HUMAN ANTIBODY TECHNOLOGY

An evaluation of the SCID-hu-PBL model

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Transplantation of human lymphoid cells into mice suffering from severe combined immunodeficiency (SCID mice) results in spontaneous production of human antibodies. We have investigated the ability of SCID-hu-PBL mice to induce antigen-specific primary and secondary human immune responses against various antigens, for the purpose of generating human monoclonal antibodies of desired specificities. SCID mice were transplanted with peripheral blood lymphocytes from tetanus toxoid (TT) immune donors, thus recruiting specific T cell help from TT specific T cells by physically coupling the B cell antigen with TT. In order to demonstrate that T cell help were retrieved from antigen-specific T cells, a TT specific T helper cell clone was established. Only mice transplanted with splenocytes and the autologous T cell clone were able elicit human antigen-specific responses. Improvement of T cell help was obtained by *in vitro* priming human B cells with a superantigen (SEA) before transplantation with autologous T cells. Human IgM and IgG responses were obtained against the hapten dinitrophenyl (DNP), ovine submaxillary mucin (OSM), the melanoma-asscociated gangliosides GM2 and GD2 and a V3 loop peptide from gp120 (HIV-1). Gangliosides were introduced to SCID-hu-PBL as a liposome construct with TT encapsulated into the liposomes and very high human IgG serotiters were obtained against the normally weak immunogenic GD2 ganglioside. Further, terminal differentiation of antigen-specific human B cells was indicated, as well as retrievingthe generated specific B cells, thus limits the use of the model with respect to generate human monoclonal antibodies. However, the ability of the SCID-hu-PBL model to elicit human immune responses emphasize the use of SCID-hu-PBL as a vaccine model. Furthermore the results suggest a potential value of the liposome construct as a melanoma vaccine in clinical trials of melanoma patients.
In order to obtain longterm culture of antibody producing B cell lines, Epstein-Barr virus is a valuable tool to immortalize the generated B cell specificity. However, EBV transformed B cells are often unstable in longterm cultures and difficult to clone. We proved the ability of the B cell growthfactor thioredoxin, secreted by the T cell hybridoma MP6, to increase the cloning efficacy EBV infected B cells.

Key words: SCID-hu-PBL mice, human antibodies, primary/secondary responses, gangliosides GM2 and GD2, liposomes, EBV, MP6.

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1. ABBREVIATIONS

A-MuLV	Abelson Murine Leukemia Virus
APC	Antigen Presenting Cell
CD	Cluster of differentiation
DNP	Dinitrophenol
EBV	Epstein-Barr Virus
GD2, GM2	Ganglioside GD2, GM2
GvHD	Graft Versus Host Disease
HIV	Human Immunodeficiency Virus
Ig	Immunoglobulin
IgH	Ig Heavy chain
KLH	Keyhole Limpet Hemocyanin
LCL	Lymphoblastoid Cell Line
LeuLeuOMe	L-leucyl-L-leucine methyl ester
mAb	monoclonal antibody
MHC	Major Histocompatibility Complex
NK cell	Natural Killer cell
OSM	Ovine Submaxillary Mucin
PBL	Peripheral Blood Lymphocytes
PCR	Polymerase Chain Reaction
RAG-1,-2	Recombinase Activating Gene-1,-2
SCID	Severe Combined ImmunoDeficiency
SCID.BG	SCID mice with the *beige* mutation
SCID-hu-PBL	SCID mice repopulated with human PBL
SCID(Ig^+)	Leaky SCID mice
SEA	Staphylococcal Enterotoxin A
TCR	T Cell Receptor
Trx	Thioredoxin
TT	Tetanus Toxoid
V_H	Ig variable heavy chain

2. INTRODUCTION

Antibodies are generated when B lymphocytes become stimulated with a particle (antigen), which the organism recognize as foreign, and constitue a very important part of the mammalian immune response. Once the body has been exposed to a foreign particle the immune system will be on the alert and protect against future exposition. The existence of protective immunity has been known for centuries. The ancient roman, Pliny the elder, suggested that livers of mad dogs were protective against rabbies, and asian physicians used crusts from the lession of smallpox patients to induce immunity in small children. More safe vaccinations were introduced by Louis Pasteur during the 1880'ies, when he showed that acquired immunity could be induced by attenuated organisms. In 1890 Behring and Kitasato used isolated diphtheria and tetanus toxin to induce immunity against these bacteria. The active substance that conferred the immunity was called anti-toxin and later given the more general term, antibody. The substance that elicited the antibody was termed an antigen, for **anti**body **gen**erator. Since then, polyclonal antisera have been applied for both therapeutical as well as analytical purposes, but the revolution came in 1975, when Köhler and Milstein introduced their technique to produce antigen specific mouse monoclonal antibodies *in vitro*. Monoclonal antibodies are derived from a single B cell clone and recognize a distinct antigenic site (epitope). The specificity of a monoclonal antibody is restricted to the epitope and not to the antigen, as the same epitope theoretically may occur on different antigens.

Monoclonal antibodies have refined many analytical procedures in immunoassays and are of potential value in industrial large scale affinity purifications (reviewed by Carlsson et al., 1991). Monoclonal antibodies have been raised against a vast number of different antigens, including bacteria, vira, parasites, toxins, and has become a valuable tool in definition of cellular markers ('CD'-nomenclature). One of the most visionary and perspective applications of specific monoclonal antibodies is in the diagnosis and treatment of cancer. Indeed many tumor-associated antigens have been identified by mouse monoclonal antibodies, and great efforts have been made to use these antibodies in the therapy of cancer. With exception of the OKT3 antibody used in immunosuppressive treatments (Goldstein, 1986) and the 17-1A antibody used for adjuvant therapy in patients with colorectal cancer (Riethmüller et al., 1994), the effect of murine antibodies

for *in vivo* therapy on humans have generally been disappointing. One explanation is the strong human-anti-mouse-antibody (HAMA) response that often follows administration of murine antibodies into patients (Schroff et al., 1985), but this problem should be possible to overcome by the use of human antibodies. Great efforts have been done to accomplish this, and considering that almost 20 years have elapsed since Steinitz and collegues in 1977 first reported the production of human monoclonal antibodies with a desired specificity, progress has been slow and cumbersome. However, human antibody technology has not been as "straight forward" as generating murine hybridomas. The major problems have been the immortalization of antigen specific human B cells in order to get stable and continously growing antibody-producing cell cultures and the generation of the desired specificity. For obvious reasons, immunization of human donors and subsequent splenectomy is not the proper choice to get specific human B cells. This has prompted investigators to develop alternative techniques like *in vitro* immunization (Borrebaeck et al., 1988a), but antibodies obtained by this technique have generally been of disappointing low affinity and primarily of IgM isotype.

New emerging techniques have in the recent years, however, given new life and new hopes to human antibody technology. The finding of the CD40 ligand on activated T cells (Armitage et al., 1992) and that anti-CD40 antibodies prevent germinal center B cells from apoptosis (Liu et al., 1989), suggested an important role for these molecules for B cell survival. Stimulation via immobilized anti-CD40 has provided new means of *in vitro* culture of human B cells (Banchereau et al., 1991; Banchereau and Rosset, 1991). Chin and coworkers (1995) generated HIV-1 neutralizing IgG antibodies, using *in vitro* immunization to obtain a primary response followed by expansion on anti-CD40 and co-culture with specific autologous T cells to obtain class-switching. Kinetic analysis of antibodies generated by this procedure showed evidence of increasing somatic point mutations in the V region genes and affinity maturation as the response progressed (Duenas et al., 1995). Recently, we also reported generation of human antibodies against the antigens DNP and GM2 by crosslinking specific surface Ig on B cells and recruiting T cell help from activated T cells by superantigen bridging (Ingvarsson et al., 1995).

The immense progress in molecular biology has opened new possibilities to select specificities from naive repertoires (Marks et al., 1991) and methods to isolate and express Ig genes in both procaryotic (Plücktun, 1991) and eucaryotic hosts (Gillies, 1992). Engineered antibodies may further be manipulated by codon-

based mutagenesis to increase the affinity (Yelton et al., 1995). The advent of immunodeficient laboratory animals, like the SCID mouse (Bosma et al., 1983) provide new means of generating a human immune system *in vivo*, that may be manipulated without the same concern that restricts to human volunteers. The papers and data presented in this thesis, provide a basis for discussion of using the SCID-hu-PBL mouse to fullfill these requirements.

However, the vast majority of human monoclonal antibodies generated today have been obtained by Epstein-Barr virus (EBV) transformation of human B cells (Henle et al., 1967; Steinitz et al., 1977), eventually followed by somatic hybridization (Kozbor and Roder, 1984). EBV has a unique ability to infect and eventually transform human B cells into continuously growing lymphoblastoid cell lines. It is important to distinguish between those B cells that in response to infection temporarily expand in culture, and those that ultimately will be transformed and immortalized. It has been shown that roughly every third B cell from peripheral blood is able to respond to EBV infection as measured by Epstein-Barr nuclear antigen (EBNA) expression (Bird et al., 1981), however, less than 1% will actually be immortalized (Tosato et al., 1985). EBV infects via the C3d (CR2) receptor, which is present on late pre-B cells and all mature B cells (Cooper et al., 1988). The virus receptor is strongly down regulated in terminal differentiation stages of antibody producing B-cells, concomittantly with the loss of sIgD expression. However, EBV is equally efficient in transforming surface μ, γand α-Ig expressing cells (Casali and Notkins, 1989) and it has been suggested that susceptibility to transformation is more dependent on entry into cell cycle than actual density of CR2 surface expression (Chan et al., 1986). The transient activation of B lymphocytes by EBV infection without subsequent transformation and difficulties in maintaining stable Ig production of transformed B cells in culture, limits the use of this technique. This may in part be overcomed by somatic fusion with a myeloma partner, resulting in more stable and easy clonable cell lines (Kozbor et al., 1982; Ohlin and Borrebaeck, 1994).

3. THE SCID MOUSE

The autosomal recessive inherited SCID defect in mice, was first reported in 1983 when Bosma and colleagues routinely tested serum Ig levels in an inbred strain of C.B-17 mice, that were kept under specific pathogen-free (SPF) conditions. Four litermates had no detectable Ig levels. Inbreeding on pedigree from these animals, showed that the defect was inheritable, and a colony that was homozygous for the defective gene was established and designated C.B-17 *scid/scid* (in the following referred to as SCID mice). The *scid* mutation was found to map to the mouse chromosome 16 near the λ light chain locus.

3.1. THE *scid* PHENOTYPE

The *scid* phenotype is characterized by the abscence of functional B- and T-lymphocytes (Bosma et al., 1983) and profound sensitivity to x-irradiation (Fulops and Phillips, 1990). Serum Ig concentrations are usually less than 20 μg/ml. The lack of functional, mature B and T cells in SCID mice results in inability to produce antibodies and reject allogeneic skingrafts (Bosma et al., 1983). SCID spleen cells do not proliferate in response to the B cell mitogen lipopolysaccharide or the T cell mitogen Concanavalin A, nor to allogeneic lymphocytes in mixed lymphocytes reactions (Bosma et al., 1983). Besides the bone marrow that appears histologically normal, the lymphoid organs are only 1/10 of their normal size and consist mostly of vascularized supportive tissue (Bosma et al., 1983). Early cells of the B and T lymphoid lineage are present in SCID lymphopoeitic tissues (Schuler at al., 1988), but are unable to differentiate into mature and functional lymphocytes. The thymus contains normal numbers of thy-1$^+$IL-2R$^+$CD3$^-$ progenitor T cells, whereas CD4 and CD8 single- or doublepositive are generally absent (Habu et al., 1987). B cell development is arrested at the stage of late pro-B cells having the phenotype of B220$^+$Thy-1^{lo}IgM$^-$ (Reichman-Fried et al., 1990), and are transformable by the Abelson murine leukemia virus (A-MuLV) (Schuler et al., 1986), that predominantly infects and transforms at the late pro-B cell stage. Schuler and colleagues also showed that hybridomas derived by fusion of adult SCID bone marrow cells, had not undergone any Ig heavy chain rearrangements. However, long term *in vitro* culture of adult SCID bone marrow showed that aberrant and nonproductive IgH gene rearrangement occured frequently (Witte et al., 1987). Myeloid cell

differentiation and function are normal and NK cell activity is also unimpaired (Dorshkind et al., 1985).

The *scid* defect is, however, not complete since around 15% of young SCID mice contain a limited number of functional T cells and measurable serum Ig levels (Bosma et al., 1988). These mice are termed SCID(Ig^+) or referred to as "leaky mice". The leakiness becomes even more pronounced in older mice, or upon antigenic stimulation (Carrol et al., 1989). The Ig levels in young SCID(Ig^+) mice vary widely (from 0.1 to >10 mg/ml), and most mice do only produce few Ig isotypes (Bosma et al., 1988). SCID(Ig^+) mice have mature B cells ($B220^+$, IgM^+) and $CD3^+$ T cells are CD4 or CD8 singlepositive (Carrol et al., 1989). However, despite no sign of serum Ig, nearly all SCID mice have low numbers of pauciclonal $CD3^+$ T cells at the age of 1 year (Carrol et al., 1989). The leaky phenotype is not inheritable, as evidenced by pedigree analysis of SCID(Ig^+) and SCID(Ig^-) mice, and selective breeding of SCID(Ig^+) mice does not increase the frequency of leaky mice (Bosma et al. 1988). Analysis of TCR rearrangement in alloreactive $CD3^+$ T cells from individual SCID(Ig^+) mice, suggested that the leaky phenotype might happen as a result of somatic reversion of the *scid* phenotype in lymphoid progenitor cells (Petrini et al., 1990).

3.2. THE *scid* DEFECT

The functional Ig and TCR genes are assembled to one functional gene from separate germline DNA sequences, the variable (V), diversity (D) and joining (J) gene elements. These elements are flanked by highly conserved heptamer and nonamer signal sequences and separated by less conserved spacer regions. The recombination event is initiated by bringing the signal sequences into proximity, followed by site specific cleavage of double stranded DNA between the coding sequences and heptamer signals. This results in an intermediate, in which the coding ends and the two flanking ends are brought into nearby contact (Figure 1, stage I). The rearrangement can then proceed by the formation of signal joints (stage III), coding joints (stage II) or a nonproductive hybrid joint (stage IV). Since the *scid* defect seemed to account for arresting the development of B and T cells at a stage just before rearrangement of their antigen receptors, and the finding of aberrant and nonproductive IgH gene rearrangements, it was tempting to speculate that the

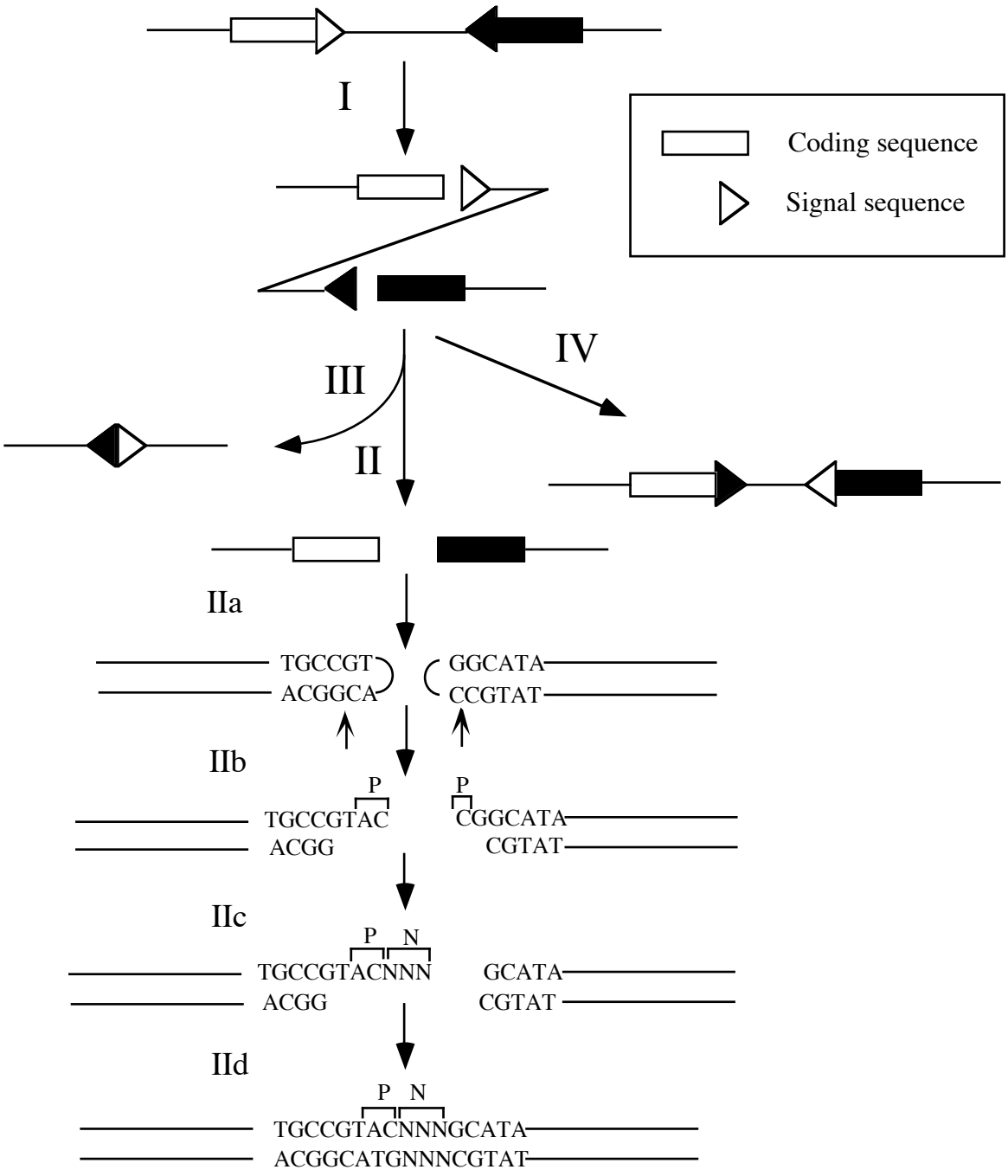

Figure 1. Lymphoid VDJ recombination. The two gene elements are brought into nearby contact, followed by double stranded breaks between the coding element and the signal sequence (I). Occasionally the signal sequences are simply inverted and results in a non-productive hybrid joint (IV). Normally the signal sequences are brought together, resulting in a coding junction (II) and a signal junction (III). The hairpinstructures are opened by single strand nicks (IIa) and results in formation of small palindromes (P nucleotides). The coding ends may undergo nucleotide addition (N nucleotides) or nucleotide loss (IIc). Finally, the ligation results in formation of the coding junction (IId). (Redrawn from Hendrickson, E.A. (1993))

scid mutation was somehow involved in VDJ recombination. Analysis of A-MuLV transformed SCID pre-B cells, showed that the VDJ recombinase recognized heptamer-nonamer signal sequences and performed endonucleolytic scissons at these sequences, but the defect was not able to join correctly the cleaved end of the coding strands (Malynn et al., 1988). Later it was shown that the *scid* defect was not only a phenomenon of lymphoid cells, but rather a general defect in the DNA repair pathway and the ability to repair double stranded breaks in all SCID cells (Fulop and Phillips, 1990). Signal junction formation appears to be normal in SCID mice (Hendrickson et al., 1990), suggesting that signal junction and coding end junctions proceed by independent mechanims. The coding ends in stage IIa (Figure 1) terminate in hairpin structures, which has been found to accumulate in the SCID thymus (Roth et al., 1992) and it was believed that the *scid* gene encodes a protein involved in the resolving of hairpin structures. However, hairpins are resolved as efficiently in *scid* cells as in wild type cells (Lewis, 1994). Recently, 3 groups have independently provided strong evidence for a candidate to the *scid* defect is a 350 kDa subunit of the DNA-dependent protein kinase (DNA-PK) (Peterson et al., 1995; Kirschgessner et al, 1995; Blunt et al., 1995), reporting that p350 levels were greatly reduced or absent in *scid* cells. DNA-PK consists of three components, Ku-70, Ku-80 and the 350 kDa catalytic subunit and binds to double-stranded DNA containing broken ends, nicks and single-stranded gaps (Carter et al., 1990).

4.3 OTHER IMMUNODEFICIENT MOUSE STRAINS

SCID mice on the C.B-17 background has normal NK cell activity. In order to improve the SCID mouse as a recipient for xenogenic transplantation experiments, the *scid* defect was introduced into BALB/c *beige/beige* mice with depressed NK activity (Mosier et al., 1993a). This strain, denoted SCID.BG, does surprisingly not suffer from leakiness to the same extent as SCID mice. At 2 month of age 24% of SCID mice were leaky (>5 μg/ml serum Ig) whereas less than 2% of SCID.BG mice were leaky. Importantly, the leakiness seems not to be age-dependent in SCID.BG mice, since only 3% were leaky at the age of 8 month, whereas virtually all SCID mice were leaky at the same age.

The *scid* mutation has similarly been introduced into the C3H strain, by backcrossing with C.B-17*scid*. In a comparative study in 3-month-old mice, 79% of the SCID mice had detectable serum levels (>0.4 μg/ml), whereas only 15% of the C3H SCID mice had detectable serum Ig but at low levels (Nonoyama et al.,

1993a). In addition, the number of leaky C3H SCID mice did not increase with age and transfer of syngeneic, normal, neonatal T cells did not affect the serum Ig levels, as has been shown to be the case with SCID mice (Riggs et al., 1991). Since the leaky phenotype is less pronounced in the C3H SCID and SCID.BG strains, these mice should theoretically offer better possibilities for cell transfer experiments.

Immunodeficient mouse strains that do not involve the *scid* gene, are the RAG-1 and RAG-2 knockout mice. The product of the RAG-1 and RAG-2 genes are involved in VDJ recombination (Stage I in figure 1) and loss of RAG-1 or RAG-2 function results in total inability to initiate and undergo VDJ rearrangement (Oettinger et al., 1990). Mice deficient in RAG-1 (Mombaerts et al., 1992) or RAG-2 (Shinkai et al., 1992) does not rearrange their T cell receptor and Ig loci and resembles phenotypically the SCID mouse, besides the defect is 100% and the mice do not become leaky with age. These mice could be very well fitted for transplantation experiments, especially a beige mutant strain could prove interesting, but they are at present not commercially available.

4. THE SCID/HUMAN CHIMERA

The impaired immune system in SCID mice, makes these animals perfect recipients of syngeneic, allogeneic and xenogeneic cell grafts. Transplantation experiments with bone marrow cells from BALB/c mice, having the same genetic background as C.B-17 *scid* but differing at the *Igh* locus, into SCID mice showed that the SCID recipients supported allogeneic B cell differentiation as evidenced by the presence of allotype Ig in serum (Bosma et al., 1983). Xenotransplantation of rat fetal liver cells caused a near complete reconstitution with restoration of thymus and spleen architecture, rat derived cells differentiating into lymphohematopoietic cells, including myeloid and erythroid cells, macrophage/dendritic lineage and mature B and T cells (Surh and Sprent, 1991). These studies indicate that the SCID cellular environment fully supports differentiation of transplanted allogeneic and xenogeneic immune systems. Special interest has been focused on the study of human immune responses in SCID mice transplanted with naive or immunocompetent human lymphoid cells. Xenogeneic transfer of human lymphoid cells has generally been accomplished in two functionally different models i) surgical engraftment of human fetal organs (SCID-hu mice) or ii) intraperitoneal injection of peripheral blood lymphocytes from healthy or diseased donors (SCID-hu-PBL). Both these models has found valuable use as a laboratory animal model to study human hematopoiesis and immune functions.

4.1. THE SCID-hu MOUSE.

Successfull engraftment of a human lymphoid system into SCID mice was reported in 1988, when McCune and colleagues implanted fetal thymus under the kidney capsule of such mice. Using this approach, they detected a transient wave of mature human T cells in the peripheral circulation with a peak level on day 40, however, the presence of human lymphoid cells was undetectable at day 64. Co-implantation of fetal lymph nodes resulted in measurable human IgG levels in serum. Since stromal microenvironment supports differentiation and proliferation of hematopoetic progenitor cells, fetal liver was co-implanted together with fetal thymus into SCID mice (Namikawa et al., 1990). Over 50% of these mice showed continously human multilineage hematopoesis for 5-11 months. The thymus grafts were indistinguishable from that of normal age-matched human fetal thymus, containing T cells with the markers CD1, CD4 and CD8 single

positive, double positive and double negative. The only difference was that the SCID-hu thymus were infiltrated with murine dendritic cells of bone marrow origin, suggesting that the human progenitors migrated from the liver graft to the thymus and became educated to recognize both human and murine MHC as self (Namikawa et al., 1990). In keeping with this, no sign of graft-versus-host disease (GvHD) has been observed in these mice, despite that the human T cells were fully functional (Krowka et al., 1991). The model sustained only low levels of human T cells in the periphery and human B cells were hardly detectable, why this model was more suited to study T cell than B cell differentiation and function. SCID-hu mice has provided a usefull tool in the establishment of an animal model to study HIV infection and therapy (McCune et al., 1991).

Recently it was reported that human fetal bone marrow cells, precultured on stromal cells, and injected intravenously into irradiated SCID recipients, migrated to the SCID bone marrow and were capable of differentiating into both human lymphoid and myeloid cells (Kollmann et al., 1994). Although human B cells were found in all the peripheral lymphoid compartments in different differentiation stages, it was not possible to detect any T cells in this mouse construct. Despite many efforts, there has currently been no reports of a SCID-hu model, with an intact and functional human immune system.

4.2 THE SCID-hu-PBL MOUSE

Almost simultaneously with the McCune paper in 1988, another approach was described by the group of Donald Mosier (Mosier et al., 1988). Injection of human peripheral blood lymphocytes from adult donors into the peritoneal cavity of SCID mice, resulted in spontaneous secretion of human Ig. The human Ig response was dependent on the initial number of cells injected. When mice were transplanted with less than 10^7 PBL, only a minority of the mice would produce human Ig. Human Ig levels might persist 5-12 month after transplantation, with a peak level round week 8 (Duchosal et al., 1992a; Mosier et al., 1988; Saxon et al., 1991). The human Ig levels are normally between 0.5-5 mg/ml in mice repopulated with PBL from EBV-seronegative donors (Torbett et al., 1991).

The fate of human lymphocytes injected intraperitoneally into SCID mice, has been very difficult to follow. Although high human Ig levels have been observed in the sera, it has specially been very difficult to locate, phenotype and quantitate

the amount of human B cells responsible for this Ig production. The many attempts to quantitate human PBL after repopulation have been diverse, but a representative study by Torbett et al. (1991) showed a major part of the PBL injected intraperitoneally, persist in the peritoneal cavity. Six weeks post implantation, 2% of the peritoneal cells were $CD20^+$ and 66% $CD3^+$ (16% $CD4^+$ and 51% $CD8^+$), whereas the spleen contained less than 1% $CD20^+$ and 8% $CD3^+$ (3% $CD4^+$ and 5% $CD8^+$). We have found the same level of B cell engraftment in our SCID-hu-PBL model (personal observation). Since we selectively remove NK cells, monocytes and $CD8^+CD11^+$ T cells from PBL prior to transplantation into SCID mice, the peritoneal cavity was found to contain 27% $CD4^+$ and 25% $CD8^+$ human T cells, whereas only 1-2% $CD4^+$ T cells were found in the spleen at week 8 post transplantation (Carlsson et al., 1992).
Only very few cells have been found in the peripheral organs as thymus, lung, liver, blood and bone marrow, which almost exclusively became repopulated with $CD3^+$ T cells (Tary-Lehman and Saxon, 1992). The use of mAbs to quantitate human lymphoid cells is, however, problematic since specially phycoerythrin-conjugated antibodies may non-specifically stain murine cells or the antibodies may bind to murine Fc receptors, leading to an overestimation of the actual values (Mosier et al., 1989; Simpson et al., 1991; Krowka et al., 1991).

More reliable results have been obtained using *in situ* hybridization on tissue sections, with a biotinylated DNA probe to human centromeres (Martino et al., 1993). Although human Ig was detected in sera, the results showed only very sparse repopulation of human cells in the lymphoid organs. Rare human cells were seen in the spleen and no cells were found in the bone marrow or Peyer's patches. It was suggested that most of the transplanted cells, resided within the peritoneal lining. Another approach was to label the human cells with a flourescent dye prior to injection into SCID mice (Ladel et al., 1993). Four weeks after transplantation, cytospin preparations showed 4% engraftment of human PBL in SCID blood, 2.7% in the spleen and 4% in thymus. In the spleen, the cells were found to cluster around the vessels, whereas only very few cells infiltrated the splenic tissue. Most cells were found to reside in the peritoneum. *In situ* hybridization with an *alu*-probe showed appearance of human T cells and rare B cells in the white pulp of the spleen, 4 weeks after repopulation (Duchosal et al., 1992a). Lymph node architecture was not restored and there were no signs of follicle generation in the spleen.

These results clearly indicate that transplantation of xenogenic, functional and mature human lymphocytes does not reconsitute SCID lymphoid organs to the same extent as has been shown with allogeneic murine cells (Hilbert et al., 1994) or rat fetal liver cells (Suhr and Sprent, 1991). Succesfull engraftment may vary from donor to donor, depending on the immune status of the donor (personal observation, Torbett et al., 1991, Duchosal et al., 1992a). However, improvements in engraftment of human PBL have been reported by different groups and the activation state of the cells may be important. Engraftment of human T cells were significantly increased when PBL transplantation was followed by treatment with human growth hormone and homing to SCID thymus was observed (Murphy et al., 1992a). Similar results were obtained after *in vitro* activation with anti-CD3 (Murphy et al., 1993). Sandhu and colleagues (1994) reported that treatment with anti-asialo GM1 and irradiation prior to transplantation with human PBL, resulted in substantial better engraftment and homing to the spleen. Removal of NK-cells from PBL with the lysosymotrophic ester LeuLeuOMe prior to transplantation produced higher amounts of human Ig (Carlsson et al., 1992), and we reported, recently, that cross-linking of Ig receptors and SEA priming of human B cells with SEA may enhance the survival of human B cells in SCID mice (Paper IV).

4.2.1 EBV induced lymphoma

Transplantation of SCID mice with PBL from EBV-positive donors, often results in large cell immunoblastic tumors (Mosier, 1991). The frequency of tumor development in SCID-hu-PBL is dependent on the initial number of cells injected. Intraperitoneal injection of 50 x 10^6 PBL resulted in tumor development within 9 weeks after transplantation and most of the mice died within 12 weeks (Mosier et al., 1988). Mice transplanted with 10 x 10^6 or less, do not establish any signs of tumors. Coincident with tumor development is a 2-3 fold increase in serum Ig levels observed (Torbett et al., 1991). The tumors are mostly oligoclonal, contain the EBV genome but differ from EBV-related Burkitt's lymphoma in chromosome alterations, c-myc rearrangement and expression of the CD10 marker (Purtilo et al., 1991; Rowe et al., 1991). The overall phenotype and genotype of the spontaneous immunoblastic B cell lymphomas in SCID-hu-PBL is analogous to the EBV-associated lymphomas that often arise in immunosuppressed- or immunodeficient patients (Mosier et al., 1991). The outgrowth of EBV tumors in SCID-hu-PBL were dependent on functional T

cells, since injection of purified B cells only produced tumors in the presence of cotransplanted T cells (Veronese et al., 1992). No differences in tumor outgrowth was observed when B cells were cotransplanted with purified $CD4^+$ or $CD8^+$ T cells. In contrast to these findings Walker and Gallagher (1994) recently reported, that depletion of $CD8^+$ cells from PBL resulted in profound outgrowth of EBV tumors in SCID-hu-PBL, thus indicating a role for NK cells or cytotoxic T cells in the surveillance of EBV induced tumors.

4.2.2 Graft-versus-Host Disease in SCID-hu-PBL

As a natural consequence of xenografting functional human immune cells into SCID mice, the appearrance of GvHD would be expected as has been found in allogeneic transplantation experiments (Hoffmann-Fezer, et al., 1993; Rudolphi et al., 1991). The reports have, however, been very conflicting on this matter, ranking from no signs of GvHD (Mosier et al., 1988; Saxon et al., 1991), to mild subclinical (Krams et al., 1989; Duchosal et al., 1992a) and lethal (Hoffman-Fezer et al., 1993; Pirruccello et al., 1992; Murphy et al., 1992b). Suggestions for the cause of GvHD is as diverse as the reports on observation of GvHD. Several reports have detected human xenoreactive antibodies in the sera of SCID-hu-PBL (Williams et al., 1992; Pirruccello et al., 1992; Pflumio et al., 1993), and especially if the donor is allergic to mouse antigens, severe GvHD develops after transplantation (Pflumio et al., 1993). In a study with transfer of semi-allogeneic mouse spleen cells (Rudolphi et al., 1991), GvHD developed only if the SCID mice had low levels of endogenous B and T cells (leaky mice), suggesting that host B and T cells were a prerequisite for the induction of GvHD. In another study, transplantation of high numbers of PBL (10^9/mouse) induced chronic GvHD (Huppes et al., 1994). The infiltrating T cells were mainly of $CD4^+$ and disease was abrogated upon administration of anti-CD4 antibodies. Injection of rIL-2 reduced the number of cells needed to induce GvHD, and the authors suggested that IL-2 may be important in regulating GvHD. In contrast, Xun et al. (1993) reported that purified NK cells injected into SCID mice induced severe acute GvHD, whereas nothing was observed if purified T cells were injected. It was suggested that IFN-γ and TNF-α were responsible for mediating the effect. The general opinion is that GvHD does not occur under normal circumstances in SCID-hu-PBL (Mosier et al., 1991a), but may be induced under special experimental conditions. In our studies (Paper II and IV) we use LeuLeuOMe treatment of the PBL before transplantation, thus depleting the donor cells for NK cells (Carlsson et al., 1992). During the experimental period of 8 weeks, we

have never observed clinical symptoms consistent with GvHD, such as ruffled fur, diarrhoea, hunched back and cachexia. However, transplantation of LeuLeuOMe treated PBL to SCID.BG mice often lead to death within 30 days, while SCID transplanted with the cells from the same donor appear normal (personal observation). In some cases the cause of death was due to development of lymphoma, but in other cases the cause of death was not obviuos, and might be attributed to GvHD. Since normal SCID mice have high NK activity (Dorshkind et al., 1985), this could indicate an effect of endogenous NK cell activity, preferentially lysing cells reactive against the host. The same observations have been reported in studies when SCID NK cell activity was reduced with anti-asialo GM1 and irradiation (Pirruccello et al., 1992; Murphy et al., 1992b; Sphitz et al., 1994) or in newborn SCID-hu-PBL with low NK cell activity (Pflumio et al., 1993). Similar results in SCID.BG were also obtained by Mosier et al. (1991), who found high levels of human antibodies associated with red blood cell agglutination. They concluded that the patogenesis in these mice was more due to xenoreactive antibodies than xenoreactive T cells and suggested that host NK cell activity was in part responsible for the lack of GvHD in adult SCID recipients. Also the high "interlaboratory" variations observed, both in reports on GvHD and on induced human immune responses, may in be due to variations in the level of NK cell activity, which again may reflect differences in exposure to environmental antigens in the household of mice.

4.2.3 Human immune responses in SCID-hu-PBL

Transplantation of human PBL into the peritoneal cavity resulted in spontaneous production of human antibodies, readily detected in mouse serum (Mosier at al., 1988). The halflife of human Ig in SCID-hu-PBL, has been estimated to 12 days for IgG and only 37 hours for IgM (Saxon et al., 1991; Smith et al., 1991). The very short halflife of human IgM may explain the low levels of human IgM normaly detected in SCID-hu-PBL sera, except in cases of lymphoma development. Isoelectric focusing of SCID-hu-PBL sera showed an oligoclonal pattern of human IgG and κ/λ usage, and PCR analysis of recovered human B cells showed a skewed and restricted V_H usage (Saxon et al., 1991). These data suggest that the Ig production in SCID-hu-PBL is caused by a small number of surviving B cells and results in a skewed and restricted repertoire. However, in search for natural occuring human antibodies in SCID-hu-PBL sera, Smith et al. (1991) found that the specific level of IgG1 antibodies directed against e.g. herpes simplex virus, seemed to parallel the total IgG concentration, and concluded that

the polyclonal human repertoire in repopulated animals is not lost when selected clones expand. Also antigen-specific human antibody responses in SCID-hu-PBL may be induced following repetitive immunizations with recall antigens as tetanus toxoid (Mosier et al., 1988, Carlsson et al., 1992; Duchosal, 1992b). This antigen-specific response mimics the normal humoral response in terms of high-dose tolerance, antigenic selection, and dose-dependent, antigen driven B cell activation (Carlsson et al., 1992).

Generation of primary responses in SCID-hu-PBL mice have been much harder to achieve. This requires functional antigen presenting cells to present antigen to naive T cells, the presence of immunocomplexes on interdigitating cells (IDC) to trigger naive B cells, cell contact between specific B and T cells, all events that normally take place in defined areas in secondary lymphoid tissues. Since human macrophages and monocytes do not repopulate in SCID (Krowka et al., 1991) the antigen presentation would rely on human B cells as the sole APC, and these have been shown not to be able to stimulate naive T cells (Lassila et al., 1988; Fuchs and Matzinger, 1992; Ronchese and Hausmann, 1993). Attempts to generate primary responses in SCID-hu-PBL have generally been disappointing. The first report appeared in 1991, when Mazingue et al. suggested that they had obtained a primary response to a recombinant schistosome antigen in SCID mice transplanted with PBL from a normal donor. No data was, however, supplied whether the donor had natural occuring antibodies or preexisting immunity against Schistosoma. Since the mice were transplanted with as low as 5×10^6 PBL, it is doubtful that any response should have been generated unless the donor had been presensitized. Presensitization of the donor before transplantation of PBL into SCID, allows generation of antigen specific human responses after subsequent immunization with antigens like KLH (Markham and Donnenberg 1992) and bacteriophage Φx 174 (Nonoyama et al. 1993b). In these studies it was not possible to detect any specific human responses when PBL from normal donors were used. Duchosal et al. (1992b) found a small proportion of SCID-hu-PBL mice producing a weak transient specific IgM response after immunization with hepatitis B core antigen. In another study a specific human IgG response against *Streptococcus pneumoniae* was generated, when SCID-hu-PBL mice were immunized with a pneumococcal polysaccharide vaccine, resulting in increased protection when challenged with live parasites (Aaberge et al., 1992). This response was probably not dependet on induction of a specific T cell response, since the vaccine is known to be a T-independent antigen. Walker and Gallagher (1994) reported the induction of specific human IgM and IgG response

against ovalbumin in SCID-hu-PBL immunized with ovalbumin entrapped into liposomes. However, 30 days post transfer immunized mice suffered from massive EBV-induced tumor development in the peritoneal cavity, and cell lines secreting ovalbumin specific antibodies were easily established *in vitro*. Sandhu et al. (1994) used irradiated and anti-asialo-GM1 treated SCID mice transplanted with PBL from normal donors. They obtained high titered specific human IgM and IgG responses against KLH, circumsporozoite malaria parasite antigen (CSP) and the carbohydrate sTn. The titers were calculated as the reciprocal of the highest serum dilution at which the optical density, using serum from vaccinated mice, was above that of the non-vaccinated. However, a representative case of an IgG specific response against KLH, showed that the optical density in ELISA did not exceed 0.25, putting the results in question. Recently, Chargui et al. (1995) reported generation of a weak (OD ~ 0.1-0.3) antigen specific human IgM response in 2 out of 4 SCID-hu-PBL mice immunized with a syntetic HIV peptide.

In our studies we have circumvented the problem of generating T cell specificity, by using abundant specific T cells present in TT vaccinated donors and coupled the B cell antigen to TT or to a syntetic TT epitope. This allowed us to generate both primary and secondary human responses against DNP, the V3 loop of gp120 (HIV-1), ovine submaxillary mucin (OSM) and the melanoma associated ganglioside GD2 (paper II). In the case of GD2, we obtained titers that normally only are seen with recall antigens like TT. In another approach we used crosslinking of the specific B cell receptor to activate the B cells and SEA to bridge between the specific B cells and unspecific T cells (paper IV). Using this approach, we obtained human responses against DNP and GM2.

The SCID-hu-PBL model certainly has its limitations and should by no means be compared to the human immune system *in situ*. It still, however, represents a valuable tool to investigate parameters that pertain to the function and regulation of the human immune system, and especially allows manipulation without the same ethical considerations that is relevant for human patients or volunteers. In the present study we have focused on generating primary and secondary responses in SCID-hu-PBL, in order to produce human monoclonal antibodies of desired specificity. The results show that it is possible to repeatedly obtain human IgG responses against primary antigens in SCID mice transplanted with PBL from normal donors. Further, our results suggest that the SCID-hu-PBL model may serve as an efficient in vivo model for the purpose of vaccine design.

In addition, the SCID-hu-PBL model has also found valuable applications in the study of human diseases, as HIV-infection (Mosier et al., 1993b), immunodeficiency (Smith et al., 1991), lymphomagenesis (Pisa et al., 1992; Purtilo et al., 1991), autoimmune disorders as SLE (Duchosal et al., 1990) and rheumatoid arthritis (Tighe et al., 1990), vaccine studies for the protection against microorganisms like *Borrelia burgdorferi* (Schaible et al., 1994), *Schistosoma mansoni* (Mazingue et al., 1991), *Streptococcus pneumoniae* (Aaberge et al., 1992) and *Toxoplasma gondii* (Beaman et al., 1994; Walker et al., 1995).

5. THE PRESENT INVESTIGATION

The aim of this study was to improve on two of the major problems in human antibody technology i) rescue and stability of EBV-transfected human B cells and ii) generation of primary and secondary B cell responses from an unprimed lymphocyte source.

In our laboratory, techniques have been designed to *in vitro* generate human monoclonal antibodies (Borrebaeck et al., 1988b). Using this protocol, specific antibodies have successfully been generated against digoxin (Danielsson et al., 1991), and the HIV-related proteins penv9 (Ohlin et al. 1989) and pB1 (Ohlin et al., 1992a). A general problem in human antibody technology, is the immortalization of the B lymphocytes, which is crucial in order to obtain stable and continously growing cell cultures secreting the desired antibody. EBV-transformation is a valuable and widely used tool in this process, but the instability of transformed B cells necessitate improved methods to maintain these cells in culture. We have consequently investigated various cell derived factors that facilitates cloning and thereby stabilization of EBV transformed B cells.

The antibodies obtained by *in vitro* immunization are often of IgM isotype and of low affinity. Sequence analysis of such antibodies, revealed near complete germline sequences (Danielsson et al., 1991), suggesting that the *in vitro* immunization method is more based on selection of the specificities rather than an actual immunization involving class-switching and somatic mutations. The present investigation was undertaken to evaluate the use of the SCID-hu-PBL model to obtain class-switched high affinity human antibodies against primary antigens.

5.1. CLONING OF EBV-TRANSFORMED B-CELLS (PAPER I)

This study was undertaken to investigate if B cell growth factors could enhance EBV infection and if these factors could facilitate cloning of LCL that otherwise were not able to be established as trioma cell lines. Earlier studies have shown that the outgrowth of EBV-infected and -transformed B cells, may be influenced by autocrine factors like IL-1β (Gordon et al., 1986), IL-5 (Baumann et al., 1992), IL-6 (Yokoi et al., 1990), sCD23 (Swendeman et al., 1987) and lactic acid (Pike et al., 1991). The growth factor investigated in this paper, was derived from a T cell hybridoma (MP6) and was first reported to be a B cell stimulating factor

(Rosén et al., 1986). It was later identified as an isoform of thioredoxin (Trx) (Rosén, A., personal communication). Trx is a 12 kDa protein, known to be present in all eukaryotic and prokaryotic organisms and catalyzes thiol-disulfide oxidoreductions. Among the known functions is the action as a hydrogen donor for ribonucleotide reductase essential for DNA synthesis (Holmgren, 1985), activation of the glycocorticoid receptor (Grippo et al, 1983) and protein disulfide reducing and refolding activities (Lundström et al., 1992). The MP6-Trx was chosen in search for a factor that could activate B cells and render these more susceptible to EBV infection. Culture of newly infected B cells on irradiated MP6 cells enhanced the outgrowth up to 2.5 times, whereas supernatant from MP6 only had a minor effect. This suggest that either direct cell contact is needed or that other factors not present in the 24 hour supernatant from MP6 are obligatory for the increased outgrowth of newly infected B cells. The most profound effect of MP6-Trx was seen on the polyclonal EBV infected cell line, LCL-29. Thymidine incorporation studies of LCL-29, showed up to 5 times increased DNA synthesis when cells were incubated with 50% MP6 supernatant. Earlier we had experienced poor clonability with these cells but addition of 50% MP6 supernatant increased the clonal outgrowth from 0 to 4% when cells were seeded at 1 cell/well. The effect of MP6-Trx depended on the presence of PBL as feeder cells. The cloning efficiency was improved significantly after co-cultivation of the B cells in a chamber insert together with live MP6 cells. This resulted in 33% outgrowth with cells seeded at 1 cell/well. The outgrowth was very slow and probably due to growth inhibitory factors found in 48-72 hours supernatant from MP6 (Rosén et al., 1986). The effect of MP6-Trx seemed to act in synergy with PBL and we reasoned that the effect could be due to an upregulation of cytokine receptors on the EBV infected B cells. IL-6 was recently reported as an important autocrine growth factor (Yokoi et al., 1990) and FACS analysis with a FITC labelled antibody against the IL-6 receptor, showed that MP6-Trx increased the expression of IL-6 receptor on LCL-29, whereas no effect was seen on the easy clonable monoclonal cell line B6. This suggests that MP6-Trx may rescue EBV infected and transformed B cells that otherwise would have been lost during cloning or expansion, thus preventing loss of cells with desired specificities.

5.2. INDUCTION OF PRIMARY HUMAN RESPONSES IN SCID-hu-PBL (PAPER II)

Previous investigations have shown that SCID-hu-PBL mice are able to respond specifically to challenge with recall antigens (Mosier et al, 1988; Carlsson et al., 1992) with the feature of normal immune parameters such as high dose tolerance and dose-dependent, antigen-driven B cell activation (Carlsson et al., 1992). These findings suggested that this animal model might be able to mimic a human immune system *in vivo* and, thus, form a fine laboratory model to generate primary and secondary affinity maturated human immune responses. From a previous study we knew that $CD45RO^+$ T cells were capable of supporting B cells to Ig production in SCID-hu-PBL (Mårtensson et al., 1994), so we devised a model that solely relied on antigen presentation on B cells to specific $CD45RO^+$ T-cells, present in the blood from TT immune donors. The B cell antigen was coupled to TT or a 15 aa peptide covering a T helper epitope of TT (Demotz et al., 1989). Repeated immunizations elicited specific responses against a hapten (DNP), a peptide (V3), a glycoprotein (OSM) and a carbohydrate (GD2), proving the generality of this approach. The response against DNP was characterized by an initial IgM response peaking on day 14 and followed by a stronger IgG response on day 28. From the OSM experiments it was evident that the specific B cell responses was dependent on T cell help mediated through the carrier protein, since it was not possible to elicit responses when we immunized with OSM alone, and no response was obtained if OSM was not physically linked to TT.

Glycolipids of the ganglio-series, such as GM2, GD2 and GD3 have received high attention as putative targets for antibody based immunotherapy against melanoma cancer. Very promising results were achieved in 1986 when Irie and Morton obtained complete remission in a melanoma patient with a human IgM antibody against GD2. The carbohydrate structure of gangliosides are poor T cell antigens, although GM2 is considered partly immunogenic in humans, indicated by the presence of naturally occuring IgM anti-GM2 antibodies in 5% of all humans (Kitamura, 1995).

In order to induce a proper response against GD2, the GD2-vaccine was introduced as a liposome construct. Liposomes themselves are effective immunological adjuvants (Gregoriadis, 1990), facilitate receptor uptake and are easily endocytosed by specific B cells (Harding et al., 1991). Only low amounts of ganglioside are necessary to induce a response, when they are incorporated into liposomes (Rapport and Graf, 1969), which lowers the costs considerably of the very expensive purified GD2. Furthermore, proteins are easily entrapped into

liposomes using the dehydration-rehydration method (Kirby and Gregoriadis, 1984).

Previous vaccine trials with GM2-expressing melanoma cells (Livingston et al., 1987) or BCG-GM2 (Livingston et al., 1989) elicited only IgM low titer responses. In our SCID-hu-PBL model we were able to elicit both IgM and IgG responses against GD2, which is less immunogenic than GM2 (Tai et al., 1985), with a final serum titer of 4000. The PBL used in this study was obtained from a patient with metastic melanoma. Although, serologic analysis revealed no seroreactivity against GD2 in ELISA, a number of GD2-reactive EBV-transformed B cells were established from the PBL. Some of these clones were sequenced, and all were found to be of (or close to) germline configuration. Accordingly, these antibodies seemed not have undergone antigen-driven somatic mutations in their V regions. No specific IgG producing EBV-clones were found and all IgM producers were of low reactivity and avidity against GD2. These findings suggested that the patient did not have an ongoing immune response against GD2, and indicates that the SCID-hu-PBL model is able to support Ig switching.

The results obtained in this paper, indicates that it is possible to induce primary specific B cell responses in SCID-hu-PBL to a variety of different antigens. Furthermore, the SCID-hu-PBL model may be very usefull for vaccine design. We have later repeatedly been able to induce high human IgG titers to GM2, using this approach from normal donors (paper III, IV and unpublished results). Furthermore, the liposome construct used in this study, may find a potential use as an efficient vaccine in melanoma patients.

5.3. IMPROVEMENT OF T CELL RESPONSES, USING CLONED T CELLS (PAPER III)

The repopulation efficacy of human lymphoid cells into SCID mice varies considerably, even when lymphocytes originates from the same donor (personal observation; Duchosal et al., 1992c; Saxon et al, 1991). Also variation between donors makes it difficult to make series of standardized experiments and compare the experiments to each other. In order to have a more defined system to study B and T cell interaction *in vitro* and *in vivo*, that would not relay on donor variations and TT immunity of the donor, we established a T helper cell clone from a splenectomized patient (Figure 2).

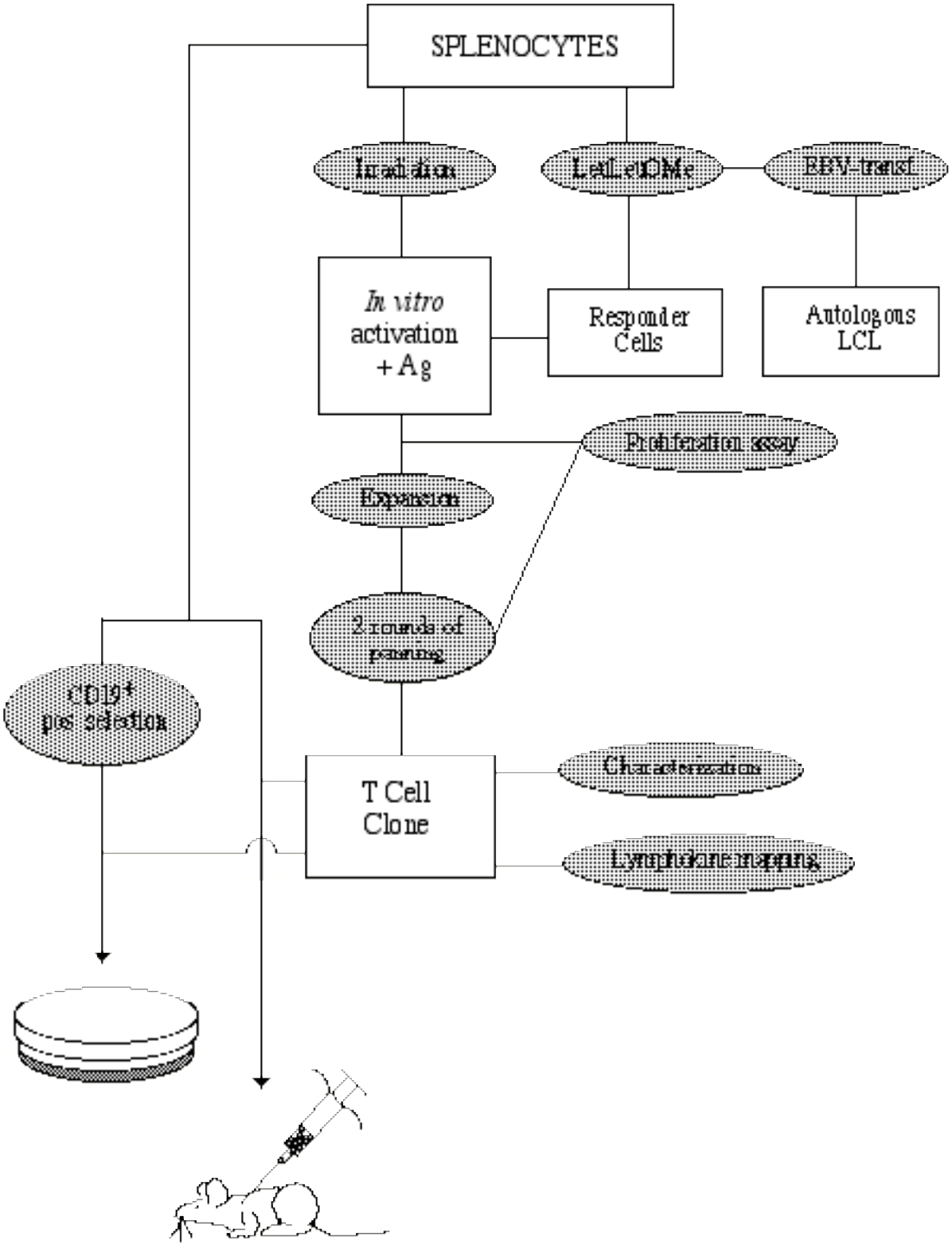

Figure 2. Schematic drawing of the experimental setup to establish and characterize the T helper clone described in paper III.

Although the patient was not boosted recently, we succeded in establishing a TT-specific T cell clone. The clone was characterized by flow cytometry to be TCR$\alpha\beta^+$, $CD4^+CD45RO^+$ and expressing the activation markers HLA-DR, IL-2R and CD38. Lymphokine mapping revealed transcripts encoding IL-1α, IL-2, IL-4, IL-5, IL-8 and IFN-γ. The T cell clone was able to stimulate isolated autologous $CD19^+$ B cells to dose-dependent IgM and IgG production when challenged with TT *in vitro*.

In vivo experiments with the T cell clone showed that only mice transplanted with splenocytes and T cell clone were able to elicit an IgG response to immunization with TT, however, the responses varied considerably among chimeras. This pattern was also observed in the *in vitro* experiment, suggesting a low frequency of TT specific B cells. The *in vivo* responses were considerably lower than normally obtained in SCID mice transplanted with lymphocytes from TT immune donors. These findings and the fact that it was not possible to elicit a TT specific response *in vivo* or *in vitro* in absence of the T cell clone, indicates that the TT immune status of the donor was very attenuated. Accordingly, splenocytes from this donor were well suited to evaluate the ability of the TT specific T cell clone to support antigen-specific B cell help, using TT to recruit T cell help

Primary responses against GM2 were obtained only in mice transplanted with splenocytes, enriched with cloned T cells and immunized with GM2+TT liposomes. Mice immunized with liposomes containing GM2 only did not elicit any antigen specific response, neither did mice not receiving the T cell clone or mice receiving T cell clone and immunized with PBS (unpublished data). All mice had human Ig production, without any significant variations between the different groups (data not shown). The fact that only mice transplanted with the T cell clone were able to respond to the primary antigen, further confirms the results from paper II: T cell help to the antigen-specific B cells are derived from TT specific T helper cells. The relatively high anti-GM2 titers obtained in mice with the T cell clone were all IgM. The data obtained in this study, suggested that the Th0 type clone certainly is able to support Ig production from both naive (IgM anti-GM2) and memory B cells (IgG anti-TT), but was not able to induce class-switching.

5.4. IMPROVEMENT OF T CELL RESPONSES, USING A SUPERANTIGEN (PAPER IV)

Establishment and characterization a T cell clone is sometimes a cumbersome work, and there will only be a limited supply of autologous lymphocytes for transplantation experiments. In order to find a more general method to get efficient T cell help, we investigated the use of SEA to recruit proper T cell help to antigen specific B cells. SEA binds strongly to HLA-DR (Fischer et al., 1988) and prior association to MHC class II on an antigen presenting cell is a prerequisite for subsequent binding to TCR Vβ (Gascoigne et al., 1991). The ability of SEA to initiate B cells to Ig production is restricted to the $CD45RO^+$ T helper cell subpopulation (Kristensson et al., 1994). This will result in a

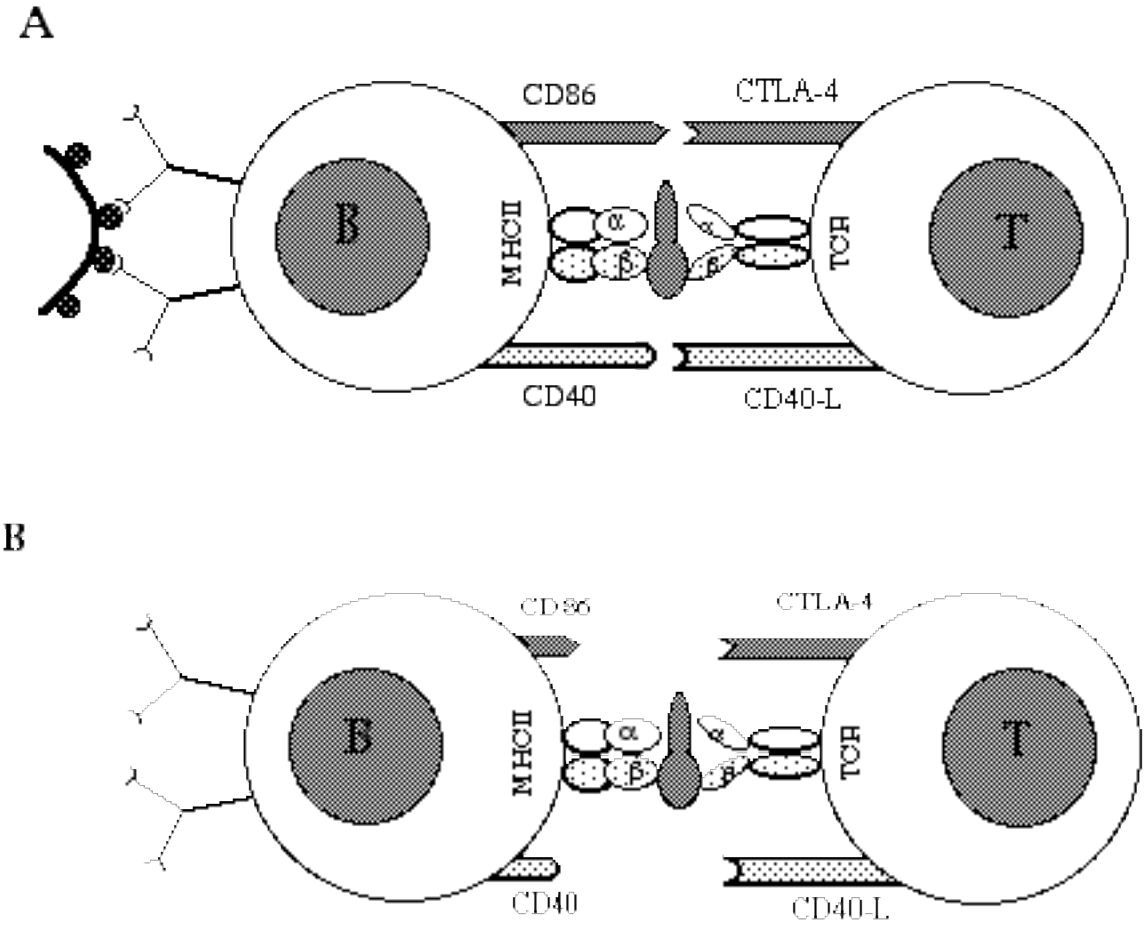

Figure 3. Crosslinking of Ig receptors on antigen-specific B cells upregulates surface expression of CD86 and CD 40 (A), whereby SEA bridging more effeciently deliver activation signals from activated T cells than B cells without receptor crosslinking (B).

non-specific polyclonal activation of the B cells. However, concommitant crosslinking of surface Ig, inducing rapid upregulation of CD40 (Ledbetter et al., 1987) and CD86 (Lenschow et al., 1994) on B cells and SEA binding to the TCR, upregulating the CD40 ligand on T cells (Kristensson et al., 1994) would suggest that antigen crosslinked B cells are more prone to be activated (Figure 3). Recently, we reported that *in vitro* inhibition of T-B cell contact by anti-CD40 ligand and anti-CD86 totally abrogated T cell induced Ig-production (Ingvarsson et al., 1995), thus showing the importance of these costimulatory molecules in activation of B cells.

A flowchart of the experimental procedure devised in this study is presented in figure 4. SEA priming of $CD19^+$ B cells prior to transplantation into SCID mice together with autologous purified T cells, resulted in more uniform and higher levels of serum IgG. The Ig production in mice receiving SEA primed B cells also displayed an uniform κ/λ ratio close to that normally observed in human plasma, whereas mice receiving non-primed B cells, displayed varying ratios between individuals. The varying success of B cell engraftment, the variable Ig levels and κ/λ ratio often obtained in SCID-hu-PBL, has been proposed to reflect that only a limited and skewed repertoire of B cells, actually survive in the SCID environment (Saxon et al., 1991). However, the activation level of the cells may also be important for proper engraftment (personal observation, Tary-Lehmann and Saxon, 1992). The more uniform Ig levels and the normal κ/λ ratio obtained in the present study, may therefore reflect that more B cells have been able to engraft properly. Immunization with both primary DNP and GM2 and secondary antigens (TT) raised high titers of antigen specific human Ig. In the case of the primary antigens, only specific IgG was detected, whereas TT immunizations also showed a specific

IgM response. The IgM anti-TT response was, however, not influenced by priming of SEA. The ratio of specific antibody to total IgG, was significantly higher in mice receiving SEA primed B cells, thus indicating a co-operative effect of antigen-specific crosslinking and SEA priming.

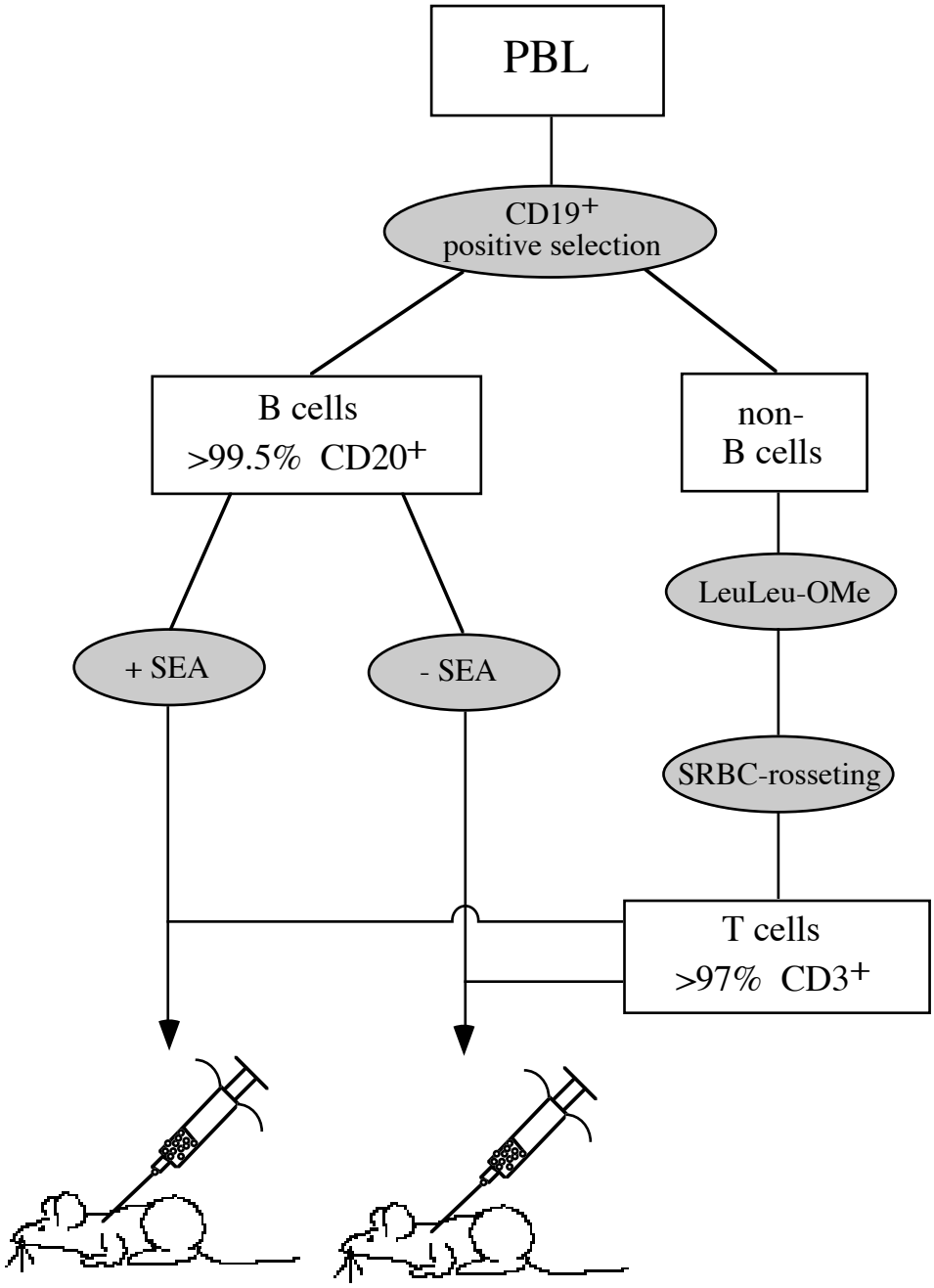

Figure 4. Schematic drawing of the experimental setup as described in paper IV

7. GENERAL DISCUSSION

The results obtained in these studies demonstrated that the SCID-hu-PBL model is fully capable of generating human B cell responses against both recall and primary antigens. We have routinely obtained human IgG responses to antigens that have been considered as primary antigens, which could indicate that SCID-hu-PBL mice also support Ig class switching. In these studies we have used immunocytes from adult donors and accordingly we have to be very careful in the interpretations of primary responses. The recirculating peripheral B cell pool consist mostly of $\mu^+\delta^+$ B cells with a distinct selection of V_H families, which indicates that some kind of ligand selection has occured during ontogeny as suggested by Gu et al. (1991). We showed (paper II) that GD2 reactive B cells had germline or near germline V_H sequences and the random distribution of the mutations indicate that the mutations in these cells were not a result of germinal center selection.

A B cell undergone even very few mutations in its Ig genes can hardly be termed as a naive B cell, but in these studies we define a primary B cell, as one that has not been exposed to antigenic challenge within a germinal center environment, resulting in the generation of memory cells and detectable specific Ig in donor serum. Furthermore, we have used antigens such as DNP, OSM and V3 (HIV-1) that normal donors are not expected to be sensitized against previously. We can not rule out that the response generated to some extent represents selection of previously sensitized cross-reactive B cells. Thus, the primary responses obtained in these studies can only be defined operationally, with the limitations regarding differentiation status of the responding cells. The very high IgG titers obtained against the gangliosides GD2 and GM2 incorporated into liposomes, were comparative in magnitude to responses obtained with TT as judged from the optical densities obtained in ELISA tests, although serologic analysis of the donors revealed no initial response against these gangliosides. Since these high responses were elicited in SCID mice transplanted with cells from apparently non-immune donors, this raises the question if the SCID-hu-PBL model is capable of inducing somatic mutations and affinity maturate the antibody response, or is it a very powerfull selection system, capable of selecting rare specificities not otherwise detected by serologic analysis of the donor or by EBV transformation of donor B cells?

We have been very interested in this particular question, and major efforts in this study have been devoted to recover and isolate specific human B cells from the chimeras. Both for the purpose of examining the progress of the immune response in the chimeras, but equally as important, to obtain the generated specificity. Numerous attempts have been made to recover B cells of various tissues from high-responding chimera, for subsequent EBV-infection or somatic cell hybridization to establish antigen-specific B cell lines. However, all such attempts failed. The same result was found when trying to salvage the recovered B cells using the CD40 or EL-4 system, known to rescue B cells and promote B cell differentiation efficiently (Banchereau et al., 1991; Wen et al., 1987). Although outgrowth of B cells were evident proved by production of human Ig, we never found any specific cells. In a representative study we followed the immune response in SCID-hu-PBL against TT during 12 weeks. Each second week, cells were recovered from bone marrow, spleen, liver, peritoneal cavity and lymph nodes of the transplanted mice. The cells were depleted for human $CD3^+$ and murine class I, and cultured under limiting dilution conditions on the EL-4 system (Wen et al., 1987). We found only human Ig being produced from cells derived from spleen and peritoneal cavity. At no point of time were any antibodies specific for the immunogen found. However, the outgrowth and frequency of Ig producing cells, increased during the experimental period, starting at week 2 with only 5% of the wells being Ig positive and at week 10 nearly 80% of the cells were Ig positive. This indicates, that human B cells with selfrenewing capabilities expand in the SCID environment during this period, as also suggested by Tary-Lehmann and Saxon (1992).

In other attempts to rescue the generated specificities, RNA were isolated from TT immunized chimeras, transcribed into cDNA followed by PCR amplification of human γ–, κ– and λ–chain sequences. Human Ig heavy and light chains were found from cells isolated from blood, liver, spleen, peritoneal cavity and lymph nodes, but we have never been able to find any antigen specific Fab's, when the isolated DNA were used to construct Fab combinatorial libraries in phages. During initial experiments low amounts of human Ig were obtained when total RNA was isolated from tissues followed by oligo dT primed cDNA synthesis. Higher yields were obtained when mRNA was captured by oligo dT beads and cDNA synthesis was initiated with specific primers. The human heavy and light constant region primers were, however, found to cross-react to the murine equivalent since this region is very conserved cross-species. Redesigning of primers with a nucleotide mis-match at the terminal 3'-end revealed that mis-

match primers yielded substantial lower amounts of Ig as visualized by the intensity of the PCR band on agarose gels. No difference between mis-match and normal primers was observed when cDNA from human B cells was used as template. This indicates that endogenous production of Ig might be a problem and careful selection of primers is obligatory to avoid contamination of human Ig sequences with murine sequences. Another problem we encountered was difficulties in amplifying human V_L sequences although, we had detected V_H sequences from the same tissue. The yields of isolated human RNA were generally low, severely limiting the amounts of cDNA available for subsequent analysis and often the PCR parameters had to be changed and optimized individually in each experiment.

In a single atypic study we were able, though, to retrieve antigen specific cells from SCID-hu-PBL. SCID mice were transplanted with PBL from a melanoma patient and immunized with the melanoma associated antigen p97 (Fig. 4). Although we found normal levels of human Ig in mouse sera, we could only detect a very low IgG response against p97 (OD <0.3). However, cells recovered from bone marrow, lymph nodes and the peritoneal cavity, proliferated vigorously on EL-4 cells and p97 specific responses from immunized mice were obtained. This study differed from the other studies, in that no measures had been taken to increase the likelihood of specific T cell help. Melanoma patients do not appear to elicit humoral or cellular immune response against p97 (Estin et al., 1988), and serologic analysis of donor serum revealed no p97 response. Accordingly, the donor PBL would not be expected to have any p97 specific T cells and the chimera environment is not expected to support generation of specific T cells as mentioned in section 5.2.3. The very low levels of specific antibodies in mouse sera, could be due to unspecific T cell help or bystander effects. The surprising finding of the recovered specific B cells, suggests that p97 immunization had rescued specific B cells from apoptosis, and made them able to survive but not differentiate within the SCID environment. If this holds true, it might to some extent explain our problems to recover the specific cells generated in experiments with specific T cell help. Since it is not possible to somatically hybridize, EBV-transform or *in vitro* stimulate plasma B cells, we hypothesize that antigen selected B cells receiving T cell help from TT specific T cells within

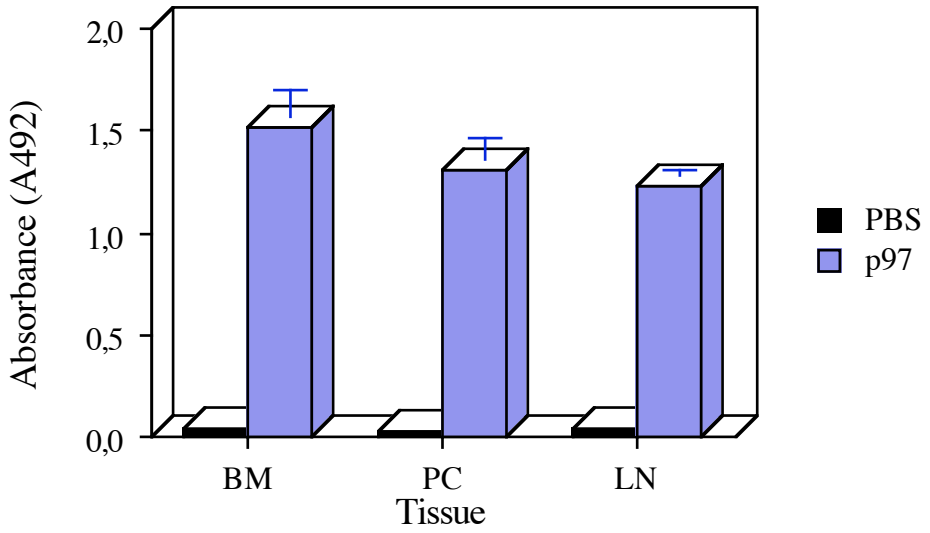

Figure 4. Specific human IgG response against p97 from B cells recovered from SCID-hu-PBL after 5 days *in vitro* culture on EL-4 cells. SCID mice were transplanted with PBL from a donor suffering from malignant melanoma. Mice were immunized with 5 μg p97 each 14 days during an 8 week period. Mice were killed and cells were recovered from peritoneal cavity (PC), lymph nodes (LN) and bone marrow (BM). The cells were cultured *in vitro* on irradiated EL-4 cells and stimulated with supernatant from PWM stimulated T cells. The culture supernatant was diluted 5 times and the antigen specific response was measured by ELISA.

the SCID-hu-PBL environment, terminally differentiate into plasma cells and not into memory cells capable of selfrenewing processes. That the B cell responses induced in our model does not evolve into a persistent B cell response is indicated by the fact that, in most of our studies the antigen specific responses peak around day 30-40 and then fades away, whereas the total Ig levels may persist for extended periods (>70 days). Direct proof of this hypothesis has been difficult to obtain as a consequence of the problems in retrieving the cells. However, in a kinetic study we found more cells staining for the plasma cell marker PCA-1 than for CD19 in the peritoneum 14 days post transplantation, whereas later on we were not able to recover PCA-1$^+$ B cells from peritoneum or other tissues. Again indicating, that some B cells terminally differentiate within SCID-hu-PBL, but it can not be excluded that the cells have migrated to a distant site where they were not accessible by peritoneal lavage. Further, it was shown that high doses of TT totally abrogated the specific response in SCID-hu-PBL, whereas late

administration of high doses of TT to responding mice were not able to inhibit the antigen-specific response (Carlsson et al., 1992), suggesting that the antigenically activated B cells had differentiated to a state that became refractory to the inhibitory doses

In normal mice, antigen-binding B cells are found within the periarteriolar lymphoid sheat (PALS) a few days after immunization (Liu et al., 1991). At this point of time clusters of B cells are recruited by specific T helper cells resulting in the development of large oligoclonal foci of antibody forming cells and it is belived that Ig class switching might occur to some extent (Jacob et al., 1991a). Later the B cells migrate to a nearby locus and form a germinal center (Jacob and Kelsoe, 1992), where it is generally accepted that somatic mutations and affinity maturation takes place (Berek et al., 1991; Jacob et al., 1991b). In SCID-hu-PBL, human B and T cells have been found to migrate into PALS together with occasional follicle development (Duchosal et al., 1992a, Hesselton et al., 1993), but no one has succeded in finding evidence of germinal centers in SCID-hu-PBL. This suggests that human B cells might be in the proper environment for inducting class switching, whereas it is highly doubtful that affinity maturation could happen outside the germinal center environment.

It has been argued that only a limited and skewed repertoire of B cells actually survive in the SCID environment (Saxon et al., 1991). The survival of B cells is, however, dependent of their state of activation. No responses are generated if immunization happens more than a few days after repopulation (personal observation; Marcus et al., 1995; Smith et al, 1991). It is possible that those B cells that survive are cells that are capable of selfrenewing, cells that are stimulated by antigen challenge or cells that become stimulated by the murine environment resulting in generation of antibodies against murine antigens as RBC (Saxon et al., 1991, Pirruccello et al., 1992). It has also been suggested that human mature T cells become anergic and refractory to stimulation in the SCID-hu-PBL (Tary-Lehmann and Saxon, 1992). T cells recovered from mice that had not been challenged with antigen besides murine endogenic antigens could not be stimulated with anti-CD3. It was also shown that these T cells recognized murine antigens, suggesting that they became anergic as a result of antigen presentation on murine cells. Many reports have, however, shown the presence of functional T cells in SCID-hu-PBL (Torbett et al., 1991; Shiroki et al., 1994; Hasui et al., 1994; Alegre et al., 1994). Duchosal et al. (1992a) suggested that the human immune

response generated in SCID-hu-PBL is a result of unspecific T cell help. The responses generated to primary antigens in our studies have, however, been dependent of help from TT specific T cells, since we could not detect any response unless the B cell antigen was physically linked to the T cell antigen. Furthermore, we showed that although mice repopulated with splenocytes had human Ig levels, we were only able to obtain a specific response when the mice were cotransplanted with a TT specific T cell clone.
As discussed in section 5.2.2, we have not observed clinical signs of GvHD among mice transplanted with human PBL. In our model we always pretreat PBL with LeuLeuOMe which depletes NK cells, and human NK-cells has been shown in part to be responsible for GvHD in SCID-hu-PBL (Xun et al., 1993). However, we have observed indications of GvHD in SCID.BG-hu-PBL, indicating that endogenous NK cells might be important to control GvHD development as also suggested by Pirruccello et al. (1992) and Pflumio et al. (1993).

The PBL used to engraft SCID mice in our studies have, unless otherwise indicated, been taken from normal donors and were not screened for EBV infection. Accordingly, most of the PBL used were probably EBV infected. This has not been a major problem in our studies, despite that LeuLeuOMe treatment removes cell populations that normally inhibit the outgrowth of EBV infected B cells *in vitro* (Ohlin et al., 1992b). Lymphomas occassionally develop in longterm studies (>60 days), but normally our studies are limited to 30 - 40 days and we have not experienced any LCL outgrowth in our transplanted SCID mice. The outgrowth of EBV induced lymphomas may to a certain extent be under control of murine NK cells, since repopulation experiments in SCID.BG with low NK cell activity, results in fatal lymphoma development within 30 days, whereas no lymphomas were observed in SCID mice transplanted with PBL from the same donor.

The very high anti-GD2 response obtained in paper II emphasize the ability of SCID-hu-PBL both to generate an immune response and as a vaccine model. The efficacy of the GD2-liposome construct to elicit a humoral response was also shown in follow-up experiments to be true for GM2 liposomes. Much attention has been payed to develop a GM2 based vaccine, since GM2 has been shown to be partly immunogenic in humans (Yamaguchi et al.,1990; Kitamura et al., 1995). As mentioned previously, vaccine trials in melanoma patients with GM2-BCG or

GM2-KLH vaccines have only shown limited results. The results obtained in our studies, suggest that a liposome construct containing encapsulated T cell antigens like TT, may prove to be very valuable as vaccines against weak immunogenic carbohydrate antigens. We have later repeatedly obtained high human IgM and IgG responses against GM2 in SCID-hu-PBL. Occasionally, we found SCID-hu-PBL only receiving PBS also contained GM2-specific human antibodies in their sera. This probably represents mice transplanted with PBL from individuals having GM2-specific autoantibodies, as has been reported to occur in 5-10% of normal donors (Kitamura et al., 1995). Surprisingly, when SCID-hu-PBL from such donors were immunized with GM2 liposomes alone without any T cell antigens, the GM2 response was abolished. Two representative independent studies, using either TT or SEA encapsulated into liposomes, are shown in fig. 5. When we compared all the studies we have done with GM2 immunizations, it became evident that, we have never observed a specific response when mice were immunized with GM2 liposomes alone without any T cell antigen as shown in figure 5. A dose-dependent inhibition was also seen in an *in vitro* study using B cells activated by GM2 crosslinking and SEA-mediated T cell help (Ingvarsson et al., 1995). These findings indicate that GM2 may be immuno-

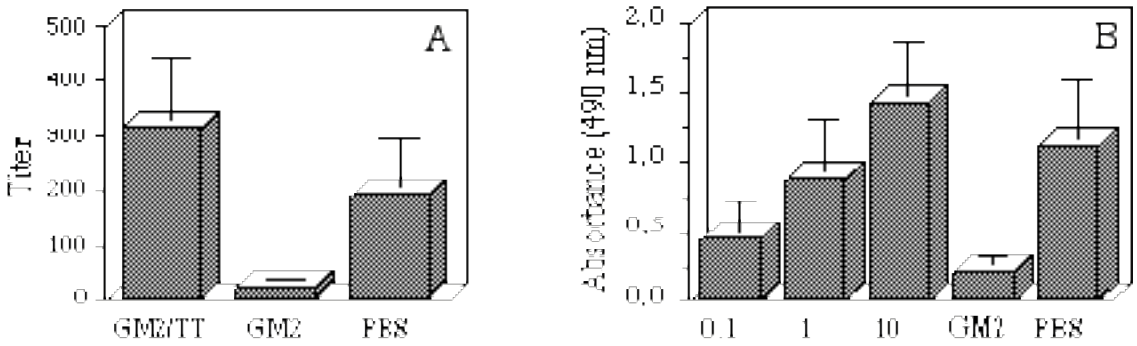

Fig. 5. Human IgM anti-GM2 responses in SCID-hu-PBL obtained in two independent studies. Mice were transplanted with PBL from normal donors and immunized with (A) GM2 liposomes containing TT, GM2 liposomes without TT and PBS, (B) GM2 liposomes containing 0.1, 1 and 10 ng SEA, GM2 liposomes without SEA and PBS. Serum was obtained on day 36 and the human response was measured by GM2 ELISA. Each bar represents the mean of (A) 5 animals and (B) 3 animals.

suppressive, which could explain the very negative results with GM2 trials in melanoma patients. It has been shown that melanomas secrete high amounts of gangliosides (Valentino and Ladisch, 1992), and it is possible that these very aggresive and malignant tumor cells evade the control of the immune system by

inducing anergy or paralyse the immune cells. Much controversy has, however, been implicated in the role of gangliosides as immunomodulatory agents. Many *in vitro* experiments have actually indicated a strong immunosuppressive effect of gangliosides, but only in the absence of serum (Ladisch et al. 1992; Kimata and Yoshida, 1994; Dumontet et al., 1994; Kaucic et al., 1994). Criticism has been raised because the *in vitro* experiments are not physiological relevant, and in the presence of serum, gangliosides very rapidly binds to serum products. Our data obtained in the SCID-hu-PBL, suggests that GM2 presented in liposomes are able to suppress GM2 specific cells, but the results also suggest that the suppression is antigen specific rather than systemic, since total Ig levels are not affected.

8. CONCLUSION

EBV technology is still one of the most efficient methods to activate human B cells *in vitro* and to obtain long term cultures of antibody producing B cells. The demand of EBV-infected or immortalized B cells to respond to various growthfactors with the purpose of preventing loss of antigen-specific cells, varies from case to case. Recently, we reported on a GM3 specific B cell clone, that could not be stabilized by either somatic fusions or cloning on MP6 cells but has been maintained in culture by frequently isolating the specific cells on GM3-coated beads (Alfonso et al., submitted). We have not been able to apply EBV technology to the SCID-hu-PBL model for the purpose of immortalizing the specific B cell responses generated in SCID-hu-PBL, possibly as a cause of a late activation state of the specific cells, rendering these cells insensitive to EBV-infection.

It has been proposed that the absence of proper secondary lymphoid environment in SCID-hu-PBL, the low numbers of widely dessiminated lymphoid cells and incomplete lymphocyte populations have hampered the attempts to generate primary responses in chimeras (Pflumio et al.,1994; Marcus et al., 1995). The studies presented in this thesis shows that we have overcome some of these problems and been able to induce human IgM and IgG responses against primary antigens. The lack of germinal center formation in chimeras, argue against affinity maturation of the responses and the observed responses are probably based more on a very efficient selection of specificities. The major pitfall of the SCID-hu-PBL model is the recovery of specific cells, if the object is to generate human monoclonal antibodies. One reason is the occasional dessimination of human cells in the recipients and as indicated in our model, specific stimulated B cells may terminally differentiate into plasma cells not capable of selfrenewing. Marcus et al. (1995) have recently reported a new approach to generate human/mouse chimeras. They transplanted human PBL into lethally irradiated Balb/c mice, radioprotected with intraveneous injection of SCID bone marrow cells. These mice have an intact structure of secondary lymhoid organs and the transplanted human cells were found to home to lymphoid organs within 24 hours, together with appearance of primary follicular centers in these organs. Using this model they were able to generate primary responses against KLH and Nef (HIV-1). Another approach is to construct transgenic mice expressing human heavy and light chain genes, which succesfully has been accomplished by constructing a

minilocus containing human μ, γ1 and κ chains (Lonberg et al., 1994, Taylor et al., 1993). These mice responded to antigenic challenge resulting in heavy chain class-switched and somatic mutated human antibodies. However, these mice does not encode the whole human Ig locus, and the antibody repertoire is accordingly restricted. Further improvement in this model as well as the model proposed by Marcus et al. (1995), may prove to be superior to the SCID-hu-PBL with respect to a model system to obtain human antibodies.

The results we have obtained in our SCID-hu-PBL model to elicit a human immune response to a cancer vaccine and the indications of a possible suppressive effect of GM2, as well as the ability to induce protective immunity against HIV-1 (Mosier et al., 1993b) and streptococcal infections (Aaberge et al., 1992), supports the use of the SCID-hu-PBL model as an *in vivo* model to dissect and evaluate the human immune response in vaccine studies.

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