Invited Review

hu-PBL-SCID mice: an in vivo model of Epstein-Barr virus-dependent lymphoproliferative disease

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Summary. The Epstein-Barr virus (EBV) exists in most humans as a lifelong latent infection established in host B cells after a primary viral encounter. In immuno-suppressed individuals, such as post-transplant patients, the presence of EBV-infected B cells may lead to lymphoproliferative disease. Injection of human peripheral blood lymphocytes from EBV-positive donors into mice with severe combined immunodeficiency induces human lymphoproliferative disease in the recipient closely resembling that of human post-transplant patients. This xenochimeric human-mouse model is increasingly being used to elucidate the mechanisms of EBV-specific lymphomagenesis and to assess novel therapeutic approaches.

Key words: Epstein-Barr virus, Lymphoproliferative disease, hu-PBL-SCID mouse

Introduction

Lymphomagenesis in humans is a highly complex process involving numerous events that ultimately lead to acquisition of the neoplastic phenotype. A particular subset of lymphoma, called lymphoproliferative disease (LPD), arises in immunosuppressed individuals as a result of the expansion of EBV-immortalized B cells. Severe combined immunodeficient (SCID) mice can be populated with human peripheral blood leukocytes (PBL, hu-PBL-SCID mice) (Mosier et al., 1988), and these xenochimeras possess many characteristics of the human immune system, including development of EBV-related LPD. Thus, hu-PBL-SCID mice provide a useful model for the *in vivo* study of the events underlying the uncontrolled growth of specific EBV-infected B cell clones.

This review describes the main characteristics of SCID mice, of the transferred human immune system, and of human lymphomagenesis in the chimeric animals. Emphasis is given to the characterization of LPD as well

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as to the current state of research regarding the treatment of LPD in hu-PBL-SCID mice.

The SCID mouse

Severe combined immunodeficient (SCID) mice were first described by Bosma and collaborators (Bosma et al., 1983). These animals are homozygous for an autosomal recessive mutation mapped to the centromeric region of chromosome 16 (Bosma et al., 1989), and display defective V(D)J recombinase system (Kim et al., 1988; Lieber et al., 1988; Malynn et al., 1988; Okazaki et al., 1988; Hendrickson et al., 1990; Carroll and Bosma, 1991) and DNA-double-stranded break repair (Fulop and Phillips, 1990; Hendrickson et al., 1991), defects associated with deficient DNA-dependent protein kinase activity (Blunt et al., 1995; Boubnov and Weaver, 1995; Kirchgessner et al., 1995). As a result, SCID mice cannot synthesize functional T cell receptors or immunoglobulins (Ig) and are thus deficient in mature T- and B-cells. The maturation arrest, however, is not absolute. A minor proportion (~15%) of young mice (< 6 weeks old) possess few functional B- and T-cells generated by somatic events, low levels of serum Ig, and are considered "leaky" (Bosma et al., 1988). The incidence of leakiness increases with age until 10 to 14 months, when virtually all SCID mice display a "leaky" phenotype (Bosma et al., 1988; Carroll and Bosma, 1988; Carroll et al., 1989; Young and Kearny, 1995). In SCID mice, lymphoid tissues are atrophic with the exception of the bone marrow (Custer et al., 1985; Bosma and Caroll, 1991). For example, the thymus is about one tenth its normal size. Monocytes, granulo-cytes, megakaryocytes, erythrocytes and natural killer (NK) cells, which do not need the expression of rearranged genes for their development, differentiate normally and are functional in SCID mice (Dorshkind et al., 1984, 1985; Bancroft et al., 1986).

Due to their profound immunodeficiency, SCID mice do not reject xenogeneic transplants. The first transfer of non-malignant xenogeneic human graft was reported concurrently by two groups. McCune and collaborators (McCune et al., 1988) surgically implanted human fetal thymus and lymph node pieces, and intravenously

injected fetal liver cells into SCID mice (SCID-hu mice). These SCID-hu chimeras supported the differentiation of mature human T cells and human Ig were detected in the chimeric mouse sera. Similarly, Mosier and collaborators (Mosier et al., 1988) populated SCID mice with purified human peripheral blood leukocytes (PBL), and the resulting hu-PBL-SCID mice had human B- and T-cells as well as detectable levels of human Ig in their sera for several months.

The hu-PBL-SCID mouse model

Since the original report of human leukocytes xenografted in SCID mice 9 years ago, hu-PBL-SCID mice have been used in various areas of research, including production of monoclonal antibodies (Carlsson et al., 1992; Duchosal et al., 1992a; Walker and Gallagher, 1994), the study of viral (Mosier, 1991), and parasitic (Seydel and Stanley, 1996) infections, and the action mechanisms of antiviral or antitumor therapeutic agents (Abedi et al., 1992; Boyle et al., 1992a,b; Garnier et al., 1993; Hersh et al., 1993). In addition, injection of lymphoid cells from patients with autoimmune diseases into SCID mice resulted in expression and subsequent *in vivo* study of primary biliary cirrhosis, rheumatoid arthritis, thyroiditis, myasthenia gravis and systemic lupus erythematosus (reviewed in Duchosal, 1992).

The successful population of SCID mice with human PBL, reflected by increasing serum levels of human Ig in the recipient, depends on the extent of manipulations of the cells prior to their transfer (Duchosal et al., 1992b), and on the number of PBL injected (reviewed in Mosier, 1991). Typically, the injection of $20\text{-}50\times10^6$ PBL/mouse yields serum levels of human IgG ≥500 μg/ml 30 days after cell transfer. The transfer of less than 10^7 cells per mouse populates successfully only a fraction of mice (Duchosal et al., 1992b). This variability remains to be fully elucidated, but probably reflects quantitative differences between small aliquots of terminally-differentiated cells, and may be amplified by the resistance of the rudimentary murine immune system to the xenografting of a limited number of human cells.

As mentioned above, SCID mice do possess functional myeloid cells, and intra-peritoneal injection of human PBL in SCID mice results in a complex immune response, including neutrophil recruitment into the peritoneal cavity and induction of a wide array of murine cytokines (Santini et al., 1995). Depletion of endogenous mouse NK cells by irradiation or pretreatment of the recipient mice with an anti-asialo G_{M1} antibody improves human cell engraftment, as assessed by the higher Ig levels in hu-PBL-SCID mouse sera than those in untreated xenochimeric animals (Sandhu et al., 1994; Shpitz et al., 1994; Lacerda et al., 1996a). SCID mice injected with PBL from donors with previous Epstein-Barr virus (EBV) contact, routinely assessed by the presence of anti-EBV antibodies in the donor's sera (EBV-seropositive donors), usually develop peak serum Ig levels higher than those obtained after transferring

similar numbers of PBL from EBV-seronegative donors (Mosier et al., 1988; Duchosal et al., 1992b).

The distribution of human cells in hu-PBL-SCID mouse tissues and organs has been reported (Duchosal et al., 1992b; Hoffmann-Fezer et al., 1992). Human cells are detectable in the peritoneal cavity and peripheral blood, but not the organs, of hu-PBL-SCID mice 15 days after PBL transfer. After 30 days, human cells are found in the non-lymphoid organs, mostly as perivascular infiltrates. In the spleen, the human cells are mainly present in the white pulp. In general, T cells expressing the CD45RO memory phenotype marker, with either the $CD3^+CD4^+CD8^-$, or the $CD3^+CD4^-CD8^+$ markers, are primarily (95%) detected in the mouse (Duchosal et al., 1992b; Tary-Lehmann and Saxon, 1992; Tary-Lehman et al., 1995). Human T cell engraftment can be enhanced by the injection of human growth hormone in recipient mice (Murphy et al., 1992). The human graft in this model contains few B cells, and their presence is routinely inferred from the detection of human Ig in the xenochimeric mouse sera (reviewed in Torbett et al., 1991). Human B cells have been found in the spleen, peritoneal cavity and peripheral blood of xenochimeric animals. In our hands, using a procedure that minimizes the time and manipulation of human cells prior to transfer, hu-PBL-SCID mice regularly develop clinical (i.e.: anemia) and/or histological (perivascular infiltration of T cells and fibrosis) signs characteristic of graft-versus-host disease (Duchosal et al., 1992b).

Initial reports indicated that hu-PBL-SCID mice possess a limited repertoire of human Ig-producing B cells 50-120 days after xenotransplantation (Saxon et al., 1991; Abedi et al., 1992; Duchosal et al., 1992c). Recently, using a highly sensitive 2D-PAGE technique, polyclonal production of human Ig was observed in hu-PBL-SCID mice during the first three weeks after cell transfer (Tissot et al., 1996), indicating that a large repertoire of human B cells populate recipient mice shortly after human PBL transfer. After this 3-week period, a progressive shift towards oligoclonal Ig production was observed. Human IgG (all four subclasses), IgM, IgA, and trace amounts of IgD and IgE (Saxon et al., 1991; Abedi et al., 1992; Duchosal et al., 1992b,c) are detected in xenogeneic mouse sera. Detectable levels of human serum IgG and IgM are present in hu-PBL-SCID mice one week after human PBL transfer. These levels gradually increase, peak at 0.5-5 mg/ml 60-90 days post injection, which corresponds to 5-50% of the donor's levels (Mosier, 1990; Mosier et al., 1990, 1992; Saxon et al., 1991; Abedi et al., 1992; Duchosal, 1992; Duchosal et al., 1992a-c), and then slowly decline without returning to zero for up to two years (Duchosal et al., 1992c, and personal communication). Thus, human Ig in xeno-chimeric mice do not result from the mere adoptive transfer of human Ig, but from a spontaneous secretion of human Ig by the transferred human B cells. Mice injected with PBL from EBV-seropositive donors are prone to develop spontaneous LPD of human B cell

origin (Mosier et al., 1990), the frequency and latency of which varies between donors and is usually associated with a continuous increase in serum human Ig levels (Picchio et al., 1992).

Injection of purified B cells in SCID mice does not induce human Ig production highlighting the requirement of a helper (CD4$^+$CD8$^-$) T cell stimulatory component for B cell function in the model (Veronese et al., 1992; Bombil et al., 1995). The cooperation between B- and T-cells in the hu-PBL-SCID mouse model is further exemplified by the capability to obtain a secondary immune response elicited upon boost with tetanus toxoid (TT), a thymus-dependent recall antigen (Mosier et al., 1988; Carlsson et al., 1992; Duchosal et al., 1992a). The levels of anti-TT antibody obtained in boosted hu-PBL-SCID mice are similar to, or higher than, those in immunized donors, indicating that the transferred human immune system in the model remains highly functional. Primary immune responses, generally of low levels, have been observed following antigenic stimulation with 1) keyhole limpet hemocyanin (KLH), 2) a protein of the circumsporozoite malaria parasite, 3) a carbohydrate antigen (AcNeu-α2-α6-Gal NAc-0) coupled to KLH (Sandhu et al., 1994), 4) a schistosomial antigen (Mazingue et al., 1991), and 5) a hepatitis B core antigen (Duchosal et al., 1992a). In the first three cases, pretreatment of the mice with an anti-asialo G$_{M1}$ antibody and irradiation to eliminate endogenous mouse NK cells were required to generate a detectable response, indicating that the primitive immune system of SCID mice partially resists or inhibits human cell engraftment and/or function.

The Epstein-Barr virus

The Epstein-Barr virus (EBV) is a ubiquitous human DNA herpesvirus that infects the majority (>90%) of the population worldwide (reviewed in Kieff and Liebowitz, 1990). Primary encounter with EBV results in a silent infection or a symptomatic, self-limiting disease known as infectious mononucleosis (IM). The virus is also the causative agent of several human malignant diseases, including nasopharyngeal carcinoma (NPC), endemic Burkitt's lymphomas (BL) in Africa, and some LPD in immunosuppressed individuals (reviewed in Miller, 1990a).

In humans, primary EBV infection occurs orally and is first established in the mucosal epithelial cells, followed by B lymphocytes. Binding of EBV to B cells is mediated through the interaction between the major viral envelope glycoprotein gp350/220 and the C3d complement receptor (CR2) on B cells (Jondal et al., 1976; Nemerow et al., 1985; Tanner et al., 1987). In immunocompetent individuals, the acute EBV infection is controlled by humoral and cell-mediated immune responses. However, a small fraction of B cells remains latently infected, and establishes a lifelong asymptomatic virus carrier state in the host. In addition, a continuous low degree of viral replication is detected lifelong in oropharyngeal epithelium of healthy carriers (Kieff and Liebowitz, 1990).

Two virus subtypes, namely EBV-1 and EBV-2, are distinguished by their divergent latent viral protein amino acid sequences (Khanna et al., 1995) and by their various transformation potentials *in vitro* (Rickinson et al., 1984). The EBV-2 subtype is commonly detected in African populations, is associated with 50% of BL, and is rarely found in individuals within western societies, where the EBV-1 subtype is primarily found.

In vitro, EBV infection of B lymphocytes induces continuous cell proliferation and gives rise to lymphoblastoid cell lines (LCL). Alternatively, spontaneous formation of LCL occurs in *in vitro* cultures of PBL from EBV-seropositive donors in the presence of cyclosporin A (CsA), an immunosuppressive drug, or following depletion of T cells (Rickinson et al., 1984). This transformation process initially requires activation of the viral lytic cycle and occurs by expansion of bystander cells secondarily infected *in vitro* rather than by proliferation of the originally infected EBV-carrying cells (Yao et al., 1991).

In infected B cells, three types of latency (Latency I, II, and III) have been defined and are characterized by expression of various latent viral genes (reviewed in Rickinson and Kieff, 1996). Latency III is typically represented by LCL, in which the viral genome is maintained in a latent episomal form, and a limited number of viral latent genes are expressed comprising the six EBV nuclear antigens (EBNA-1, -2, -3A, -3B, -3C, and -LP), the three integral membrane proteins (Latent membrane protein: LMP-1, -2A, and -2B), and two small nonpolyadenylated RNAs (EBV-encoded RNA: EBER-1 and -2) (reviewed in Khanna et al., 1995). Five of these gene products, EBNA-2, -3A, -3B, -3C, and -LP, are necessary for transformation *in vitro*, while EBNA-1 is the only protein required to maintain episomal (latent) infection. Typically, 1 to 50 episomal copies of the viral genome are contained in LCL cells and are replicated during the cell cycle by the cellular DNA polymerase (Gregory et al., 1987). Latency I is observed in BL and NPC with limited expression of viral latent genes comprising the EBNA-1 protein and the two EBERs. Latency II is found in NPC where EBNA-1, the two EBERs, and LMP proteins are expressed. Both Latency I and II have been observed in virus-infected tumor cells and have been reproduced experimentally *in vitro* (reviewed in Rickinson and Kieff, 1996).

Activation of the lytic cycle occurs spontaneously or can be triggered in latently-infected cells by transient *in vitro* exposure to drugs such as 12-O-tetradecanoyl-phorbol-13-acetate (TPA) (Hudewentz et al., 1980), calcium ionophore (Faggioni et al., 1986), or anti-Ig antibodies (Takada, 1984; Takada and Ono, 1989). The first step in the induction of the lytic cycle is the expression of the *Bam*HI Z EBV replication activator protein (ZEBRA) from the BZLF1 gene (Miller, 1990b). The ZEBRA protein is a transactivator that subsequently activates the expression of other viral genes important in

the lytic cascade. The prime importance of BZLF1 in the induction of the viral lytic cascade has been demonstrated *in vitro*. Inhibition of BZLF1 expression following treatment of cells with antisense oligo-deoxynucleotides directed against the specific transcript also inhibited EBV lytic infection (Daibata et al., 1996).

Control of primary and latent EBV infection

Individuals suffering from IM develop strong humoral and cell-mediated immune response against EBV (reviewed in Rickinson and Kieff, 1996). Antibodies (Ab) to numerous viral antigens are observed during acute IM, an immune response first characterized by the appearance of IgM Ab and followed by a switch to IgG, this latter usually concomitant with convalescence. The Ab observed are directed against various viral antigens, including latent EBNA proteins, the viral-encoded nucleocapsid (VCA) expressed during lytic infection, and viral membrane antigens (MA) expressed on the surface of lytically-infected cells. Neutralizing IgG Ab are predominantly directed against the viral gp350 protein responsible for the attachment of the virus to B cells. Healthy EBV-carriers have lifelong titers of anti-VCA, anti-gp350, and anti-EBNA-1 Ab at levels that vary widely between individuals.

The cellular immune response observed during IM and in healthy carriers is predominantly mediated by CD8$^+$ cytotoxic T lymphocytes (CTL), although some CD4$^+$ T cells, and NK-like responses have been observed (Rickinson, 1986; Rickinson and Kieff, 1996). *In vitro*, EBV-specific HLA class I-restricted CTL can be generated by exposing T cells from EBV-seropositive individuals to autologous EBV-transformed lymphocytes (Rickinson et al., 1980). These EBV-specific CTL: 1) target eight of the latent viral proteins expressed in LCL (EBNA-2, -3A, -3B, -3C, -LP, LMP-1, -2A, and 2B) (Khanna et al., 1995), and 2) control the expansion of EBV-infected cells, as demonstrated *in vitro* by the fact that such CTL allow regression of EBV-infected B-cell outgrowth in cell cultures (Rickinson, 1986). Specific CTL also inhibit EBV-infected cell proliferation *in vivo*. In post-transplant patients under immunosuppressive therapy who develop LPD (Cleary et al., 1986), the injection of the donor's EBV-specific CTL expanded *in vitro* induces disease regression (Rickinson et al., 1984; Papadopoulos et al., 1994; Rooney et al., 1995; Lacerda et al., 1996b).

Although various forms of latency have been described, little is known about the nature of the persistence of the EBV infection in healthy carriers. A recent report indicates that, in the peripheral blood of healthy individuals, EBV persists predominantly in resting CD19$^+$CD23$^-$CD80$^-$ B cells (Miyashita et al., 1995). Therefore, cells latently infected with EBV *in vivo* are different from proliferating EBV lymphoblastoid CD23$^+$ B cells observed *in vitro*.

hu-PBL-SCID mice as an *in vivo* model for EBV-dependent lymphoproliferative disease

EBV-specific B-cell lymphoproliferative disease

Transfer of high numbers of PBL from EBV-seropositive donors to immunodeficient mice results in LPD development in the recipient (Mosier et al., 1988). These LPD resemble those occurring in immuno-suppressed individuals. It is now widely accepted that tumor formation in hu-PBL-SCID mice arises as a direct consequence of the presence of EBV-infected human B cells (reviewed in Mosier, 1991). Molecular analysis of tumor DNA using EBV-specific probes confirmed the presence of human EBV DNA in all tumors tested (Torbett et al., 1991; Duchosal et al., 1992b; Picchio et al., 1992). Moreover, no LPD was observed in SCID mice receiving PBL from EBV-seronegative donors. Human PBL from EBV-seronegative donors injected to SCID mice could be infected *in vivo* by EBV. This led to tumor development of human B cell origin in the animals (Boyle et al., 1992a,b; Bombil et al., 1995).

A great variability in the appearance and kinetics of LPD is observed when SCID mice are populated with PBL derived from various EBV-seropositive donors (Mosier et al., 1990; Picchio et al., 1992). Accordingly, donors have been classified into "high-incidence" and "intermediate-low incidence" groups with regard to their propensity for generating LPD in the animals. Injection of SCID mice with PBL from "high incidence" donors results in rapid appearance of LPD in all mice engrafted. A longer latency period is observed when PBL from "low-intermediate incidence" donors are used, while some EBV-seropositive donors fail to give rise to tumor formation in xenochimeric animals. The number of PBL engrafted is important for LPD development (Mosier et al., 1989; Duchosal et al., 1992b), i.e., the higher the number engrafted, the sooner hu-PBL-SCID mice develop fatal LPD. Typically, 100% of mice injected with 50x10^6 human PBL develop LPD 6-15 weeks after engraftment. In most cases, the increased human serum Ig parallels tumor formation (Table 1). At autopsy, tumors were most commonly found in the hepatic hilus, but were also seen at other sites, such as mesenteric lymph nodes, pancreas, spleen, thymus and lungs (Table 2), and tumor masses could spread to more than one organ. Interestingly, tumoral B cells obtained from hu-PBL-SCID mice tumors can be cultured *in vitro* and retain full tumorigenicity after re-injection into SCID mice (Cannon et al., 1990). Similarly, LCL generated *in vitro* from either a spontaneous outgrowth of EBV-infected B cells from EBV-seropositive donors or from active *in vitro* EBV infection of EBV-seronegative donor PBL induced tumor formation after injection into SCID mice (Picchio et al., 1992).

Histopathology of LPD

In our hands, histological analysis of enlarged lymph

Table 1. Human IgG levels in 31 hu-PBL-SCID mice[a].

DONOR	MOUSE	D15[b]	D30	D60	D90	D120	TUMOR	DAY[e]
A	1	160	1100	9700	3000	-[c]	Y[d]	D108 (3150)
A	2	50	328	2950	5800	12500	Y	D128 (12475)
A	3	75	623	3200	-	-	Y	D82 (5100)
A	4	220	1700	-	-	-	Y	D50 (2500)
A	5	320	1300	-	-	-	Y	D53 (2700)
A	6	230	1100	-	-	-	Y	D50 (3100)
A	7	210	1100	1700	2100	3800	Y	D159 (4100)
A	8	80	163	1100	2500	7000	Y	D157 (7700)
A	9	57	269	700	7200	-	Y	D109 (9000)
A	10	40	226	453	580	400	N[f]	D220 (350)
A	11	130	830	820	1000	-	N	D95 (1300)
A	12	350	1300	2400	5100	-	Y	D94 (5120)
A	13	450	1150	995	1950	-	Y	D111 (2700)
B	14	154	1800	3500	-	-	Y	D69 (5400)
B	15	220	1300	5400	-	-	Y	D69 (5600)
B	16	100	2500	3500	-	-	Y	D69 (4000)
B	17	83	1200	-	-	-	Y	D44 (2000)
B	18	320	-	-	-	-	Y	D28 (4200)
B	19	130	2400	-	-	-	Y	D41 (3800)
B	20	405	1200	-	-	-	Y	D56 (5100)
B	21	300	1900	1800	-	-	Y	D69 (1840)
B	22	145	2200	4800	-	-	Y	D64 (5400)
B	23	210	1750	-	-	-	Y	D49 (3200)
B	24	130	3500	-	-	-	Y	D37 (4000)
B	25	370	2400	4000	-	-	Y	D64 (3980)
B	26	180	840	2000	-	-	Y	D69 (2200)
B	27	50	250	2490	-	-	Y	D69 (2510)
C	28	511	2050	-	-	-	Y	D51 (2400)
C	29	334	1690	-	-	-	Y	D52 (4450)
C	30	917	1460	-	-	-	Y	D58 (5900)
C	31	148	1680	-	-	-	Y	D68 (3200)

[a] : human IgG levels in µg/ml; [b] : day post 50x10^{6} PBL transfer; [c]: not done (mouse dead); [d]: tumor was observed at autopsy; [e] : day at which autopsy was performed and, in brackets, human IgG levels in µg/ml; [f] : no tumor was observed at autopsy.

Table 2. Site of tumor[a].

TUMOR SITE	% MICE
Liver	86
Lung	4
Kidney	16
Pancreas	25
Spleen	7
Mesenteric lymph node	36
Thymus	44
Gonad	6
Intestine	10
Surrenalis	2

[a]: the analysis comprised 100 mice populated with 50x10^{6} PBL from 9 EBV-seropositive donors and with a tumor at one or more anatomical sites.

nodes from mice with LPD demonstrated a diffuse proliferation of lymphoid cells that destroyed the normal architecture of the organ and infiltrated the capsula and surrounding tissues. At high magnification, cellular composition was polymorphic, comprised of immunoblasts with abundant mitoses, lymphoplasmacytoid- and plasma-cells, as well as some rare lymphocytes (Fig. 1A,

B). Necrotic areas were observed within large lymphoid masses. This picture is compatible with that of high-grade large B cell immunoblastic lymphomas with scattered plasmacytoid cells, as previously reported by others (Cannon et al., 1990; Okano et al., 1990; Nakamine et al., 1991).

In organs, the diffuse proliferation of lymphoid cells was clustered around vessels and infiltrated the adjacent parenchyma, a pattern particularly evident in liver, lung, and kidney (Table 2, Fig. 1D-F). Extensive areas of necrosis were observed primarily in the liver afflicted with LPD at the hilus. The spleens affected by LPD were large (up to 1.2 g), and, upon histology, the splenic tissue was either infiltrated with immunoblasts in the white pulp or completely replaced by a diffuse proliferation of blast cells (Fig. 1C). *In situ* hybridization using a human ALU-specific RNA probe labeled with ^{35}S confirmed the human origin of the tumoral cells (Fig. 2).

Immunohistochemistry analyses revealed that the large majority of cells in LPD were stained with an anti-human leukocyte common antigen antibody, and with anti-human Ig antibodies, confirming the human B cell origin of the tumors (Fig. 3A, C, D). Human T cells bearing a human memory phenotype marker (CD45RO,

UCLH1) were scattered in variable proportions (1-5%) among human B cells (Fig. 3B).

*Characterization of EBV-induced lymphomagenesis*

In vitro, EBV-transformation of B lymphocytes results in viral latent protein expression and upregulated expression of cell adhesion molecules CD11a (LFA-1), CD54 (ICAM-1) and CD58 (LFA-3) (Gregory et al.,

1988; Rowe et al., 1991), as well as of the B cell activation marker CD23 (Wang et al., 1987, 1990). Expression of this latter marker is known to be induced by EBNA-2 (Wang et al., 1987). Human B cell LPD that arise in hu-PBL-SCID mice resembles to, but is not identical to, the *in vitro*-transformed LCL from EBV-positive PBL (Rowe et al., 1990, 1991; Mosier et al., 1992; Rochford et al., 1993; Rochford and Mosier, 1995). Phenotypic characterization of LPD from hu-
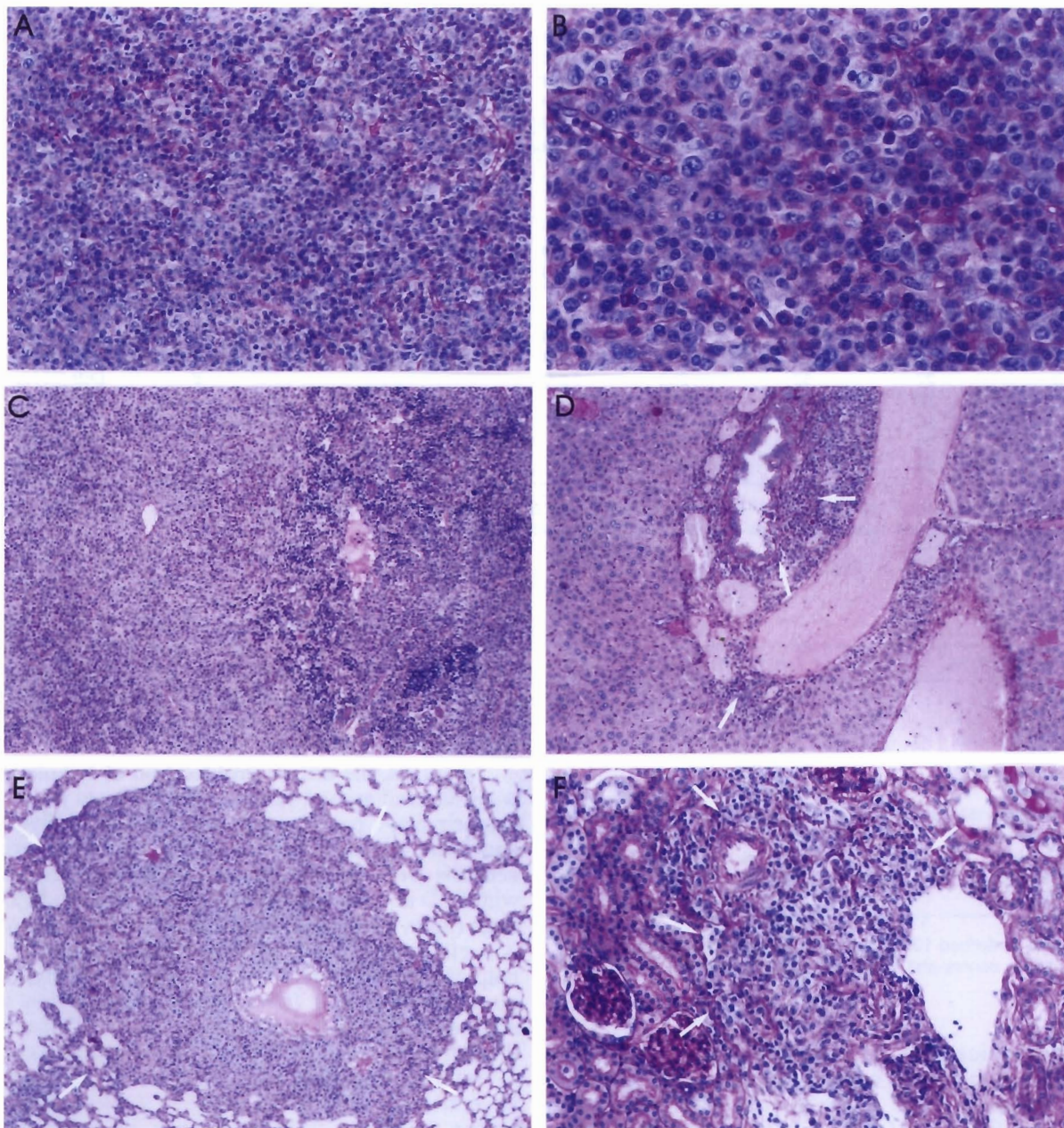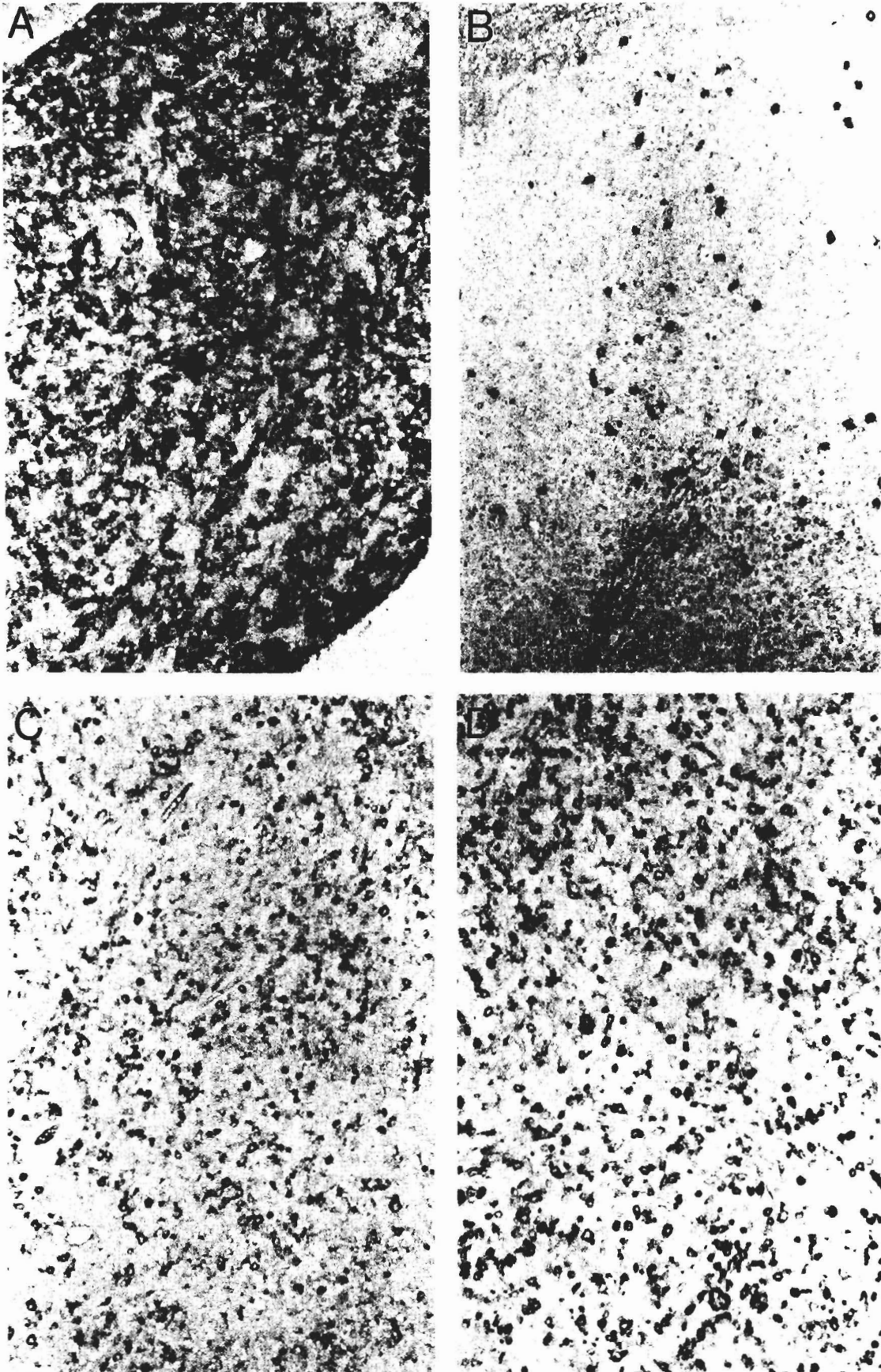

Fig.1. Photomicrographs of PAS-stained sections from an abdominal lymph-node **(A,B)**, spleen **(C)**, liver **(D)**, lung **(E)**, and kidney **(F)** of a mouse injected with 50x10^{6} PBL from an EBV-seropositive donor 52 days previously. The LPD massively invade the lymph node and white pulp of the spleen (left side, C), and are clustered in the portal area of the liver and perivascular areas of the lung and kidney (arrows). A, F, x 64; B, x 128; C-E, x 32
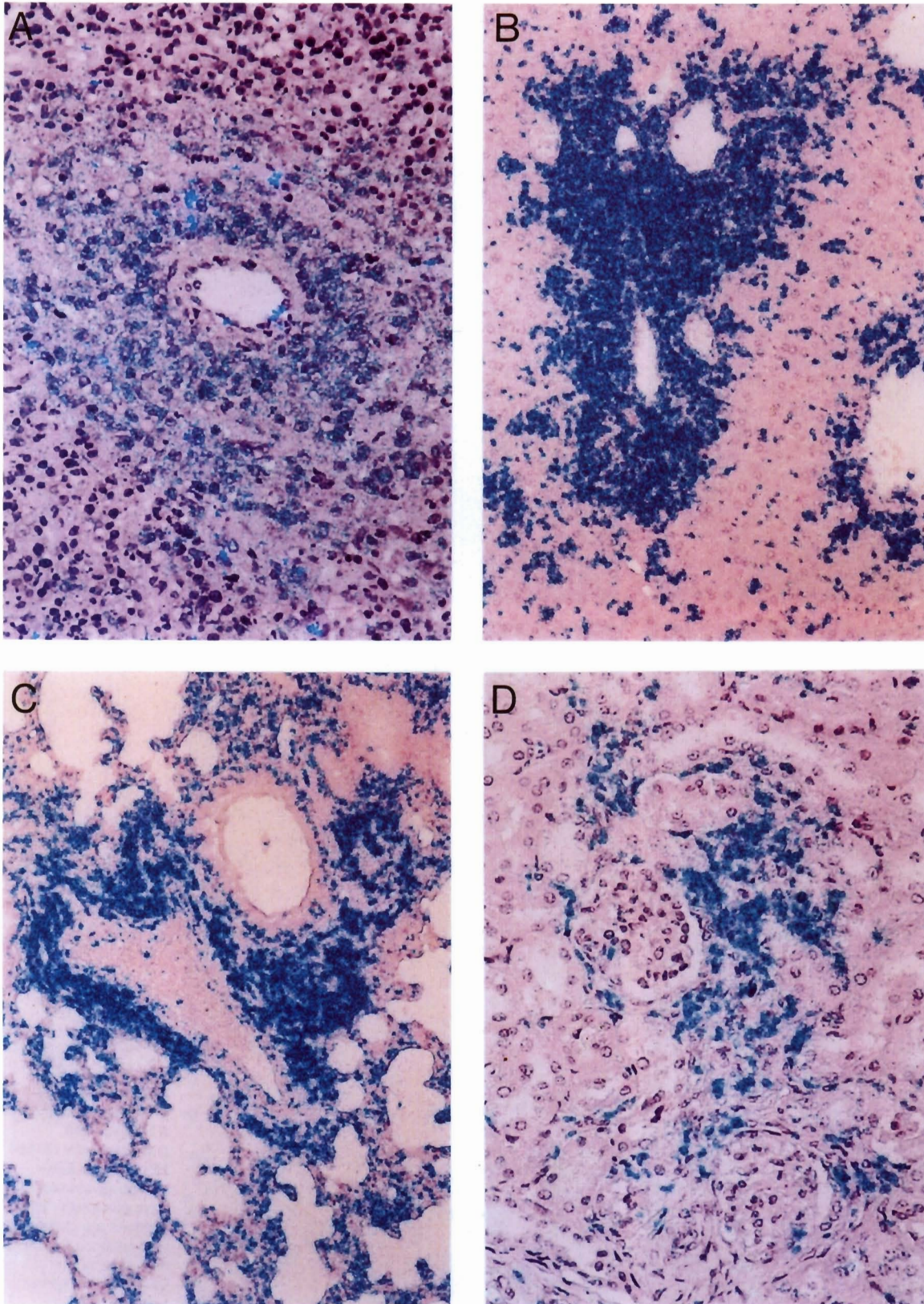
Fig. 2. Photomicrographs of sections from a lymph-node (**A**), liver (**B**), lung (**C**), and kidney (**D**), hybridized *in situ* with a human-specific ALU repeat family probe (turquoise dots) and PAS-stained. The sections are from two SCID mice populated with 15×10^6 PBL from an EBV-seropositive donor 52 (A) and 57 (B, C, D) days previously and presenting EBV-dependent lymphoproliferative diseases. A, x 64; B-D, x 32

Fig. 3. Photomicrographs of a tumor from a SCID mouse populated with 15x10^6 PBL from an EBV-seropositive donor 59 days previously. The sections are stained with antibodies to human LCA (**A**), CD45RO (**B**), IgG (**C**), and IgM (**D**). x 40

PBL-SCID mice indicated that they are composed, in addition to a lymphoblastoid cell population similar to LCL, of plasmacytoid cells. These two populations can be differentiated by respective levels of CD23 and CD38 (plasma cell-associated marker), as well as by their distinct EBV gene expressions (Rochford and Mosier, 1995). Lympho-blastoid cells express intermediate levels of both surface markers (CD23^{int}CD38^{int}) and express viral gene mRNAs associated with latency (EBNA-1, -2 and LMP-1). Plasmacytoid cells display low and high levels of CD23 and CD38, respectively (CD23^{low} CD38^{high}), express EBV genes required for lytic cycle replication (BZLF1, BRLF1), and no EBV latent gene transcript. Interestingly, reinjection of lymphoblastoid tumoral B cells or LCL into SCID mice results in some cells switching to the plasmacytoid phenotype, with decreased and increased CD23 and CD38 expression, respectively (Rochford and Mosier, 1995), as well as a slight decrease in adhesion molecule expression (Rowe et al., 1991; Rochford and Mosier, 1995). These results indicate that, under environmental constraints, plasma-cytoid cells most probably arise from the transformation of the lymphoblastoid cell population. In addition, the two tumoral cell subpopulations present in hu-PBL-SCID mice display distinct biological activities when purified and cultured *in vitro*. Lymphoblastoid cells have a high proliferative index and do not secrete human Ig, while plasmacytoid cells proliferate slowly, produce high levels of human Ig and die quickly *in vitro*. In post-transplant patients, B cell differentiation towards a plasmacytoid phenotype, as assessed by CD38 expression, correlates with the progression of LPD (Garnier et al., 1993).

Southern blot analyses of Ig heavy chain re-arrangement as well as of fused terminal repeats of the EBV genome indicate that these tumors are primarily oligoclonal (Picchio et al., 1992). The clonal complexity of rapidly growing LPD is higher than that of slow-onset tumors.

Lymphoproliferative disease and EBV replication

In vitro, EBV-specific B-cell transformation is preceded by the reactivation of the EBV lytic cycle. New viral particles are generated and transform bystander B-cells (Yao et al., 1991). The role of EBV replication in the LPD development *in vivo* in the xenochimeric mouse model is still unclear, although several reports suggest that viral replication may be an important step in tumor formation in hu-PBL-SCID mice (Rowe et al., 1990; Picchio et al., 1992; Pisa et al., 1992; Rochford et al., 1993; Rochford and Mosier, 1995). Southern blot analyses of hu-PBL-SCID mice tumors using a probe specific for EBV-terminal repeats show that some tumors contain both the replicative (linear) and latent (episomal) form of the virus, while others contain only the latent form (Picchio et al., 1992). The presence of replicative viral DNA is generally associated with a more rapid onset of lymphomagenesis in the xenogeneic environment.

Factors influencing the development, and treatment, of EBV-induced LPD in hu-PBL-SCID mice

No LPD is observed following immunosuppression of hu-PBL-SCID mice using CsA or methylprednisone (Boyle et al., 1992a,b; Veronese et al., 1992), probably reflecting the requirement of T cell help to ensure proper engraftment and function of B cells in the xenogeneic model, as described above. *In vitro* culture of PBL from EBV-seropositive donors in the presence of CsA leads to spontaneous outgrowth of transformed B-lymphocytes. (Rickinson et al., 1984). Treatment of hu-PBL-SCID mice with imexon, an immunomodulator that exerts inhibitory action on LPD without affecting engraftment of human B cells, has been reported (Hersh et al., 1993). Further investigation of the action mechanism of this immunomodulator may elucidate important steps leading to lymphomagenesis in hu-PBL-SCID mice, which may in turn help identify LPD origins in immunosuppressed individuals.

Cytokines play a major role in regulating the immune system. In particular, interleukin 2 (IL-2), released by CD4$^+$ T-cells, aids proliferation of B cells and Ig secretion. Repeated injection of low doses of IL-2 into hu-PBL-SCID mice significantly reduces fatal LPD in the xenochimeric animals (Baiocchi and Caligiuri, 1994). The effect of human IL-2 on the development of LPD in hu-PBL-SCID mice is partly dependent on the presence of mouse NK cells, which are known to modulate the engraftment of human PBL in SCID mice and bear IL-2 receptors on their surface (Shpitz et al., 1994; Lacerda et al., 1996a). Thus, human IL-2 may function by activating the mouse NK cell population, thereby reducing graft viability in the xenochimeric mice.

Interleukin 10 (IL-10) is produced by the T$_{H2}$ effector arm of CD4$^+$ T-cells and is a pleiotropic factor that enhances B lymphocyte proliferation and stimulates the production and secretion of high amounts of Ig (Rousset et al., 1992). *In vitro*, Epstein-Barr virus transformation of B cells induces increased production of human IL-10 (hIL-10) correlating with cell growth (Burdin et al., 1993). Interestingly, hu-PBL-SCID mice with EBV-specific B-cell LPD have higher serum hIL-10 levels than those in LPD-free mice (Baiocchi et al., 1995). In addition, EBV expresses an analogue of the human interleukin, viral IL-10 (vIL-10), that shares numerous functions with the human molecule (Moore et al., 1990; Vieira et al., 1991). *In vitro*, vIL-10 is expressed during the lytic viral cycle and the initial EBV infection, and plays a central role in B cell trans-formation following EBV infection *in vitro* (Miyazaki et al., 1993). Although a direct role for hIL-10 and vIL-10 in lymphomagenesis has not been confirmed, these proteins may contribute to the prevention of cell death and the promotion of EBV-infected B cell proliferation *in vivo*.

The CD40 surface marker is present both on normal and neoplastic B cells, and bears homologies to the nerve growth factor receptor-tumor necrosis factor α receptor family (Stamenkovic et al., 1989). Cross-linking of CD40, either by its ligand (gp39) on activated T cells or by antibodies, induces B cell activation, proliferation and differentiation into IgM-secreting plasma cells (Banchereau et al., 1991). Recently, it was reported that treatment of hu-PBL-SCID mice with anti-CD40 antibodies delayed the onset of EBV-induced LPD in the xenogeneic mice (Fulop and Phillips, 1990; Funakoshi et al., 1994, 1995; Murphy et al., 1995). Human Ig was still detectable in treated animals, indicating that anti-CD40 treatment did not inhibit survival of human grafts. In contrast, treatment of hu-PBL-SCID mice with an anti-CD20 antibody, which also prevented LPD, inhibited engraftment of functional B cells, as reflected by the absence, or very low levels, of human B cells and Ig in treated mice (Murphy et al., 1995). Recently, injection into hu-PBL-SCID mice of a monoclonal antibody (mAb 2E1) directed against the co-stimulatory molecule effector cell protease receptor-1 (EPR-1) present on T cells protected the xenochimeric mice from EBV-induced LPD (Duchosal et al., 1996). This treatment also inhibited human Ig production in the mouse model.

Antiviral agent treatment of xenogeneic animals to reduce incidence of EBV-induced LPD have been reported. As mentioned above, reactivation of the EBV lytic cycle may have an important function in LPD. Boyle et al. (1992a) reported that the anti-herpetic drugs ganciclovir and acyclovir (ACV) had little or no effect, respectively, on the survival of hu-EBV$^+$-PBL-SCID mice. However, the routes of administration and drug doses have not been systematically studied in this model. We found that ACV injected i.p. into hu-PBL-SCID mice had a half life of about 6 hours, and was not detectable after 24 hours (Fuzzati-Armentero et al., unpublished results). In addition, the action of ACV on EBV replication *in vitro* is fully reversible, and viral replication resumes almost immediately after removal of the drug from cultures of EBV-infected B-cells (Lin et al., 1983). Therefore, higher and continuous doses of ACV may be required to inhibit EBV replication *in vivo* in hu-PBL-SCID mice.

In immunocompetent individuals, EBV infection is controlled by the host's CTL response (Rickinson, 1986), and immunosuppressive therapy can result in the appearance of LPD. The *ex vivo* expansion of autologous EBV-specific CTL followed by their reinjection into respective bone marrow-transplanted patients with LPD induced lymphoma regression without significant treatment-related toxicity (Rooney et al., 1995). Similarly, Lacerda et al. (1996b) reported that inoculation of autologous, but not HLA-mismatched, EBV-specific CTL infiltrated autologous tumors, and significantly increased the survival of hu-PBL-SCID mice compared to that of untreated animals. Finally, SCID mice engrafted with B cell from post-transplant LPD, and subsequently treated with autologous EBV-specific CTL, lived significantly longer than animals injected only with LPD cells (DiMaio et al., 1995). Therefore, it appears that the development and treatment of LPD are similarly influenced by manipulations of the EBV T cell response in humans and in the murine model.

Conclusions

In recent years, the rapid growth of transplantation medicine and the AIDS epidemic, have resulted in an increased population of immunosuppressed individuals at risk for development of LPD. The xenochimeric hu-PBL-SCID mouse model has provided a convenient animal system to study EBV-driven human B cell LPD development. Indeed the EBV-induced LPD in hu-PBL-SCID mice, and its developmental processes closely resemble LPD that arises in immunosuppressed individuals. The pathways leading to the outgrowth of LPD are still unclear and will require more detailed analyses of LPD in hu-PBL-SCID mice to clarify the tumorigenic processes initiated by EBV. Such analyses may also facilitate development of novel therapeutic approaches.

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