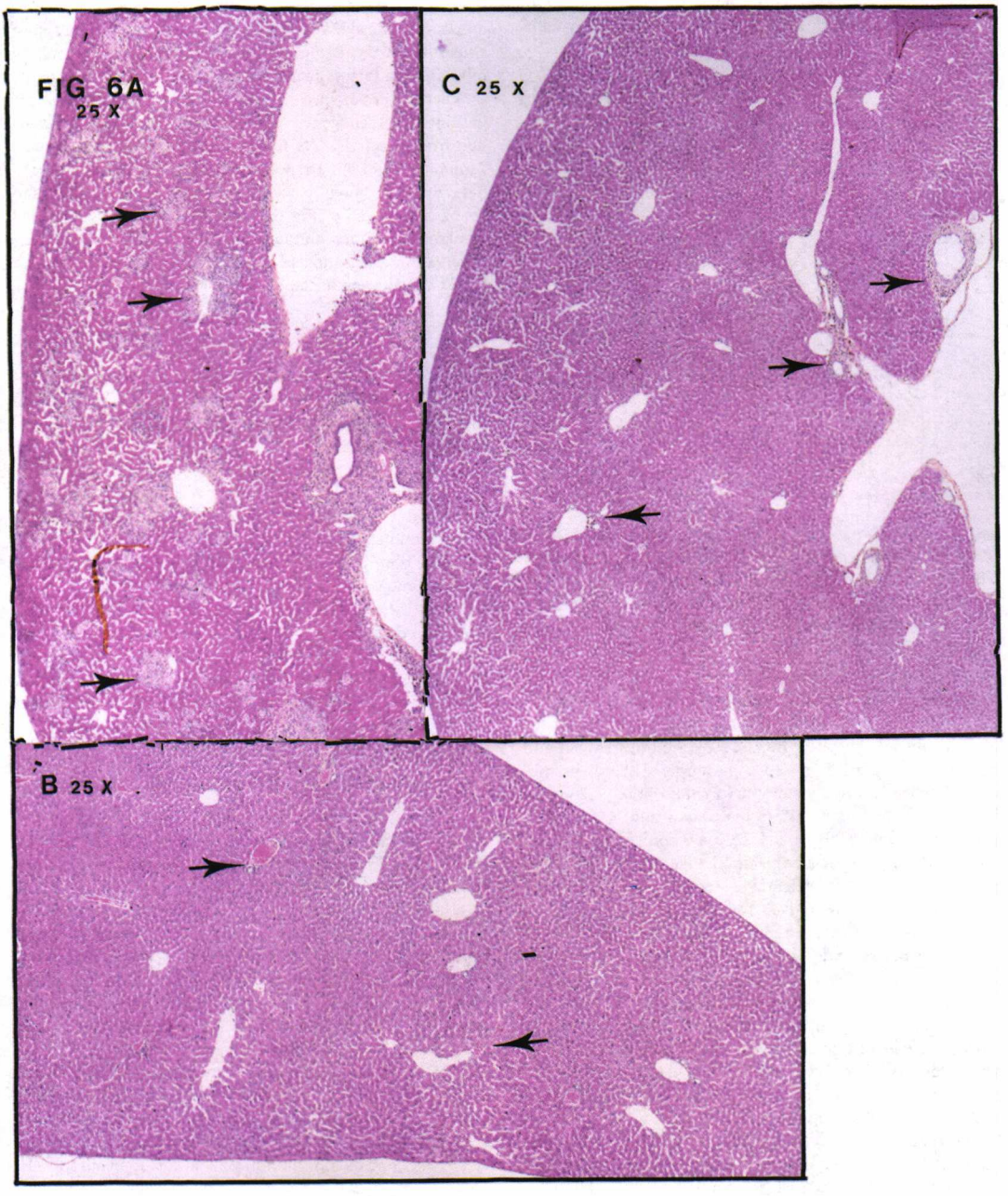


FIG 6A
25 X
C 25 X
B 25 X

$CD4^+$ CB15 cells were tested for induction of GvH-like disease in heavily conditioned CBA/N mice in presence of exogenous IL-2. The number of clonal CB15 cells needed to induce lethal desease was 1.3×10^6/g, instead of the 4×10^6/g needed for PBL grafts (Fig. 3). The three-fold difference of the cell numbers required to induce acute disease seems insignificant, because it can be attributed to the CD4 content of the grafts, which is about 40% for the PBL graft [6] and 100% for the CB15 graft. Nearly identical behaviour of PBL or CB15 grafts in mice shows again the pronounced similarities of Herpesvirus saimiri transformed human T cells to activated primary T cells.

Applications of the xenogeneic GvHD model system

Chimeric mouse models are necessary for experimentally studying human immune reactions *in vivo*, if agents are analysed which are toxic in humans and if primate experiments have to be avoided. During acute xenogeneic GvHD, massive infiltrates of cytokine producing human $CD4^+$ T cells were observed. Thus, large amounts of activated human $CD4^+$ T cells are available in an animal system. The spontaneous course of the disease being lethal, effects on human $CD4^+$ T cells can be read out easily in survival rates. As the GvHD can already be induced in newborn mice of about 1 g of bodyweight [6] (unpublished results), the murine model can be applied for studies of the human immune system with as few as 4×10^6 human PBL per mouse, if human IL-2 is supplemented. Consequently, about 500 tests can be done with the PBL of just one blood bank donation. The transplantation of the permanently growing clonal T cell line CB15 allows constant features of the grafts in chimeric animals. Such might be relevant, if the system was applied for preclinical drug testing or in AIDS research. Human immunodeficiency virus, and especially poorly replicating strains, have been shown to replicate efficiently on CB15 cells [24].

After transplantation of large numbers of either human PBL or of the human cell line CB15 in conjunction with daily injections of human IL-2, the mice suffered from generalized human $CD4^+$ lymphoblastic perivasculitis (unpublished observations). Also, Hoffmann-Fezer [18, 19] had demonstrated that the engraftment of human PBL in non-conditioned SCID mice induced severe generalized human $CD8^+$ lymphoblastic perivasculitis. This indicates that circulating activated lymphoblasts and high levels of cytokines are sufficient disease conditions. The adherence of the human lymphocytes to the murine endothelium cannot be explained by the binding of murine endothelial molecules CD54, CD11a/CD18 or CD2, which are species-specific and not active on human T cells. Instead, the species unrestricted cytokines TNFα, IL-1 and IL-8, which are known to induce the adluminal procoagulant state [35, 36], might cause adherence of the human cells. The present understanding of the aetiology of generalized vasculitis, like Wegener's disease, is incomplete [37]; $HgCl_2$-induced necrotizing vasculitis is the only available animal model and it is too complex to dissect individual aetiological factors [38]. Therefore we propose our model systems for vasculitis research.

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Chapter 6

Peripheral Blood Leukocytes that Induce Human to Mouse Graft versus Host Disease, Reject Allogeneic Human Skin Grafts

Peripheral Blood Leukocyte Grafts That Induce Human to Mouse Graft-vs.-Host Disease Reject Allogeneic Human Skin Grafts

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Allogeneic graft-versus-host disease is characterized by skin, gut, and bile duct destruction by relatively few donor type lymphocytes. In contrast, we can now show that human-to-mouse xenogeneic graft-versus-host disease is characterized by vasculitis and tumorlike infiltrations of the murine lymphohemopoietic organs with many human CD25$^+$, HLA-DR$^+$, CD4$^+$ lymphoblasts. Using the technique of serial transplantation, it appears that at least 90% of the human lymphoblasts were unreactive to murine tissues. It is demonstrated consistently that the donor type lymphoblasts induced typical allogeneic rejection of distantly located full thickness human unmatched fetal skin grafts. The fact that the human grafts show primary immune responses in vivo indicates that the graft-versus-host disease murine model may be suitable for vaccination studies. (Am J Pathol 1995, 147:1708–1714)

At present, many human vaccines have been developed. However, these are not easily tested, because *in vitro* primary immunization is laborious and cumbersome. Also, experiments on primates are not encouraged and the human cells in the HuPBL-SCID (severe combined immunodeficient) mouse, as described by Mosier et al,[1,2] show only secondary immune responses. In this latter model human peripheral blood leukocytes (PBLs) are transferred just below the critical number necessary to induce graft-*versus*-host disease (GvHD).[3] This results in a few, mostly CD8$^+$, human lymphocytes populating the murine hemopoietic tissues.[4] These cells have been shown to be anergic to T cell receptor activation.[5,6]

In contrast, transplantation of elevated numbers of human peripheral blood cells in mice results in xenogeneic GvHD.[7] Dissimilar to allogeneic GvHD, which is characterized by epithelial infiltrates with relatively few cells, the xenogeneic GvHD consists of large tumorlike infiltrations in the lymphohemopoietic tissues.[7–10] As these large infiltrates could be observed as early as 2 weeks after transplantation,[7,8] they were thought to contain many human leukocytes not reactive against mouse antigens. Therefore, we studied the possibility of such xenografted human PBL reacting against third-party antigens, thereby creating a model that would allow for vaccination studies.

Materials and Methods

Conditioning of Mice

Inbred BALB/c-SCID/Rij, CBA/N/Rij, and Bg/Nu/XID/Rij were kept and bred under specified pathogen-free conditions in our own breeding colony.[7]

Conditioning was performed by total body irradiation (TBI) at doses previously published to be optimal for transplantation purposes: 9 Gy for the CBA/N mice at 4 weeks of age and 7 Gy for the Bg/Nu/XID mice at the age of 8 weeks.[7] The SCID mouse is abnormally sensitive to irradiation.[11] Therefore, the 3-week-old female SCID mice, used for the serial transplantations, received 2.5 Gy TBI (maximum tolerated dose).

Serial Transplantations

Infiltrate analysis was performed to determine the relative number of human cells reactive to the murine

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tissues, with serial transplantations. For this purpose we used CBA/N mice with GvHD 8 days after the reception of i.p. grafts of 4×10^6 human PBL/g. The acute GvHD could be assessed clinically by progressive dyspnea. When obtaining the tissues, gross necropsy would reveal emaciated, anemic animals with small hemorrhages, most commonly in the lymphohemopoietic organs and in the synovium of the peritoneum. The ascitic fluid cells of these mice were transplanted i.p. into other heavily conditioned CBA/N or SCID mice, in a dose range of 4×10^6 to 4×10^4 cells/g. All these mice received daily injections of 10^4 IU of human interleukin-2 (proleukin, a kind gift from Eurocetus Benelux B.V., Amsterdam, the Netherlands).[8]

Histopathology

The pattern of growth and activation of the various human cells in the murine tissues was studied with immunohistochemistry using human specific antibodies.[9,10] Complete gross necropsy was performed on 10 mice that had received 4×10^7 human PBL/g i.p.[8] and on 10 mice transplanted with 2×10^7 human PBL/g i.v.,[7] with GvHD 7 to 14 days post-transplantation. These necropsies were compared with heavily conditioned CBA/N mice, which had also received 5×10^5 syngeneic bone marrow (BM) cells i.v., but no human PBL. Most tissue samples were fixed in phosphate-buffered 4% formaldehyde and paraffin-embedded (Hypercentre XP, Shandon, Zeist, the Netherlands). The samples were processed to 3 μm sections and stained with either hematoxylin, phloxine, and saffron,[7] (HPS) or with hematoxylin (Fluka Chemika, Buchs, Germany) and eosin-yellow (H&E) (Merck, Darmstadt, Germany). H&E slides of alternating tissue sections were prepared. Every other slide was incubated with antibody to human CD45, the leukocyte common antigen (LCA, Dakopatts, Glostrup, Denmark) in conjunction with the peroxydase technique.[12] Tissues with LCA staining were also hematoxylin-counterstained. In mice, which had not received human tissues, no human LCA^+ cells were observed.

From four CBA/N mice with GvHD 7 days after the i.v. human PBL grafts, the tissue samples were snap-frozen and stored in liquid nitrogen. Immunohistochemistry was performed on 5 μm thick cryostat sections.[9,10] Biotin-labeled monoclonal mouse antibodies were used to prevent background staining of CBA/N mouse tissues. Peroxidase activity was shown with 3-amino-9-ethylcarbazole (Sigma Chemical Co., St. Louis, MO), alkaline phosphatase activity with Fast Blue BB (Sigma Chemical Co.). $CD4^+$ or $CD8^+$ human lymphocytes were double-labeled for measurement of activation. Rat anti-human CD4 or CD8 were used as biotin-labeled antibody,[9,10] followed by peroxidase-labeled avidin (Vector Laboratories, Burlingame, CA). The mouse anti-human CD25 (interleukin-2 receptor; Immunotech S.A., Marseille, France) and anti-HLA-DR[9,10] antibodies were stained with alkaline phosphatase labeled goat anti-mouse immunoglobulin (Jackson ImmunoResearch Laboratories Inc., West Grove, PA).

Transplantation Procedure of Human Fetal Tissues

In vivo immune response analysis of human PBL grafts was performed with the histological study of co-transplanted human allogeneic unmatched skin and gut into 8-week-old Bg/Nu/XID mice. Human fetal tissue from suction abortions at ~14 weeks of gestation was collected in Hepes-buffered Hanks' salt solution. Fetal skin with a diameter of 1 cm was grafted to the dorsal side of these mice, as published by Coligan et al.[13] The protocol had two modifications; 1) narcosis was with 2,2,2-tribromethanol (Avertine, Sigma Chemical Co., Bornhem, Germany), and 2) dehydration of the wound was prevented with OpSite (Smith and Nephew, Hall, UK). Fetal gut with a length of 1 cm was subcutaneously grafted into a dorsal site. One month later five mice with human fetal skin grafts and six mice with fetal gut grafts were conditioned with 7 Gy TBI and subsequently i.p. transplanted with 4×10^7 cells human PBL/g. These animals had GvHD 11 days later and were killed for histological analysis. The mice that served as controls had a tissue graft, but were not transplanted with PBL. Extra controls were performed, because it had been previously published that SCID mice with allogeneic GvHD contained fewer gastrointestinal and skin lesions than non-immune deficient control mice.[14] Therefore, three Bg/Nu/XID mice conditioned with 7 Gy TBI and transplanted i.v. with 10^6 BM and 10^6 CBA spleen cells were also histologically analyzed.

Results

Serial Transplantations

To enable an estimate of the number of murine unreactive human lymphoblasts in the infiltrates of mice with xeno-GvHD, serial transplantations from CBA/N mice toward CBA/N and SCID mice were performed. The results are shown in survival curves as depicted

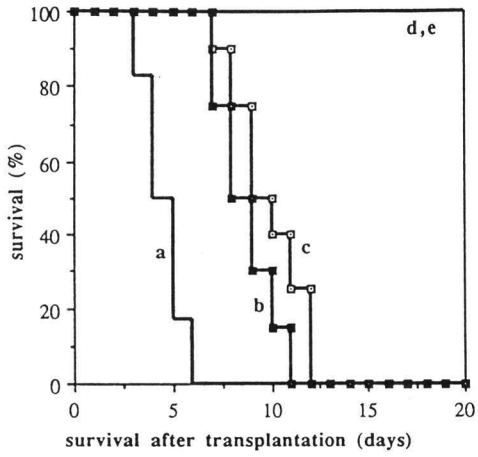

Figure 1. *Survival percentages of mice after serial transplantation of human peripheral blood leukocytes.* (Line a) *Six CBA/N mice grafted with 4 × 10^6 ascitic fluid cells/g body weight.* (Line b) *Fifty CBA/N mice grafted with 4 × 10^6 fresh human lymphocytes/g.* (Line c) *Twenty CBA/N mice grafted with 4 × 10^5 ascitic fluid cells/g.* (Line d) *Four SCID mice grafted with 4 × 10^5 ascitic fluid cells/g.* (Line e) *Ten CBA/N mice grafted with 4 × 10^4 ascitic fluid cells/g.*

in Figure 1. It is shown that the number of cells needed to induce lethal GvHD was reduced by a factor of 10 by using the serial transplantation in the syngeneic inbred H2K^k CBA/N mice. This reduction did not occur after transplantation into the H2-incompatible inbred H2K^d SCID mice.

Pathology of Acute Xenogeneic GvHD

The most striking phenomenon of the acute GvHD was the generalized acute perivasculitis of both the small and the large arteries and veins. Human LCA$^+$ lymphoblasts were observed, and seen attached and passing through the endothelium (Figure 2). These inflammations, with varying degrees of infiltration of the mouse stroma, were occasionally observed in the majority of the murine tissues. In contrast, perivasculitis and engraftment were invariably observed in the murine lymphohemopoietic tissues. Small bleedings at these sites were often seen. Figure 2 demonstrates that the human cells grew tumorlike in the murine lymphohemopoietic tissues. It is observed that the outer rims of these tumorlike infiltrates are more dense, with aggregated human cells, when compared with the inner parts. Necrosis of the center was not seen. The expansions of human cells from these tumorlike areas appear mostly as diffuse infiltrates, with few signs of cell death or apoptosis of murine cells. The areas most profusely infiltrated with human cells were the paracortex of the lymph nodes and the white pulp of the spleen. In most cases the infiltrate occupied most of these murine organs (Figure 2) and predominantly consisted of human T cells (see below). This process was accompanied by depletion of murine lymphohemopoietic cells.

The architecture of the spleen had changed, with the white pulp enlarged, and the borders between the white and red pulp could no longer be distinguished. There was no correlation between the extent of infiltration of the white pulp and the dissemination into the red pulp. The red pulp contained varying numbers of LCA$^+$ cells. Immunohistochemical analysis[9,10] showed spleen sections with many human CD3$^+$ cells in both in the red and white pulp. Most of the CD4$^+$ cells were large blasts (Figure 2), whereas the CD8$^+$ cells were lymphocytes. In three out of four mice the CD4$^+$ cells were more frequent in the white pulp than in the red pulp. The other case showed an equal distribution between the two compartments. The CD8$^+$ cells were more frequent in the red pulp in half of the mice, whereas the other half showed an equal distribution. Double-labeling experiments showed that about 30 to 50% of the CD4$^+$ cells were also CD25$^+$, whereas CD8$^+$ CD25$^+$ dou-

Figure 2. *Photomicrographs of tissue sections of CBA/N mice with acute GvHD. Staining was with human-LCA/peroxidase on paraffin tissue sections, with counterstaining of the nuclei with hematoxylin to increase the contrast with the dark brown signal. Cell population-specific humanimmunoperoxidase identification was done with frozen tissue sections. In the peritoneal lymph node many human LCA$^+$ cells were observed* (A; 25×). *The boundary of the infiltrate with dark brown human color appears more dense than the central part. The thymus is sparsely repopulated and shows diffusely some LCA$^+$ cells* (B; 10×). *A detail of this is shown in C* (C; 50×). *The BM, within the left upper corner mouse granulopoiesis, shows many paratrabecular human LCA$^+$ cells* (D; 10×). *A detail of murine lymphohemopoietic cells surrounded by human LCA$^+$ cells is shown in* E. *Depicted are nuclear rests of a megakaryocyte* (arrow) *and an LCA$^+$ cell* (arrow) *attached to another megakaryocyte* (E; 250×). *The spleen shows the most dense human LCA staining on the boundary of the infiltrate* (F; 25×). *The photomicrographs of the white pulp of the spleen depict the many human CD4$^+$* (blue), *CD25$^+$* (dark brown) *lymphoblasts* (arrows) (N; 250×), *but no CD8$^+$* (blue), *CD25$^+$ lymphoblasts* (O; 250×). *The liver also shows many LCA$^+$ cells in the triangles with perivasculitis. Depicted is an LCA$^+$ cell attached to the vascular endothelium. The bile duct endothelium appears intact. In the sinusoids are some LCA$^+$ cells visualized* (G; 50×). *The dark brown signal of human CD11c$^+$ stains histiocytic cells, mostly in the triangles* (J; 100×). *The next two photomicrographs of serial tissue sections depict the interrelatedness of these human CD11c$^+$ histiocytic cells* (K; 100×) *and the CD3$^+$ lymphoblastic cells* (L; 100×) *in the infiltrations of the liver. The photomicrographs of the lung* (H *and* I; 50×) *show in the center a large vessel surrounded by many LCA$^+$ cells, indicative of perivasculitis. The human LCA$^+$ cells in the interstitial tissue show the interstitial pneumonitis. A detail is depicted with staining for human CD3* (M; 250×). *The bronchus epithelium appears intact, with absence of human LCA$^+$ or CD3$^+$ cells.*

Figure 3. *Photomicrographs of tissue sections of human fetal skin grafts of Bg/Nu/XID mice, co-transplanted with unmatched human peripheral blood leukocytes. The human skin grafts show infiltrates typical for allogeneic GvHD, with the dark brown immunohistochemical signal of human LCA$^+$ lymphoblastic cells in the skin rete ridges* (A; 100×). *In comparison, the mouse skin of the same animal is depicted. The epithelium appears devoid of LCA$^+$ cells* (B; 100×). *The mice that served as controls* (C; 100×) *had human skin grafts and TBI, but were not transplanted with human PBL. These grafts show a few LCA$^+$ cells around vessels, but no skin infiltration.*

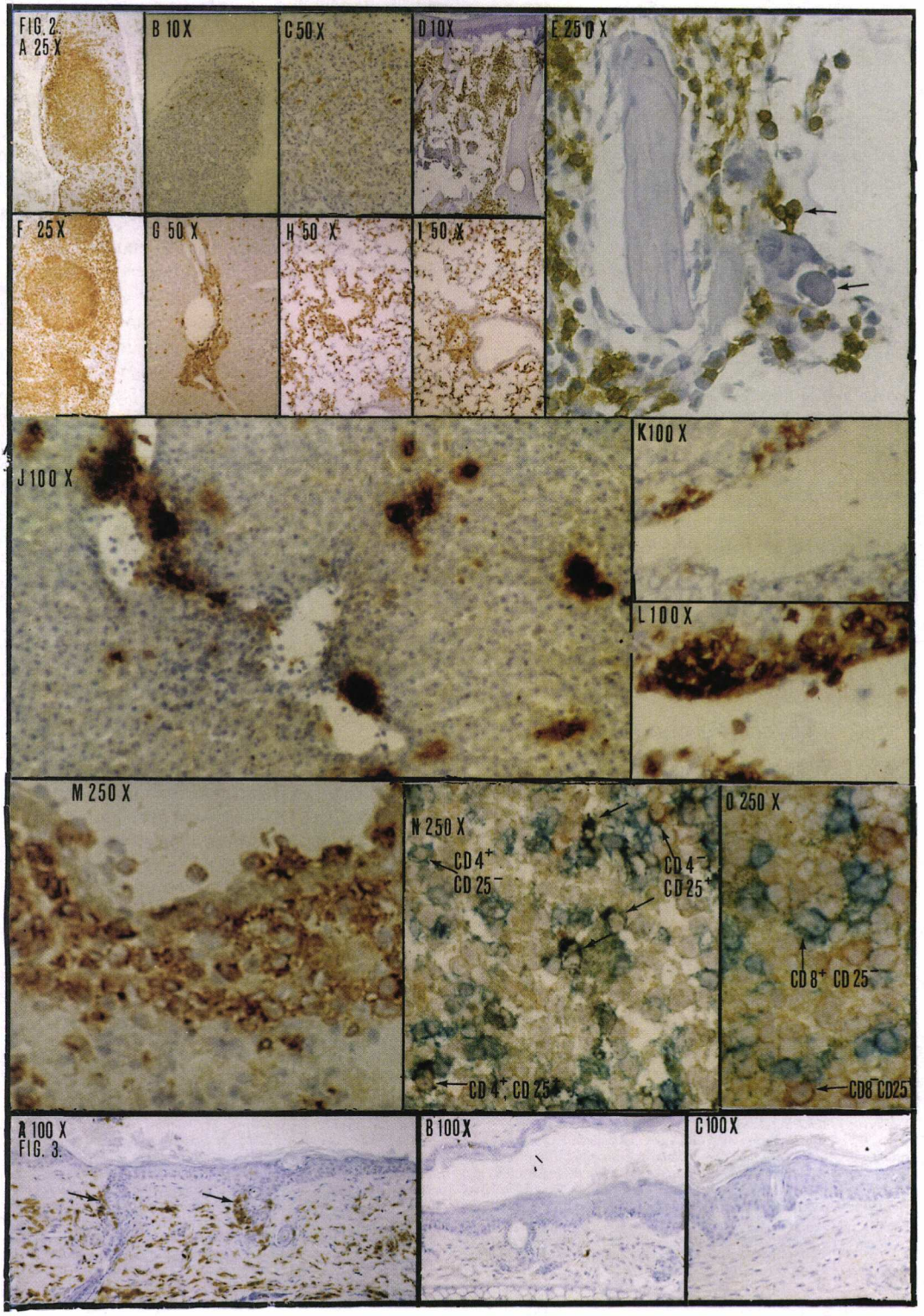
FIG. 2.
A 25 X
B 10 X
C 50 X
D 10 X
E 250 X
F 25 X
G 50 X
H 50 X
I 50 X
J 100 X
K 100 X
L 100 X
M 250 X
N 250 X
CD 4+
CD 25−
CD 4−
CD 25+
CD 4+ CD 25−
O 250 X
CD 8+ CD 25−
CD8 CD25
A 100 X
FIG. 3.
B 100 X
C 100 X

ble-positive cells were very rare. Most of the observed human cells were also HLA-DR$^+$. In all mice, both in the red and white pulp, a few human Ig$^+$ cells with broad irregular cytoplasmic Ig$^+$ rims were observed. These cells were scattered throughout the compartments and showed no organized localization.

The mesenteric lymph nodes contained many CD3$^+$, HLA-DR$^+$ cells in the paracortical region (Figure 2). The CD4$^+$ cells were more numerous than were the CD8$^+$ cells. The few human Ig$^+$ cells were localized both in the cortical regions and the medulla. The plaques of Peyer were invariably infiltrated with many LCA$^+$ lymphoblasts. Here, CD4$^+$ and CD8$^+$ cells occurred in approximately equal amounts.

The most dramatic loss of the murine lymphohemopoietic system was seen in the bone marrow, in accordance with earlier findings.[7] This did not only result from a displacement by human LCA$^+$ cells, but also observed was extensive cell death of the murine lymphohemopoietic cells. This is depicted in Figure 2, in which the vicinity of the human LCA$^+$ lymphoblasts is also shown. This paratrabecular site of the human infiltrate was the site most commonly observed. The murine BM is characterized by differing degrees of repopulation and degeneration. Often seen was the reaction of the mouse stroma to the human graft. It consisted of fibrin deposition and proliferation of the mouse stromal cells.

The thymi contained varying numbers of human LCA$^+$ lymphoblasts. The range was from a few LCA$^+$ cells to diffuse infiltration of both the cortex and the medulla with many LCA$^+$ cells. Immunohistochemistry showed rare mouse CD3$^+$ cells, which did not form a normal cortical and medullary architecture. Intermingled with these mouse cells a few human CD3$^+$ cells were observed.

The liver infiltration also varied interindividually. The range was from a few LCA$^+$ cells in the triangles to large infiltrates (Figure 2) with obstruction of some of the cholangiae, perivasculitis, and diffuse dissemination into the sinusoids. Immunohistochemistry showed in two out of four mice medium grade (III/IV[15]), and in two out of four low grade (II/IV) infiltration of the portal triads with many CD3$^+$ cells. More of these were CD4$^+$ than CD8$^+$. Some of the CD4$^+$ cells were CD25$^+$, and almost all of both the CD4$^+$ and CD8$^+$ cells were HLA-DR$^+$. In three out of four mice CD11c$^+$ large cells with a histiocytic appearance were observed as single cells or as small clusters. In Figure 2 it is shown with serial tissue sections that these human histiocytes had intimate contact with the human CD3$^+$ cells.

In the lung peribronchiolar infiltrates, perivasculitis and varying degrees of pneumonitis were always present. The cause of death appears to be perivasculitis with hemorrhages of the pulmonary vessels (Figure 2). The perivascular and peribronchiolar infiltrates consisted of human CD3$^+$ cells, the majority of them CD4$^+$ (Figure 2) than CD8$^+$, with some human Ig$^+$ cells.

Infiltration and destruction of the peritoneum and the omental tissue with many human LCA$^+$ blasts was observed in the mice, which had received i.p. human PBL grafts. This was sometimes associated with obstruction of the gut. Also, engraftment of the LCA$^+$ blasts was seen along the sites that communicate with the peritoneum. Examples of these were along the esophagus (often with pleuritis) along the ureters, the ovarian tubes, the femoral vessels, and the hilus of the pancreas.

After both i.p. and i.v. inoculation of the graft, small groups of human LCA$^+$ cells were observed in the lamina propria of the intestinal villi of the gut, increasing in number from the stomach to the coecum. In the lamina propria single human CD3$^+$ cells occur, most of them CD8$^+$. CD4$^+$ cells were extremely rare. In the epithelium of the skin a few solitary human LCA$^+$ cells were observed.

Two other CBA/N mice not suffering from GvHD, which had been grafted i.p. with 4×10^7 human PBL/g, were analyzed histologically with HPS staining on day 6 posttransplantation. Gross inspection of these mice showed no abnormalities. However, with microscopy an atypical spleen in some areas of the white pulp was observed. It consisted of stretches of enlarged white pulp that contained many human lymphoblasts. There was no evidence of vasculitis.

In summary, the acute GvHD is characterized by tumorlike infiltrates of the murine lymphohemopoietic tissues, consisting mostly of human CD4$^+$ lymphoblasts and severe generalized lymphoblastic perivasculitis.

Human Tissue Grafts in Conjunction with Unmatched Human PBL Grafts

Microscopic analysis of fetal skin grafts of five Bg/Nu/XID mice co-transplanted i.p. with human PBL showed epithelial infiltration of the human skin. The infiltrates contained numerous human LCA$^+$ cells, typical for allogeneic GvHD in four out of five of the animals. The morphology is depicted in Figure 3. The epidermis shows grade III/IV rejection,[15] with parakeratosis, acanthosis, irregularity of rete ridges, a poorly delineated basal layer, and a dense human

LCA^+ lymphoblastic infiltrate, which extends into the subcutis and involves the adnexa.

Tissue sections of a control Bg/Nu/XID mouse, treated with human fetal skin graft and 7 Gy TBI, showed normal human skin with some vacuolization of the parabasal cells, without LCA^+ cells (Figure 3). Three more controls with a human fetal skin graft were analyzed, which received no TBI and had no human PBL either. Tissue sections show no LCA^+ cells in the epidermis either. However, tissue sections of the control mice showed some human LCA^+ histiocytic cells in the subcutis and fat tissue underneath it. These human LCA^+ histiocytic cells were also observed as single cells in the murine spleen, lymph node, and lung.

Allogeneic resection was presumably also seen in subcutaneous human fetal gut transplantations, but these tissues were affected from the adluminal site in varying degrees by necrosis and infiltration with macrophages, which are LCA^- and therefore probably of murine origin. These degenerations had also been seen in syngeneic fetal gut transplantation.[16] The vital parts of the human fetal gut grafts were affected by grade II/IV rejection, with diffuse endothelial infiltration with human LCA^+ lymphoblasts. These infiltrates extend into the lamina propria. In contrast, tissue sections of two control mice, which had subcutaneous human fetal gut grafts and 7 Gy TBI, showed only a few human LCA^+ cells.

The HPS-stained tissue sections of three other Bg/Nu/XID control mice grafted with 10^6 BM and 10^6 spleen cells of a completely allogeneic CBA/Rij mouse showed typical allogeneic GvHD of skin and gut.

Discussion

The many activated human lymphohemopoietic cells in the CBA/N mice after the grafting of elevated numbers of human peripheral blood leukocytes suggest the successful transplantation of a functional human immune system. It was observed that large numbers of human $CD4^+$ and $CD8^+$ T cells and some B cells were present in the reticuloendothelial areas of the murine lymphohemopoietic tissues (Figure 2). The number of both the human $CD4^+$ and $CD25^+$ cells (Figure 2) in these mice with acute GvHD was higher than in the ones with chronic GvHD[9,10] (G. Hoffmann-Fezer, unpublished results). The large number of human $CD4^+$ lymphoblasts is consistent with previous findings that human $CD4^+$ T cells are the most significant cells in the induction of the xenogeneic GvHD.[7,8]

In addition to grafted human lymphocytes, we also found grafted human histiocytic cells, which were restricted to the peritoneal cavity after i.p. engraftment of the PBL.[9,10] Now after the i.v. transplantation of the graft, these histiocytic cells were observed as small infiltrations in the portal triads of the liver. These consisted of many $CD11c^+$ histiocytic cells, intermingled with $CD3^+$ lymphoblastic cells (Figure 2).

In the late stage of the disease, the spongelike lymphohemopoietic tissues appear to shed human lymphocytes into the circulation. This was shown in our previous papers, by the demonstration of circulating human cells in end-stage GvHD.[7,10] Now, it is shown histopathologically that these mice have perivasculitis, containing human $CD4^+$, $CD8^+$, and B cells (Figure 2). Dissemination of the human $CD11c^+$ histiocytic cells was not observed.

There may be an objection that the human T cells do not function well in xenogeneic GvHD, because the affected target tissues are dissimilar from allogeneic GvHD. In the tissue sections of mice with xeno-GvHD, human lymphoblast aggregates were observed in the stroma of all the murine tissues, but not in the epithelia.[7] The high concentration of donor type cells in the outer rim of the infiltrates and the cell death or apoptosis of the mouse BM cells in the vicinity of human lymphoblasts (Figure 2) both suggest that some of the human lymphoblasts in the mice were reactive to the mouse tissues. This can also be concluded from the fact that the number of cells needed to induce lethal GvHD was reduced by a factor of 10 by the serial transplantation in the syngeneic inbred $H2K^k$ CBA/N mice and the fact that this reduction did not occur after transplantation into the inbred $H2K^d$ SCID mice (Figure 1). Therefore, we can speculate that the human cells function well in the murine environment but are generally unable to home in on the epithelial tissues. The inability of the circulating human lymphoblasts to infiltrate the murine epithelia may be due to evolutionary differences in homing receptors. However, it is now shown that xenografted human fetal skin and possibly also human fetal gut, which can be expected to have had some human blood vessels anastomized with the murine circulation, were affected by typical allogeneic GvHD (Figure 3).

The fact that distantly located unmatched allogeneic grafts were rejected can be explained by presuming that of the large pool of proliferating human cells some are unreactive to the murine tissues. With serial transplantation (Figure 1) it appears, once more, that not all of the grafted human cells are reactive with murine epitopes. If they had all been

reactive, the percentage of cells needed to induce xeno-GvHD in the second series of mice would be expected to be in the range of the number of reactive cells. The number of lymphocytes reactive with allogeneic major histocompatibility complex is between 0.01 and 1%[17] of the total number and is widely accepted to be identical with the number of xenoreactive lymphocytes. However, the number of needed cells declined just one magnitude, from 4×10^6 cells/g to 4×10^5 cells/g (Figure 1). If all the human cells had been reactive with the murine cells, a decline of at least one order of magnitude more to 4×10^4 cells/g would have been expected. Therefore, it seems that at least 90% of the human lymphocytes were not reactive with murine tissues.

As the GvHD murine model induces an activated proliferating human immune system capable of primary immune-responses, it may be suitable for vaccination studies.

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Chapter 7

Efficient HIV-1 and Measles Virus Replication in a Human to Mouse Graft versus Host Disease Model Permits Immunization Research. Corrigendum

Efficient replication of human immunodeficiency virus type 1 and measles virus in a human-to-mouse graft versus host disease model permits immunization research

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An acute graft versus host disease (GvHD) murine model was developed to study the pathogenic and protective mechanisms against viruses that replicate in cells of the human immune system. The model allowed efficient replication of lymphotropic, macrophage and amphitropic strains of human immunodeficiency virus type 1 (HIV-1) and measles virus (MV). Cytopathic lymphotropic strains of HIV-1 and a wild-type MV strain replicated in a 'burst'-like manner, whereas a non-cytopathic lymphotropic HIV-1 strain and all macrophage-tropic HIV-1 strains caused persistent infection of the graft. The replication kinetics of infection with these viruses were highly reproducible and were very similar to those observed in natural infection of humans. Infection with these viruses, with the exception of HIV-1_{SF2}, led to a significant delay and abrogation of the GvHD, indicating a direct immunosuppressive effect. Interestingly, infection with the lymphotropic HIV-1_{SF2} strain was rapidly and spontaneously abrogated. The model was also shown to be suitable for the evaluation of passive immunization strategies. Administration of a combination of antibodies against the HIV-1 V3 loop and the HIV-1 CD4 binding sites prevented subsequent infection with HIV-1_{IIIB}. In contrast, administration of CD4 binding site specific human monoclonal antibody at a concentration that would neutralize the virus *in vitro* enhanced *in vivo* infection with HIV-1_{IIIB}. The model also allowed evaluation of *in vivo* immunization studies. Immunization with a live attenuated measles vaccine resulted in protection from a wild-type MV challenge, whereas immunization with a subunit candidate vaccine appeared to give partial protection.

Introduction

One of the major problems in studying the pathophysiology of human immunodeficiency virus type 1 (HIV-1) infection is the lack of a suitable animal model. Although HIV-1 has been shown to replicate in some other primates, like the chimpanzee, the susceptibility to the various phenotypes of the virus is different and these animals do not appear to develop the acquired immunodeficiency syndrome (AIDS) (Nara *et al.*, 1989; Saxinger *et al.*, 1987).

So far, two murine models for HIV-1 have been developed based on transplanted human cells. The first model, as described by D. E. Mosier and co-workers is based on human peripheral blood leukocytes (PBL) injected intraperitoneally (i.p.) into non-conditioned severe combined immunodeficient (SCID) mice. These human PBL SCID mice do not develop signs of graft versus host disease (GvHD) due to the limited numbers of transplanted cells (Torbett *et al.*, 1991). It was shown that T cell line adapted lymphotropic HIV-1 strains replicated in these human PBL SCID mice (Mosier *et al.*, 1991, 1993*a*), enabling passive immunization studies to be done (Vittecoq *et al.*, 1992). However, the human graft was shown to have defective immunological properties, because the T cells, which are mostly activated $CD8^+$ cells, are anergic to T cell receptor activation (Simpson *et al.*, 1991; Tary-Lehmann & Saxon, 1992).

The other model, developed by McCune *et al.* (1991) is based on human fetal thymus grafts in the subcapsular space of the kidney of non-conditioned SCID mice. HIV-1 primary isolates were shown to infect grafted lymphocytes and histiocytes (Namikawa *et al.*, 1988; Bonyhadi *et al.*, 1993; Aldrovani *et al.*, 1993). This method is not only very laborious, but the human grafts are also immunologically deficient, as they do not significantly populate the murine tissues (Krowka *et al.*, 1991).

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In search of a more satisfactory rodent model for the study of the pathogenesis of HIV-1 we explored a human-to-mouse acute GvHD model (Huppes *et al.*, 1992). This model is characterized by the development of human lymphocyte CD4/CD8 ratios of $\geqslant 1$ (Huppes *et al.*, 1994) and the generation of donor-type immune function (W. Huppes & G. Hoffmann-Fezer, unpublished results). We speculated that in this model the abundant presence of human $CD4^+$ lymphocytes in the murine lympho-haemopoietic tissues would serve as an efficient substrate for the replication of lymphotropic virus. In addition, the presence of large numbers of activated macrophages would provide an optimal substrate for macrophage-tropic viruses (Hoffmann-Fezer *et al.*, 1992, 1993). Infection of these cells is thought to be important for the persistence of HIV-1 after primary infection (Schuitemaker *et al.*, 1993*a*, *b*). Here we present data on the kinetics of HIV-1 and measles virus (MV) replication in this model and the effects of immunological interventions.

Methods

Production of virus stocks. Virus stocks of the primary HIV-1 isolates HIV-1_{Q17} (Schuitemaker *et al.*, 1993*a*, *b*) and HIV-1_{BAL} (AIDS Research Reference Reagent Program) and the molecular clone HIV-1_{SF2} (Cheng-Majer *et al.*, 1990) were propagated on stimulated human PBL. The stimulation was carried out with phytohaemagglutinin (PHA) and the Epstein–Barr virus transformed B cell lines APD and BSM (VanDeGriend *et al.*, 1984). Stocks of the primary isolates HIV-1_{PR} and HIV-1_{M31} and of the PHA-PBL culture adapted HIV-1_{MN} strain (Popovic *et al.*, 1984) called HIV-1_P were produced in PHA-stimulated PBL. The HIV-1_{PR} strain was obtained from an asymptomatic patient (gift of J. C. C. Borleffs, Academical Hospital Utrecht, the Netherlands). Stocks of the HIV-1 molecular clones HIV-$1_{320.2A.1.2}$ and HIV-$1_{320.3.1}$ (Groenink *et al.*, 1991) were prepared in the CB15 T cell line, within 3 weeks of culture (Nick *et al.*, 1993). The CB15 cell line was infected with the supernatant of transfected HeLa cells. The supernatant obtained after the transfection of HeLa cells with the HIV-$1_{320.2A.2.1}$ molecular clone (Groenink *et al.*, 1991) was used directly. The stocks of the T cell line adapted HIV-1 strains MN, RF and IIIB (Gallo *et al.*, 1984) were produced on H9 cells. Characteristics of the tropism and the ability of the virus strains to form syncytia are given in Table 1. Stocks of the wild-type MV-BIL strain were produced in the Epstein–Barr virus transformed B cell line JP (VanBinnendijk *et al.*, 1990, 1992, 1994).

Conditioning of mice. Inbred BALB/c-SCID/Rij and CBA/N/Rij mice were kept and bred under specified pathogen-free (SPF) conditions in our own breeding colony (Huppes *et al.*, 1992). The CBA/N mice used for experiments were 4 weeks old. The SCID mice were either newborn or 8 weeks old.

Total body irradiation (TBI) was carried out with a ^{137}caesium source, type gamma-Cell 20 (Atomic Energy of Canada, Ottawa, Canada), at a dose rate of 0·87 Gy/min. The conditioning scheme was 9 Gy for the CBA/N mice, with supportive care of 5×10^5 syngeneic bone marrow cells intravenously (Huppes *et al.*, 1992). The newborn and 8-week-old SCID mice received 1 Gy and 3 Gy TBI, respectively.

Transplantation procedure. Human PBL for xenografting were isolated from fresh buffy coat cells of healthy blood donors using lymphocyte separation medium (LSM; Organon Teknica) (Huppes *et al.*, 1992). CBA/N mice grafted intraperitoneally (i.p.) with 2×10^7 human PBL/g (Huppes *et al.*, 1992) were used for survival studies. The remaining experiments were carried out with i.p. PBL grafts of 4×10^6 lymphocytes/g (xeno-GvHD mice) (Huppes *et al.*, 1994). In each experiment four CBA/N mice conditioned with 9 Gy gamma irradiation served as irradiation procedure controls. These mice invariably died at day 11–16 after transplantation. Also, in each experiment, four CBA/N mice conditioned with 9 Gy and intravenously transplanted with 5×10^5 syngeneic bone marrow cells served as transplantation procedure controls. These mice invariably lived throughout the observation period of 30 days.

Immunization procedures. Mice were immunized passively 1 h after the cell graft by i.p. injection with 250 µg antibodies or with 500 µg serum. The monoclonal antibodies were F58H3 to the HIV-1_{IIIB} V3 loop, 257-D to the HIV-1_{MN}-like V3 loop (Gorny *et al.*, 1993) and GP13 to the HIV-1 CD4 binding site (Schutten *et al.*, 1993). F58H3 neutralizes HIV-1_{IIIB} specifically (VanBinnendijk *et al.*, 1990, 1992, 1994; Devash *et al.*, 1990). The mice that served as negative controls were injected with the K14 monoclonal antibody, which has the same isotype as F58H4 and 257-D. This antibody does not recognize the glycoprotein complex of HIV-1, nor HIV-1 epitopes on virus infected cells (Teeuwsen *et al.*, 1990). The antiserum used was the anti-HIV-1 immunoglobulin (HIVIG) (Schuitemaker *et al.*, 1993*a*, *b*). This contains polyclonal specific anti-HIV-1 antibodies (Schuitemaker *et al.*, 1993*a*, *b*; Prince *et al.*, 1988, 1991).

Active immunization studies are described for the MV vaccines because HIV-1 vaccines have not been shown to give effective protection. Xeno-GvHD mice were immunized 1 h and 3 days after the xenograft by i.p. injection with the vaccine. One thousand infectious units (i.u.) of live virus Schwarz vaccine, i.e. the MV-Edmonton B strain (VanBinnendijk *et al.*, 1990, 1992, 1994), or 5 µg of non-live immunostimulating complexes (ISCOM) vaccine, containing the F and H proteins (VanBinnendijk *et al.*, 1990, 1992, 1994) were used. The mice that served as negative controls received empty ISCOMS. After 8 days, ascitic fluid cells of the xeno-GvHD mice were harvested in culture medium containing 100 i.u. of Thromboliquine/ml (heparin; Organon Teknica). The culture medium was RPMI 1640 (Gibco) with 10% decomplemented bovine calf serum (Hyclone). Subsequently, these ascitic fluid cells were serially transplanted at a dose of 4×10^5 cells/g recipient body weight into heavily conditioned CBA/N mice. The mice received a challenge i.p. of 1000 $TCID_{50}$ (50% tissue culture infective dose) of MV-BIL 1 h after all the previous procedures. Again, 3 days later, the infectious centre (IC) was determined.

Detection of virus. The mice were sacrificed and the ascitic fluid cells were collected in culture medium to which 100 i.u. IL-2/ml had been added. Subsequently, IC assays were done to determine the number of these cells needed to demonstrate virus infection. The cells were titrated in 5-fold dilutions from 2×10^6 to 0 cells, using 96-well plates with 2×10^4 CB15 cells (Nick *et al.*, 1993), SUPT1 cells (Aids Research and Reference Reagent Program) or JP cells (VanBinnendijk *et al.*, 1990, 1992, 1994) per well. The IC read-out was done 5–7 days after plating, respectively, either with a p24 HIV-1 ELISA (a kind gift of Organon Teknica) or, when analysing MV infection, by analysis of syncytia using a microscope (VanBinnendijk *et al.*, 1990, 1992, 1994). The IC was determined as the lowest dilution at which virus could still be detected. Flow cytometry of the harvested cells was done after labelling them with the FITC labelled anti-human CD4 antibody (Becton and Dickinson) (Huppes *et al.*, 1994).

Culture and transplantation of CB15 cells. Before transplantation, the human $CD4^+$ cell line CB15, derived from herpesvirus saimiri transformed cord blood (Nick *et al.*, 1993), was propagated in culture medium, with the addition of 100 i.u. IL-2/ml. To obtain mice without

Table 1. *Characterization of 12 HIV-1 stocks*

HIV-1 strain	Passages*	Composition†	Syncytia	T cell tropism	Macrophage Tropism‡	$TCID_{50}$/ml
BAL	< 10	Pr	+/−	+	+	200
Q17	< 10	Pr	+	+	+	1000
PR	< 10	Pr	+	+	+	ND
320.2A.1.2	< 10	Cl	+/−	+	−	40
320.2A.2.1	< 10	Cl	−	+/−	+	40
320.3.1	< 10	Cl	+	+	+	200
SF2	< 10	Cl	+	+	−	600
M31	< 10	Cl	+	+	−	ND
IIIB	< 10	Ol	+	+	−	1000
RF	< 10	Ol	+	+	−	60
MN	< 10	Ol	+	+	−	600
P	< 10	Ol	+	+	−	ND

* Number of *in vitro* passages of the virus.
† Composition of the stock, either as mono- (Cl), oligo- (Ol) or polyclonal (Pr) virus.
‡ Tropism of the virus for histiocytic macrophage cells.
ND, Not determined.

immune capacities the CB15 cell line was transplanted i.p. into heavily conditioned SCID mice at a dose of $1{\cdot}3 \times 10^6$ cells/g (CB15-SCID mice) (Huppes *et al.*, 1994).

Histopathology. Gross necropsy was carried out on mice in the various experiments. Also, at each time point three mice which had received neither human cells nor virus and of three mice that had received human cells but no virus (negative controls) were examined. Tissue samples were fixed in phosphate-buffered 4% formaldehyde and were then paraffin embedded. Tissue slides were stained with haematoxylin (Fluka Chemika) and eosin-yellow (HE) (Merck). HE slides were prepared from alternating tissue sections. The intervening slides were double labelled *in situ* for HIV-1 Gag RNA and human CD4, respectively with *in situ* hybridization and immunohistochemistry (Embretson *et al.*, 1993).

Results

Replication and dissemination of HIV-1 and MV in the xeno-GvHD mice

Primary isolates and molecular clones of lymphotropic, macrophage and amphitropic HIV-1, T cell line adapted $HIV\text{-}1_{IIIB}$ and the wild-type MV-BIL were shown to replicate efficiently in the xeno-GvHD mouse model (Fig. 1). The kinetics of the infectivity patterns were not significantly influenced by the use of different human donors. Between day 5 and day 8 post-infection with the primary HIV-1 isolates a 10–100-fold increase in the percentage of infected cells was observed. The percentages of infected cells after infection with molecular clones of HIV-1 were largely identical to those of the primary isolates at day 5 post-infection. However, infection with these molecular clones gave no significant increase in the virus load after that day (Fig. 1).

Histological analysis revealed that the peritoneum of infected xeno-GvHD mice was invariably heavily infiltrated with lymphoblastic cells which showed strong staining with anti-human CD4 (Table 2). The percentage of these cells also showing an HIV-1 Gag signal largely coincided with the results obtained with the IC test (Fig. 1, Table 2). The ability to form syncytia coincided with the *in vitro* abilities of the virus strains. In contrast to earlier reports (Schuitemaker *et al.*, 1993*b*), small syncytia were observed after infection with $HIV\text{-}1_{BAL}$, both *in vitro* (Table 1) and *in vivo* (Table 2). The peritoneum also contained histiocytic cells with weak staining for human CD4. Although the slides from some of the mice showed few histiocytic cells, it was generally observed that HIV-1 with macrophage tropism had infected most of these cells (Table 2). Interestingly, the lymphotropic molecular HIV-1 clone 320.2A.1.2 did not show hybridization with human macrophages, whereas the macrophage-tropic strains 320.2A.2.1 and 320.3.1 did (Table 2). The mice that were not grafted with human cells (negative controls) showed no human CD4 or Gag signal. The xeno-GvHD mice that were not infected with virus were positive for human CD4, but negative for HIV-1 Gag.

The high level of infection with the primary isolates at day 11 led to the dissemination of virus to various tissues. The peritoneum showed many double positive lymphoblastic cells. Many of these cells were also seen at perivascular sites in the lung, the periportal sinusoids of the liver and in lymph nodes. Few double positive lymphoblastic cells were observed in the thymus. Human $CD4^+$ histiocytic cells were not observed outside the peritoneum.

Virus infection results in delay and abrogation of the GvHD

Consistent with the dissemination of HIV-1, it was shown that HIV-1 infection of the 2×10^7 PBL/g grafts suppressed the GvHD significantly. It appeared that

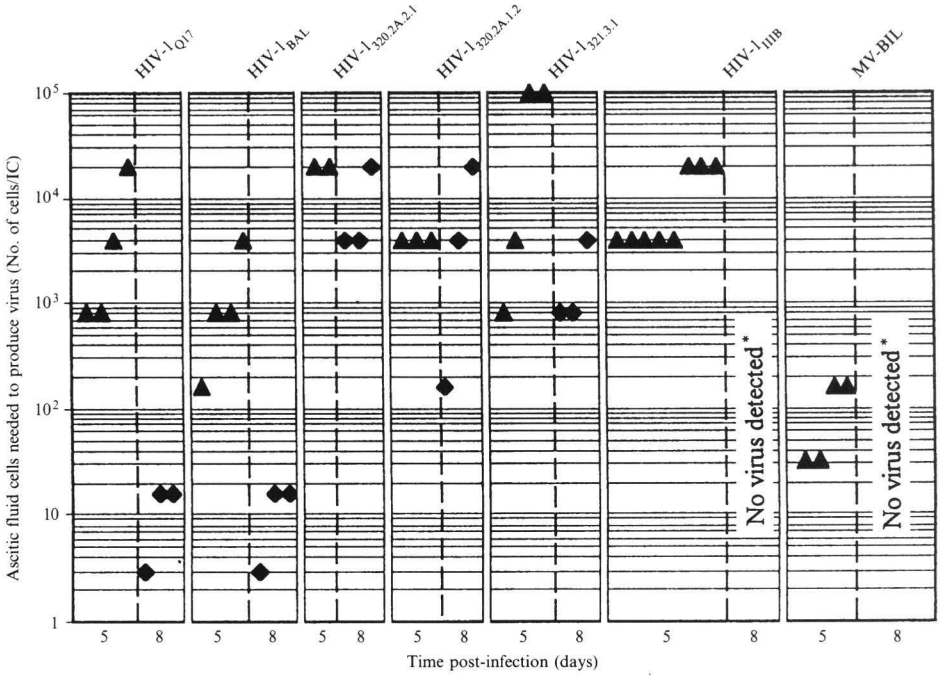

Fig. 1. Virus production with xeno-GvHD mice. Heavily conditioned CBA/N mice (see Methods) received 4×10^6 human PBL and subsequently 10 $TCID_{50}$ of virus. The results show the number of ascitic fluid cells from an individual mouse needed to give an IC in the *in vitro* read-out test. The mice analysed at day 5 post-infection had blood cells from different human blood donors. The same donors were used for the mice analysed at day 8 post-infection. * The 2×10^6 ascitic fluid cells of the four mice analysed gave no IC. The ascitic fluid cells contained > 20% human $CD4^+$ cells by flow cytometry.

Table 2. *Histological analysis of HIV-infected xeno-GvHD mice**

HIV-1 strain	Time post-infection (days)	HIV-Gag$^+$ Hu-CD4$^+$ cells† (%)	HIV-Gag$^+$ Hu-Macrophage cells‡ (%)	Syncytia
320.2A.1.2	6	0·1–1	0	+
	11	0·1–1	0	+
320.2A.2.1	6	0·1–1	100	–
	11	0·1–1	100	–
320.3.1	6	0·1–1	100	+
	11	0·1–1	100	+
BAL	6	0·1–1	100	+
	11	10–100	100	+
Q17	6	0·1–1	100	+
	11	10–100	100	+

* Necropsies of the CBA/N mice were done on day 6 or 11 post-infection with HIV-1. Before the infection these mice had been conditioned heavily and had subsequently been transplanted with 4×10^6 human PBL/g of recipient body weight (see Methods).

† The immunohistochemistry of human CD4 and HIV-1 Gag (p24) RNA was done on alternating slides of the peritoneum. The lymphoblastic cells and their syncytia were high in human $CD4^+$ expression.

‡ The histiocytic macrophage cells were low in human $CD4^+$ expression.

$HIV\text{-}1_{IIIB}$ delayed the GvHD for 1–3 days and abrogated it completely in about 10% of the mice (Fig. 2). Statistical analysis of data presented in Fig. 2 showed that the results are significant (Table 3). A comparable effect on survival after infection with $HIV\text{-}1_P$ and primary isolates $HIV\text{-}1_{Q17}$ and $HIV\text{-}1_{PR}$ was observed (Table 3).

It was also shown that MV (MV-BIL) infection delayed and abrogated the GvHD. Finally, the GvHD was also suppressed by MV-BIL infection of newborn SCID mice transplanted with rhesus monkey PBL (Table 3).

Abrogation and enhancement of HIV-1 infection with HIV-1 antibodies

It was observed that the lymphotropic $HIV\text{-}1_{IIIB}$ strain infected many human PBL by day 5, but at day 8 no infected PBL were observed. This pattern was also observed with MV-BIL infection (Fig. 1). $HIV\text{-}1_{IIIB}$ infection was enhanced by treatment of the xeno-GvHD mice with GP13, the passively transferred monoclonal

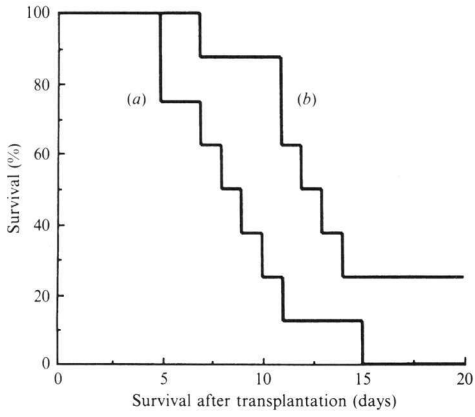

Fig. 2. Suppression of human-to-mouse GvHD with HIV-1_{IIIB}. Heavily conditioned CBA/N mice received 2×10^7 human PBL grafts/g of recipient body weight and were subsequently infected with HIV-1_{IIIB} (see Methods). Survival of these mice was plotted against time. Line (*a*), sham treatment of mice with i.p. injection of 250 µl of culture medium ($n = 8$). Line (*b*), i.p. injection of 10 $TCID_{50}$ of cell-free supernatant of the HIV-1_{IIIB} stock in 250 µl of culture medium ($n = 8$).

Table 3. *Virus infection significantly enhances the survival of mice with human-to-mouse GvHD**

Virus strain	No. of Mice per group	*P* value	Significance†
HIV-1IIIB	8	0·0493	+
HIV-1MN	7	0·1778	–
HIV-1_{P}‡	6	0·0447	+
HIV-1_{RF}	6	0·8719	–
HIV-1_{M31}	5	0·1885	–
HIV-1_{PR}‡	6	0·0209	+
HIV-1_{Q17}	12	0·0048	+
HIV-1_{SF2}¶	23	0·3974	–
MV-BIL	6	0·0048	+
MV-BIL§	6	0·0384	+

* Human PBL were transplanted into heavily conditioned CBA mice at a dose of 2×10^7/g of recipient body weight. Two groups of mice were identified. One individual human donated PBL to one mouse of each group. Subsequently, the mice of one group were injected with 10 $TCID_{50}$ of virus. The other group had sham injections with culture medium. The survival of these mice was plotted against time and was subsequently analysed using the Kaplan-Meier method. Each row represents a different experiment.

† Statistical significance is $P < 0{\cdot}05$.

‡ The P and PR strains were injected as 10^5 PBL of cell-bound virus.

§ In this experiment, instead of 4-week-old CBA/N mice and human PBL, newborn SCID mice and rhesus monkey PBL were used.

¶ HIV-1_{SF2} was dosed at 100 $TCID_{50}$.

antibody to the CD4 binding site (Fig. 3). HIV-1_{IIIB} infection was abrogated completely by the combination of monoclonal antibodies to the V3 loop and the CD4 binding site (Fig. 3). Infection with the primary isolate HIV-1_{Q17} was not prevented by these monoclonal antibodies or the HIVIG.

HIV-1_{SF2} infection is sponteneously abrogated in vivo

While validating the model, a surprising phenomenon was found. The *in vivo* i.p. injection of as many as 100 $TCID_{50}$ of HIV-1_{SF2} did not cause infection of PBL grafts of the xeno-GvHD CBA/N chimeras (Table 4). To distinguish between a failure of virus replication *per se* and immunological control of the infection, we modified our model to one without any immunological response, using heavily conditioned SCID mice with CB15 cell grafts (CB15-SCID mice). Interestingly, *in vivo* infection of these mice with HIV-1_{SF2} could not be established either (Table 4), although replication of HIV-1_{SF2} on the CB15 cell line readily occurs *in vitro*. To investigate whether replication of HIV_{SF2} could occur at all *in vivo*, CB15 cells or PBL were infected *in vitro* before engraftment. It appeared that CB15 grafts supported HIV-1_{SF2} infection for at least 8 days. In contrast, PBL grafts became negative for HIV-1_{SF2} 3 days after transplantation (Table 4). Consistently, no abrogation of the GvHD was observed in a large group of 23 xeno-GvHD mice, with PBL grafts that had been infected *in vitro* with HIV-1_{SF2} (Table 3).

Prevention of MV-BIL infection by pre-exposure to MV vaccines

The effects of pre-exposure of the xeno-GvHD mice to MV vaccines were also studied. Fig. 4 shows that MV-BIL infection of PBL grafts in CBA/N mice was prevented by pre-exposure to the Schwarz live vaccine. The MV ISCOM subunit vaccine appeared to induce partial protection. However, the empty ISCOMS may also have had some protective effect, as the number of infected ascitic fluid cells was one-quarter (one dilution step) less with these empty ISCOMS when compared to control mice without ISCOMS. The challenge MV-BIL was found in large quantities in all these sham immunized mice (Fig. 4).

Discussion

Quantitative analysis showed efficient replication of primary isolates of lymphotropic, macrophage and amphitropic HIV-1 strains and of the MV-BIL strain in the acute xenogeneic GvHD model. Consistent with the fact that human $CD4^+$ lymphocytes are the main initiator of the GvHD (Huppes *et al.*, 1992, 1994), direct cytopathic effects of HIV-1 and MV-BIL are implied by the significant delay and complete abrogation of the GvHD (Fig. 2, Table 3).

The *in situ* hybridization data for HIV-1 RNA indicate that the peritoneum of the acute xeno-GvHD mice contains many HIV-1 infected human T cells and macrophages. As each chimeric mouse was made with

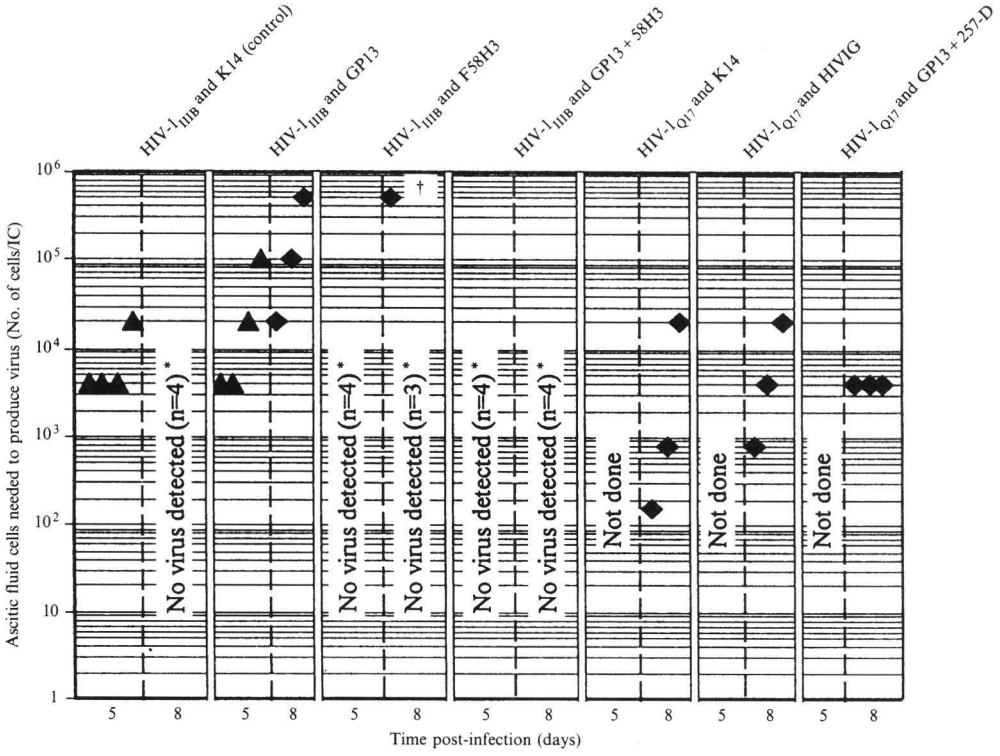

Fig. 3. Passive vaccination for HIV-1 infection in xeno-GvHD mice. Heavily conditioned CBA/N mice (see Methods) received 4×10^6 human PBL/g. The mice analysed at day 5 post-infection had blood from different human blood donors. The same donors were used for the mice analysed at day 8 post-infection. Passive vaccination was with 250 μg of anti-HIV-1 CD4 binding site GP13 and/or anti-HIV-1 V3 loop F58H3 or 257-D monoclonal antibody or with 500 μg of HIVIG. The controls had 250 μg of K14, a non-neutralizing anti-HIV-1 GP41 monoclonal antibody. Subsequently, the xeno-GvHD mice were infected with 10 $TCID_{50}$ of HIV-1. The results represent the number of ascitic fluid cells from an individual mouse needed to give an IC in the *in vitro* read-out test. * The 2×10^6 ascitic fluid cells of the mice analysed gave no IC. The ascitic fluid cells contained > 20% human $CD4^+$ cells by flow cytometry. † The xeno-GvHD mouse produced an IC centre with 5×10^5 ascitic fluid cells.

the PBL of a different human donor, it was remarkable that the number of cells needed to induce an IC after a particular virus infection showed little variation. As it is easy to generate these chimeras, the model provides a convenient tool for the study of host-virus interaction. Interestingly, human macrophages in mice infected with the lymphotropic HIV-$1_{320.2A.1.2}$ did not show staining for HIV-1 RNA, whereas they did show staining in mice infected with the macrophage-tropic strains 320.2A.2.1 and 320.3.1. This, in addition to the highly cytopathic lymphotropic HIV-1 strain IIIB showing 'burst'-like replication, indicates that during the initial infection macrophage-tropic HIV-1 strains play an important role (Fig. 1, Table 2). This is similar to human HIV-1 infection, as it was found that lymphotropic syncytia inducing strains are cleared soon after infection [J. Goudsmit (Academical Centre, Amsterdam), personal communication]. The fact that infection with the primary isolates resulted in a 1–2 log higher number of HIV-1 infected cells as compared to the HIV-1 strains and clones also indicates synergism of infection through polyclonality of the virus in the initial phase of infection (Figs 1 and 3; Table 2).

In contrast to all the other viruses used, it appeared that the highly cytopathogenic full-length molecular clone HIV-1_{SF2} is rapidly eliminated *in vivo* (Table 4). Consistently, HIV-1_{SF2} is highly sensitive to neutralization by complement and antibodies *in vitro* [S. Zolla-Pazner (Neuviëme Colloque de Cents Gards, Marne la Coquette, France) personal communication]. The fact

Table 4. *HIV-1_{SF2} is eliminated in vivo*

Mouse strain*	No. of mice	Human graft	Method of infection	HIV-1 strain	Day of sacrifice†	HIV test result
SCID	20	CB15	*in vivo*	SF2	8	Negative
SCID	5	CB15	*in vivo*	Q17	8	Positive
SCID	6	CB15	*in vitro*	SF2	8	Positive
SCID	3	PBL	*in vitro*	SF2	2	Positive
SCID	3	PBL	*in vitro*	SF2	4	Negative
SCID	8	PBL	*in vitro*	SF2	8	Negative

* The human $CD4^+$ CB15 cell line ($1{\cdot}3 \times 10^6$ cells/g) or the human PBL (4×10^6 cells/g) were transplanted into heavily conditioned SCID mice. Subsequently, the mice were infected *in vivo* with 100 $TCID_{50}$ of HIV-1_{SF2} or with 10 $TCID_{50}$ of HIV-1_{Q17}. The *in vitro* infection was by co-culture of the graft with the virus for 1 h with 100 $TCID_{50}$ of HIV-1_{SF2}.

† At the indicated time after cell transfer, the mice were sacrificed and 5×10^6 ascitic fluid cells were cultured with the same number of CB15 cells. One week later the Gag (p24) HIV-1 ELISA test was performed.

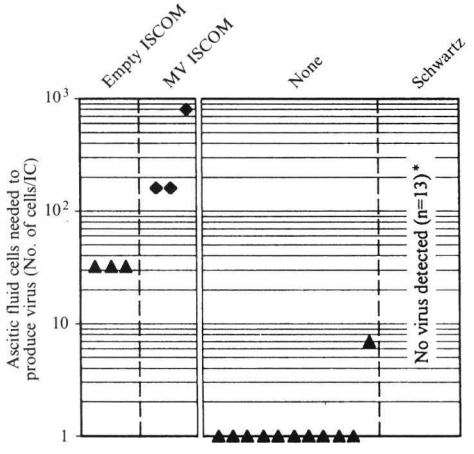

Fig. 4. Vaccination against MV infection. Heavily conditioned CBA/N mice received 4×10^6 human PBL/g and were subsequently immunized with either 5 μg of subunit vaccine (MV ISCOM) or with 1000 i.u. of live virus vaccine (Schwartz; see Methods). After 8 days the ascitic fluid cells were transferred into heavily conditioned CBA/N mice at a dose of 4×10^5 cells/g. Subsequently, these mice were challenged with 1000 $TCID_{50}$ of MV-BIL. Three days later an IC test was done to determine the number of ascitic fluid cells needed to give an IC in the *in vitro* read-out test. * The 2×10^6 ascitic fluid cells analysed gave no IC. The ascitic fluid cells contained > 20% human $CD4^+$ cells by flow cytometry.

that elimination also occurred after *in vivo* infection with HIV-1_{SF2} of heavily conditioned SCID mice grafted with the CB15 T cell line (Table 4), shows that this effect is probably caused by a plasma factor. Elimination by plasma is consistent with the fact that complement has anti-HIV properties (Spear *et al.*, 1991). These data do not support the use of HIV-1_{SF2} to test vaccine efficacy (Ada, 1993). In contrast, after *in vitro* infection of the CB15 cell line with HIV-1_{SF2} prior to transplantation, these CB15-SCID chimeric mice showed persistent HIV-1 infection. Interestingly, human PBL grafted SCID mice eliminated the HIV-1_{SF2} infection under these conditions (Table 4). This shows that human PBL grafts are able to participate in the elimination of HIV-1_{SF2}. Together, this indicates that HIV-1_{SF2} may be useful for intervention strategies. In addition, the reproducible replication of the HIV-1_{SF2} strain in these CB15-SCID mice indicates that these chimeras are a valuable extension of the model.

In agreement with the findings using human PBL SCID mice (Mosier *et al.*, 1988; Vittecoq *et al.*, 1992; Safrit *et al.*, 1993), our results with xeno-GvHD mice demonstrate the feasibility of passive immunization studies with lymphotropic HIV-1 strains (Fig. 3). However, it can be speculated that enhancement through Fc-mediated macrophage infection contributes to prolonging the 'burst'-like *in vivo* replication of the lymphotropic HIV-1_{IIIB} strain by *in vitro* neutralizing concentrations of the HIV-1 CD4 binding site specific antibody GP13 (Fig. 3). Furthermore, neither the combination of V3 loop specific and CD4 binding site specific human monoclonal antibodies nor the HIVIG prevented infection by the primary isolate HIV-1_{Q17} (Fig. 3). This is consistent with studies of patients, where HIVIG did not clear HIV either (Prince *et al.*, 1991). However, some beneficial effect cannot be excluded, as in our murine model and in patients these antibodies appear to reduce the number of infected cells significantly (Figs 1 and 3; Prince *et al.*, 1991). It is concluded that the study of passive immunization for post-exposure treatment should include *in vivo* infection with primary isolates.

With the human PBL SCID mouse, secondary antibody recall responses of recently vaccinated PBL donors were detected (Mosier *et al.*, 1993*b*). However, aspecific antibody responses and naturally occurring red blood cell xeno-reactive antibodies could not be excluded (Hesselton *et al.*, 1993; Williams *et al.*, 1992; Nonoyama *et al.*, 1993). In contrast, we recently found, using distantly located allogeneic skin grafts, that induction of cellular immunity in the xeno-GvHD mice was indeed a primary cellular immune response (W. Huppes & G. Hoffmann-Fezer, unpublished results). In this paper, active protection by immunization with live attenuated MV vaccine has been demonstrated. Partial protection may have been conferred by immunization with subunit MV vaccine (Fig. 4). The complete protection conferred by the live attenuated MV vaccine is not likely to be caused by natural killer cells, as these cells could not be found in the model (Huppes *et al.*, 1994). Neither did it appear to be caused by anti-MV antibodies, as none of the sera from the mice were positive using an MV ELISA (VanBinnendijk *et al.*, 1990, 1992, 1994; results not

published). Protection by plasma factors like gamma-interferon, is not likely either, because empty ISCOMS did not significantly protect against MV infection. As the protection was obtained by transfer of a cell fraction and within 2 weeks of the transplantation procedure (Fig. 4), it is ascribed to T cell immune mechanisms.

In contrast to earlier SCID mouse models (Mosier *et al.*, 1991; McCune *et al.*, 1991), the technologically simple and economic xeno-GvHD murine model allowed replication of HIV-1 and MV. Modification of the model to one without any immunological capability (CB15-SCID mice) allowed virus replication and immunological interaction to be differentiated. The model also enabled the study of cytopathological effects of virus on T cells and macrophages of the human immune system. In addition, these xeno-GvHD chimeras enabled both passive and active immunization studies.

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Corrigendum

Efficient replication of human immunodeficiency virus type 1 and measles virus in a human-to-mouse graft versus host disease model permits immunization research

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It has emerged that there are several inaccuracies in the above paper. These are corrected below.

Summary, line 16: The word 'delay', should be added after 'led to a'.

Methods, page 2708, line 31: HIV-1_{PR} was obtained from a *symptomatic* not *a*symptomatic patient.

Methods, page 2708, line 72: the reference to F58H3 should be 'Åkerblom *et al.*, 1990'. [Åkerblom, L., Hikula, J., Broliden, P., Mäkitalo, B., Fridberger, T., Rosen, J., Villacres-Eriksson, M., Morein, B. & Wahren, B. (1990). Neutralizing cross-reactive and non-neutralizing monoclonal antibodies to HIV-1 gp120. *AIDS* **4**, 953–960.]

Methods, page 2709, line 12: *in situ* hybridization was done with a probe that spanned about 90% of the HIV-1 genome.

Table 1: HIV-1_{M31} should be 'Pr', not 'C1' and is positive for macrophage tropism. The T cell-adapted HIV-1 strains, MN, RF and IIIB, had been passaged more than 10×. The ACH codes for the primary isolates of HIV-1 (Q17, BAL and M31) are ACH 168.10, ACH 172.Ba-L and ACH 105.31 respectively. Owing to a misunderstanding, immuno-histochemistry with CD4 was reported. However, immuno-histochemistry with CD4 had not been performed. HIV-1 RNA^+ cells were categorized on the basis of morphology only. Consequently, in Results the phrases lymphocyte-*like* and macrophage-*like* cells should be substituted for lymphocytes and macrophages, respectively.

In retrospect, Georg Kraal and Klara Tenner-Racz have withdrawn co-authorship since they feel their contributions to the work were insufficient. Instead Victor Smit (Trojan Horms', Vinkenlaan 29, 2623 GG Delft, The Netherlands) is included as second author. The paper should now be cited as:

Efficient replication of human immunodeficiency virus type 1 and measles virus in a human-to-mouse graft versus host disease model permits immunization research

Wim Huppes and Victor Smit

Journal of General Virology (1995), **76**, 2707–2715

Chapter 8

General Conclusion

General conclusion

Hypothesis

In this dissertation support for two working hypotheses was found:

1. Cellular xenografts need to circumvent the natural antibody barrier.
2. Donor-type cytokines create immune function of discordant xenografts.

Circumvention of natural antibody cellular xenograft barrier

First of all it has been shown that successful transplantation of human peripheral blood leukocytes (PBL) strongly relates to absence or low levels of natural antibodies. Matched normal non-immune deficient mice and CBA/N mice with X-linked immuno-deficiency (XID) appeared to have low levels of natural antibodies that react with human peripheral blood grafts. Subsequently, it was demonstrated that these mice could be used for cellular xenografts instead of Severe Combined Immuno-deficient (SCID) mice[1] (Chapter 2).

This successful xenografting to the CBA/N mouse is due to the delayed B lymphocyte differentiation of this strain, leading to late development of immuno-globulin M production[2,3,4]. Experimentally, these mice have a deficient antibody response towards pneumococcol polysaccharide type III[5]. However, this immune deficiency is minor and opportunistic infection was not shown. The generally accepted view is that mice of the CBA/N strain can be kept and bred in a colony just like any other non-immune deficient rodent, without having to invest in micro-biological isolation to prevent opportunistic infection. In that way expensive rodent facilities are not needed. This would not only allow many more laboratories to use mice for this kind of medical research, but would also allow larger scale experiments in an economical way.

Secondly, successful use of the normal non-immune deficient BCBA (F1 of Black and CBA) mouse (Chapter 3) was demonstrated. This is interesting, because F1 strains combine constitutional strength of outbreeding with the identical genetic constitution of the inbred parents. By matching human PBL and murine sera, it was found that the anti-human ABO (H) blood group antigens of erythrocytes, granulocytes, lymphocytes, monocytes and $CD34^+$ hemopoietic stem cells were not opsonized by some of the mouse sera. Subsequently, it was shown for human PBL that this was determining a take, for instance in these BCBA mice.

Thirdly, it was demonstrated that normal non-immune deficient BCBA mice could be constructed that were reconstituted devoid of natural antibodies. This was done by thymectomy and subsequent allogeneic transplantation with SCID bone marrow (Chapter 4). Since then, the group of Reisner has shown that instead of thymectomy and single high level dose of total body irradiation (TBI), fractionated high level dose can be used to condition Balb/c mice for xeno-transplantation[6]. The interval between TBI and xeno-transplantation the group of Reisner used was one day. At that day the mice were also reconstituted with SCID bone marrow cells. We did not get takes after one day with single high level dose TBI in BCBA mice using non-matched PBL (Chapter 3). We conjectured that the half life of polyreactive IgM of about half a day[7] would not allow consistent takes. It needs to be determined whether other groups can repeat the results of the group of Reisner.

The finding that natural antibodies are the major barrier in discordant xeno-transplantation (Chapter 2) initiated research into the further application of this knowledge. It was investigated whether the natural antibody barrier for the *cellular* PBL xeno-graft could be circumvented. It was shown that such could be done by conditioning the recipient non-immune deficient mice with Cobra Venom Factor (COF) and liposomes containing dichloromethylene diphosphonate (Cl_2MDP) (Chapter 3). If the further follow-up of this invention with the use of discordant xenogeneic *solid tissue grafts* would also proof successful, this concept would be even more interesting, because it could contribute to solving the problem of shortage of donor tissues for clinical application.

Advantages and disadvantages of the new model

The major disadvantage of the presented model using mice that are heavily conditioned before transplantation is that experiments that would require observation periods longer then two weeks would necessitate serial transplantation of the graft (Chapter 7). Serial transplantation is necessary, because acute graft versus host disease (GvHD) is a lethal disease at the end of the second week. Obviously, it would be better if the model would allow for longer observation periods without the need of serial transplantation.

In chapter 2 incremental conditioning regimens with high dose total body irradiation (TBI) of CBA/N mice showed that a more chronic form of GvHD could not be induced. However, chronic GvHD could be induced in recipient mice with the SCID mutation[1]. Hoffmann-Fezer et al.[8,9], and others[10] have shown that chronic xenogeneic GvHD up to three months after transplantation could be achieved in about 40-60% of the recipient SCID mice after the transplantation of 10^8 human PBL. Interestingly, clear evidence of HIV-1 infection in this type of chimera was also in the range of 40-75%[11,12,13,14,15,16,17]. (Occasionally, small groups of mice have shown a 100% rate of infection[14].)

In this thesis it is shown that transplantation of 10^9 human PBL (Chapter 5) increased the relative number of SCID mice with chronic GvHD. However, this method appears not to be practical, because the result was also that the spread of the

onset of lethal disease became much larger (17-77 days). In addition, it was shown that chronic GvHD in SCID mice lead to just the selective proliferation of xenoreactive clones. This favors cytotoxic $CD8^+$ lymphocytes more than $CD4^+$ lymphocytes and B lymphocytes[8,9,10]. Consequently, this would not be practical for the long term study of human immune function or of the human immuno-deficiency virus in these SCID mice[18].

However, recently it was shown that SCID mice that also have been crossed with non-obese diabetic (NOD) mice are more ideal recipients of human leukocyte grafts than SCID mice[19,20]. NOD mice spontaneously develop auto-immune insulin-dependent diabetes mellitus[21]. Development of diabetes is T cell dependent, and NOD-SCID mice fail to develop diabetes[21]. In addition to absence of B and T lymphocytes, these mice exhibit defective macrophage function, NK cell activity, and hemolytic complement activity[22]. The disadvantage of this strain is, however, that NOD-SCID mice spontaneously developed lethal thymomas after the age of three months (up to the age of 6 months)[19,23].

It was also shown that $2x10^7$ human PBL show increased engraftment and that the graft induced chronic GvHD in *all* recipient NOD-SCID mice (Personal communication D.L. Greiner)[22]. However, the $CD4^+/CD8^+$ ratio in these chimeras is 0,5 or less. This is lower than in man and the acute xeno-GvHD model presented in this thesis. Both man and the model show ratios of mostly above 1. This level of $CD4^+$ lymphocytes of 0,5 or less in these PBL transplanted NOD-SCID mice seems sufficient for the study of AIDS, as these chimeras were proven to be highly susceptibility to HIV-1 infection[19]. Immune function of the graft was demonstrated by the fact that they caused clearing of human lymphatic filariasis[23]. Consequently, it appears that NOD-SCID mice may be much better recipients of human grafts for observation periods up to three months than SCID mice or heavily conditioned mice undergoing serial transplantations. Future research could be directed at finding middle ground between the high dose TBI conditioned CBA/N mice with $CD4^+/CD8^+$ ratios of the human PBL graft above 1 and non-conditioned NOD-SCID mice with $CD4^+/CD8^+$ ratios of the human PBL graft of 0,5 or less. Possibly this could be done using the NOD-SCID strain in a matrix experiment with the human PBL dose on the X-axis and the conditioning dose on the Y-axis. Subsequently solutions should be sought for the principal limitations of the presented xenogeneic human to mouse transplantation system, i.e., the uncomplete human immune system developed in the recipient mice and the incompatibility of many costimulatory mechanisms between murine and human cells.

At first (Chapter 1-4) another disadvantage of the presented acute xenogeneic GvHD model was the requirement to handle large volumes of blood. When fewer discordant xenogeneic T cells were used, peripheral tolerance appeared to result in a lack of GvHD. Such has also been demonstrated in Mosier's model, where 10^7 human PBL are transplanted into non-conditioned SCID mice[28,29]. Supposedly, this peripheral tolerance occurred via primary immunization, secondary litigation of the human CD28 receptor would be excluded. Proteins like murine B7 and LFA-3 are species specific and therefore unable to deliver costimulary signals of antigen presenting cells. It is generally accepted that a secondary signal is necessary to induce full interleukin-2 secretion, allowing efficient T cell proliferation[24]. As high affinity Il-2 receptors are

expressed after stimulation of T cells, exogenous human Il-2 might be able to induce proliferation of grafted human lymphocytes within the murine environment.

In contrast to pre-activation *in vitro* prior to transplantation, daily injections of human Il-2 *in vivo* into chimeric mice indeed allowed a 10-fold reduction of the cell number to inflict GvHD, from 4×10^7 to 4×10^6 human PBL per gram body weight of recipient (Chapter 5). This solved the problem of having to handle large volumes of human blood. Without Il-2 half a liter of human blood was sufficient for the production of just 1-4 chimeras, whereas with daily injections of recipients with human Il-2 this number went up to 10-40 chimeras. A similar conclusion was recently drawn by Gritsch et al., who found that species specific stromal factors could produce growth of discordant xenogeneic blood cells[25].

The major advantage of the presented human to mouse xenograft model, compared to the models of the group of McCune[26,27] and Mosier[28,29,30,31,32], is the presence of donor type immune-function. In addition, it appears that the graft is easy to analyze because of abundance of donor type (human) immune-cells. In chapter 2, 5, 6 and 7 it is shown that *xenogeneic* GvHD generated large infiltrates of apparently functionally active donor type cells in the murine lymphohemopoietic tissues and generated lymphoblastic vasculitis. In these chapters it is also demonstrated that the human helper lymphocyte is the major contributor to the acute discordant xenogeneic GvHD. It was shown that these cells, but also the $CD8^+$ cells were activated and responsive, being HLA-DR^+ and $CD25^+$. In addition to engraftment of human lymphocytes, many human $CD11^+$ histiocytic cells were observed, which were intermingled with human $CD3^+$ lymphoblastic cells (Chapter 6). Functional activity of human PBL grafts was also indicated by their rejection of distant allogeneic skin grafts (Chapter 6). These human skin grafts were at the back of the mice, a distant site from the intraperitoneal human PBL graft.

Modification of the xeno-GvHD model to one without any immunological capability, using Herpesvirus saimiri C488 immortalized cell clones, allowed pinpointing of immunological interaction of the human graft from other processes (Chapter 5 and 7). After transformation by Herpesvirus saimiri, human T cell lines show the phenotype of mature activated lymphocytes[33]. Immortalized cell clones grow in the presence of Il-2, but without need for antigen or mitogen stimulation. Many features of the parental cultures are retained after transformation. The karyotypes were normal[34]. Biochemical signal transduction patterns and immunological functions like antigen specificity were found identical to untransformed counterparts[35,36,37]

The cell line CB 15 has been analyzed in detail[33,35,36,37]. In chapter 5, Herpesvirus transformed human $CD4^+$ CB 15 cells were shown to have nearly identical behavior of PBL grafts when tested for induction GvH-like disease in heavily conditioned mice in presence of exogenous Il-2. It appeared that the chimeras suffered from generalized human lymphoblastic vasculitis, just like after human PBL transplantation. The adherence of the human lymphoblasts to the murine endothelium cannot be explained by the binding of murine endothelial molecules CD54, CD11a/CD18 or CD2, which are species specific and not active on human T cells. Instead, the species unrestricted cytokines TNFα, Il-1 and Il-8, which are known to induce the adluminal procoagulant state[38,39], might cause adherence of the human cells.

This is consistent with the finding of Hoffman-Fezer et al. that the engraftment of human PBL in non-conditioned SCID mice induced severe generalized human CD8+ vasculitis[8,9]. The present understanding of the etiology of generalized vasculitis, like Wegener's disease, is incomplete[40]; $HgCl_2$-induced necrotizing vasculitis is the only available model, which is too complex to dissect individual etiologically factors[41]. Therefore we propose that our model could be useful for vasculitis research.

Another advantage of the acute xenogeneic GvHD model is that it appears suitable for virology. In chapter 7 it is demonstrated that both the human PBL graft and the Herpesvirus derived CB 15 human cell line graft allow replication of human virus. In this chapter it is also shown that the model allows vaccination studies, with the successful prevention of measles virus infection with the Schwartz vaccine. Partial protection may have been conferred by immunization with sub-unit measles virus vaccine. As the protection was obtained by transfer of a cell fraction and within two weeks after the transplantation procedure, it is ascribed to human T cell immune function.

In addition and in agreement with the findings with the model of the group of Mosier[42,43], our results demonstrate the feasibility of passive immunization studies (Chapter 7). However, the presented model has the advantage of achieving HIV infection in all recipients, and not just in 40-75% of the recipients[11,12,13,14,15,16,17]. It is shown that *in vitro* neutralizing antibodies caused enhancement of HIV-1 infection in the chimeras. This suggests that HIV-1 specific antibodies may play an important role in the persistence of HIV-1 infection and long term *in vivo* elimination of HIV-1 specific antibodies could counteract HIV-1 infection.

Furthermore, neither the combination of *in vitro neutralizing* V3 loop specific and CD4 binding site specific human monoclonal antibodies nor of *in vitro neutralizing* polyclonal hyper-immune globulin (HIVIG[44]) prevented infection by the primary isolate HIV-1Q[17] (Chapter 7). It is therefore concluded that the study of passive immunization should include *in vivo* infection with primary isolates, because *in vitro* results of passive immunization appear to be non-predictive for the *in vivo* outcome.

While testing the many HIV-1 pseudo-strains in the xeno-GvHD model, the surprising phenomenon of the elimination of HIV-1_{SF2} by murine plasma and naive human PBL was found. This suggests that HIV-1_{SF2} may be useful for intervention strategies (Chapter 7). In this chapter it is also shown that HIV-1_{SF2} was eliminated by murine plasma and naive human PBL, but was propagated in a human leukemia cell-line. This suggests that HIV-1_{SF2} may be a safe product for HIV-1 vaccination and for leukemia therapy.

It can be concluded that the xeno-GvHD mouse is a convenient model for research of human virus. Inherent to increasing high density of humans on global scale, viral threats like the human immuno-deficiency virus type 1 and 2 will make the presented models ever more important[45], because viral pandemics can cause extinction of species, as has been shown for algae[46]. Therefore, it will be exciting to observe the further growth of this research area in the next decade.

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Summary

The battle against AIDS and cancer is hampered by lack of a model that allows their integrated study with the pathophysiology of the human immune-system. The presented model that is characterized by human to mouse graft versus host disease is proposed as a solution to this problem.

In chapter 2 it is demonstrated that the major problem to transplantation of human cells into mice is the natural antibody barrier. These antibodies exist naturally in animals and lyse grafted discordant cells. They were found to be absent or at a low level in newborn mice, and in mice with hereditary severe combined (SCID) and X-linked (XID) immuno-deficiency.

In chapter 3 it is shown that human blood cells and murine sera could be matched and that matches were predictive for successful engraftment. It was found that some adult mice, e.g., BCBA mice, had absence or low titer of these natural antibodies against the human blood group types O (H) and A.

In chapter 4 it is shown that normal adult non-immune deficient mice can be constructed into laboratory animals that have absence of natural antibodies. The described method is thymectomy and transplantation with SCID mouse bone marrow.

In addition, it is shown in these chapters 2-4 that large numbers of primate peripheral blood leukocyte grafts cause graft versus host disease (GvHD) in severely conditioned recipient mice lacking natural antibodies. This graft versus host disease is histologically characterised by large donor type infiltrates in the murine lympho-hemopoietic tissues and by vasculitis (Chapter 2, 5 and 6). It is shown that the donor type $CD4^+$ T cells are the principal causative cell type of the disease.

In chapter 6 the *in vivo* human immune-system was shown to be functional; it was demonstrated that human peripheral blood leukocyte grafts rejected distantly located non-matched third party skin grafts. This is the first time that a functional *in vivo* human immune-system was described. In addition it shows that the model has the option of combined organ grafts.

Subsequently, the model was also applied to virology. In chapter 7 it is shown that human virus replicated and had immunological interactions with the engrafted human immune system. It is shown that live measles vaccination prevented subsequent measles infection. In this chapter it is also shown that HIV-1_{SF2} was eliminated by murine plasma and naive human PBL, but was propagated in a human leukemia cell-line. This suggests that HIV-1_{SF2} may be a safe product for HIV-1 vaccination and for leukemia therapy.

Samenvatting

De strijd tegen AIDS en kanker wordt vertraagd door het ontbreken van een model dat de pathofysiologie van het menselijke immuun-systeem integreert. De voorgestelde oplossing is het in dit proefschrift beschreven muizen-model, dat is gebaseerd op transplantatie van menselijke bloedcellen op zo'n manier dat zij een afstotingsreactie tegen de muis vertonen.

In hoofdstuk 2 wordt beschreven dat er van nature in plasma eiwitten (antilichamen) voorkomen die een belangrijke barrière vormen voor getransplanteerde cellen van dieren van andere families. Deze antilichamen kunnen die cellen uiteen laten vallen (lyseren). Het bleek dat deze antilichamen niet of in zeer geringe mate aanwezig zijn bij pas geboren muizen. Tevens bleken ze niet aanwezig bij muizen met bepaalde aangeboren afwijkingen van het immuun-systeem, zoals de X-gebonden immunodeficientie (XID) en de ernstige gecombineerde immuno-deficientie (SCID).

In hoofdstuk 3 wordt beschreven dat het mogelijk bleek combinaties van mens en muis te selecteren die transplantatie van mens naar muis toelieten. Die selectie werd gedaan door muizen-sera te kruisen met humane bloedcellen. Muizen van het type BCBA bleken menselijke cellen van de bloedgroepen O (H) en A niet of slechts in geringe mate te lyseren en succesvolle transplantaties toe te laten.

In hoofdstuk 4 wordt beschreven dat van volwassen non-immuun-deficiente muizen proefdieren gemaakt kunnen worden, die geen natuurlijke antilichamen bevatten. De beschreven methode is het weghalen van de zwezerik en het doen van een transplantatie met SCID beenmerg.

In hoofstuk 2-4 wordt ook beschreven dat bij afwezigheid van deze natuurlijke antilichamen en de voor transplantatie gebruikelijke conditionering van de muizen, primaten witte bloedcellen getransplanteerd kunnen worden in die muizen. Na de transplantatie van grote aantallen van deze primaten bloedcellen treedt er een afstotingsreactie op tegen de muis, die karakteristiek is voor de methode. Zij bestaat uit een ontsteking met veel donor type cellen, waardoor het niet moeilijk is die cellen uit de muis te nemen voor verder onderzoek.

In hoofdstuk 5 wordt beschreven dat van de menselijke afweer, met name de zogenaamde helper T cellen, de omstandigheden in de muis bepalen. Tevens wordt er beschreven dat 10 maal zo weinig donor type cellen de afstotingsreactie ook kunnen geven als de muizen met het donor type groeifactor interleukine-2 worden behandeld.

In hoofdstuk 6 wordt beschreven dat zodoende de getransplanteerde menselijke afweercellen uit één muis kunnen worden doorgespoten in meerdere volgende muizen. Bovendien wordt daar beschreven dat de transplantatie van de menselijke afweercellen gecombineerd kan worden met een orgaan-inplantatie. Dit alles laat functioneel onderzoek van het model toe. Zo wordt er aangetoond dat de bloed-cellen van de éne mens de in de muis op een afstand gelegen huid van een ander mens kunnen afstoten. Dit toont aan dat het menselijke afweer-systeem functioneel is in de muizen.

In hoofdstuk 7 wordt tevens aangetoond dat het model de groei van menselijke virussen toelaat en dat het getransplanteerde immuun-systeem die virussen vervolgens aanpakt. Zo wordt er aangetoond dat het model voor vaccinatie studies gebruikt kan worden. Het blijkt dat de vaccinatie van de chimere muizen met het Schwartz mazelen vaccin een latere mazelen infectie kan voorkomen.

Er werd ook aangetoond dat er AIDS veroorzakende virussen zijn die niet persisteren in het model. Mogelijkerwijze zijn dit de AIDS vaccins van de toekomst. Het bleek namelijk dat HIV-1_{SF2} geëlimineerd werd door muizen plasma en naïve menselijke bloedcellen. Een interessante bevinding hierbij was bovendien dat het HIV-1_{SF2} wel menselijke leukemie cellen infecteerde. Dit suggereert dat HIV-1_{SF2} wellicht ook gebruikt kan worden voor de behandeling van leukemie.

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Curriculum Vitae

Willem Huppes was born in Oranjestad, Aruba, The Netherlands Antilles, on the 25th of August 1954. In 1974 he finished secondary school (Atheneum B, with economy as an extra subject) at the Krimpenerwaard Lyceum Krimpen a/d Ijssel, The Netherlands. In 1975 he finished military service and started to study medicine at the University of Utrecht. In the years 1976-77 he teached histology. In the summer of 1977 he did clinical research of stomach ulcers at the St. Laurentius Hospital Roermond. In the years 1977-78 he was member of a commission to improve the education in pathophysiology at the University Hospital Utrecht. In 1978 he was a nurse at the mental hospital "Zon en Schild" Amersfoort. In 1978 he started to study philosophy at the University of Utrecht. In 1980 he teached epistemology at the faculty of Social Sciences. After passing Medical Finals in 1982 he started specialisation in internal medicine at the Harbour Hospital in Rotterdam under supervision of prof.dr. P.C. Stuiver. It included general medicine, tropical medicine and pneumonology. In 1983 he obtained the M. Phil. degree in philosophy. In 1984 he teached nursing at the "Zuster Engelbert Stichting" Rotterdam. From 1985 to 1988 he continued his medical specialisation at the University Hospital Utrecht, under supervision of prof.dr. A. Struyvenberg. The subjects were haematology, general medicine, immunolgy and rheumatology, nephrology, oncology, intensive care medicine, cardiology and endocrinology. The education in haematology included laboratory work into melanoma tumour immunology, under supervision of prof.dr. G.C. de Gast. In 1985 he studied the formation of oxygen radical in rats under supervision of dr. B.A. Asbeck. From 1986-87 he studied the relation between the clinical aspects and the laboratory CD-classification of non-Hodgkin lymphomas, under supervision of dr. H.J. Schuurman. In 1988 he studied the immunolgy of hapten exclusion in the rat, under supervision of dr. H.J. Schuurman and prof.dr. A. Berrens. In 1987 he was certified for animal experiments. In 1988 he was certified for handling radionuclids. In that year he also became a specialist in internal medicine. From 1988-91 he worked at the Institute for Applied Radiobiology and Immunology (TNO), Rijswijk, on brain transplantation in mice with lysosomal storage disease (head: dr. G. Wagemaker). From 1990-97 he is a member of SOWEG, an advice company for employee councils. From 1991 to 1994 he worked at the TNO Institute and at the National Institute of Public Health and Environmental Protection (RIVM) on the present dissertation. From 1993 to 1994 he worked with dr. J.C.C. Borleffs of the University Hospital Utrecht on the project "xenografts for AIDS". From 1993 he worked with drs. ing. V. Smit, Pharma Key Delft, on a fast and easy method for structure-function analysis of biologically important amino-acids of proteins. In December 1996 he passed with credit as "Master of Business Administration" at the European University the Hague. From then until October 1997 he was commissioner of the Health Maintenance Organisation Rinos Health Care Amsterdam. In 1997 he also was senior consultant at Bakkenist Management Consultants Amsterdam. From December 1997 until May 1998 he is senior consultant at the Plexus Medical Group Amsterdam. Since than he is executive officer of Hera Health Consultants that advises on medical data management in collaboration with NCR and Microstrategy.