Brief Definitive Report

Lymphoproliferative Disease in Human Peripheral Blood Mononuclear Cell–injected SCID Mice. I. T Lymphocyte Requirement for B Cell Tumor Generation

By Maria Luisa Veronese,* Arianna Veronesi,* Emma D'Andrea,* Annarosa Del Mistro,* Stefano Indraccolo,* Maria Rosaria Mazza,* Marta Mion,* Rita Zamarchi,* Chiara Menin,* Marina Panozzo,*‡ Alberto Amadori,* and Luigi Chieco-Bianchi*

*From the *Institute of Oncology, Interuniversity Center for Research on Cancer, University of Padova; and the ‡Istituto Scientifico Tumori Biotechnology Section, I-35128 Padova, Italy*

Summary

Mechanisms of tumor development were studied in SCID mice injected with human lymphoid cells from Epstein-Barr virus–positive (EBV^+) donors. About 80% of peripheral blood mononuclear cell (PBMC)-injected animals developed a lymphoproliferative disease associated with oligoclonal EBV^+ tumors of human B cell origin. No change in tumor development rate occurred when monocyte-depleted PBMC were inoculated. No tumors developed when purified B cells were injected. B cell lymphoproliferative disease was also prevented in most cases when PBMC-injected animals were treated with agents that prevent T cell activation, such as cyclosporin A. Both $CD4^+$ and $CD8^+$ T cell subpopulations were able to provide putative factor(s) necessary for EBV^+ B cell expansion and progression to tumors. These data suggest that the transfer alone of potentially tumorigenic human cells into an immunodeficient environment, such as the SCID mouse, might not be sufficient for cell progression to tumor, and raise the possibility that chronic activation events could play a major role in the pathogenesis of some EBV^+ lymphomas in the immunocompromised host.

The mutant C.B-17 mouse strain (SCID) has a chromosome 16 defect involving the VDJ recombinase system that prevents correct TCR and Ig gene rearrangement, and results in a virtually complete functional T and B cell deficiency (1, 2). Thus, SCID mice behave as relatively inert recipients, and can be successfully reconstituted with human fetal lymphoid tissues (3), or adult lymphocytes. If the latter come from donors with serological evidence of previous EBV infection, the mice often develop a fatal lymphoproliferative disease (4), associated with the presence of EBV^+ tumor masses within the abdomen. Such lesions are of human B cell origin, mostly oligoclonal, and contain the EBV genome, but differ from classical EBV-related Burkitt's lymphoma in that chromosomal alterations, *c-myc* rearrangement, and expression of the common acute lymphocytic leukemia antigen (CALLA) (CD10) surface marker could not be demonstrated (5–7). These manifestations therefore instead recall some EBV^+ tumors arising in immunosuppressed humans, for which the term "opportunistic tumors" seems to be appropriate (8).

Thus, the SCID mouse model could constitute a useful tool to explore the pathways leading to lymphoma development in the immunocompromised host. It is widely accepted that lymphomagenesis is a multistep process, where several consecutive events accumulate until the full malignant phenotype is acquired. In this setting, the role of chromosomal translocations and protooncogene activation is relatively well-defined, but early incidents promoting B cell deregulation and expansion are unclear, and it is possible that several triggering events are involved. Tumors arising in human PBMC-reconstituted SCID mice have been extensively characterized for phenotype and genotype (5–7), but the pathways leading to lymphoproliferative disease are not defined. Our studies showed that the presence of functional T cells in the injected PBMC population was absolutely necessary for the progression of latently EBV-infected B cells into tumor masses. The implications of this observation within the frame of human immunodeficiency are far-reaching.

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Materials and Methods

Cell Source and Preparation. PBMC were obtained from eight healthy adult volunteers undergoing lymphapheresis after their informed consent. Prior EBV infection was confirmed by testing for the presence of serum anti-viral capsid antigen (VCA) and anti-Epstein-Barr nuclear antigen (EBNA) IgG. Mononuclear cells were isolated by Ficoll-Hypaque gradient centrifugation as reported elsewhere (9), washed four times with RPMI 1640 medium, counted, and either used as such, or separated further.

T and non-T cells (hereafter referred to as "B") were purified by double-rosetting with neuraminidase-treated SRBC (10). Cytofluorographic analysis of B cell populations with mAbs disclosed <1% contaminating T cells ($CD2^+$), and 60–80% B cells ($CD19^+$). Purified T cell populations contained <1% B cells.

Monocytes were removed from unfractionated PBMC by adherence to plastic, followed by leucine-*O*-methyl ester (Leu-*O*-ME; Sigma Chemical Co., St. Louis, MO) treatment, as reported (11). Residual monocytes were then eliminated by immunomagnetic treatment with Leu-M3 mAb (Becton Dickinson & Co., Mountain View, CA) and anti-mouse Ig-coated magnetic beads (Dynabeads; Dynal, Oslo, Norway), as detailed elsewhere (12). Subsequent cytofluorographic analysis disclosed <1% contaminating monocytes in these populations, and virtual abrogation of the proliferative response to anti-CD3 mAb (courtesy of F. Malavasi, University of Turin, Italy). Nonetheless, cell viability was not affected, and monocyte-depleted populations responded to anti-CD3 stimulation in the presence of irradiated accessory cells (data not shown). As previously reported (11), Leu-*O*-ME treatment of PBMC was also associated with virtual abrogation of non-MHC-restricted cytotoxic activity.

CD4- and CD8-depleted PBMC were obtained by immunomagnetic separation of unfractionated PBMC with anti-CD4 and anti-CD8 mAbs (Ortho Diagnostics, Raritan, NJ), respectively, as reported above. Residual contamination of the depleted phenotype in the recovered populations did not exceed 1%.

Mouse Injection. SCID mice were purchased from IFFA Credo (L'Abresle, France), and maintained in our animal facilities under pathogen-free conditions. Prophylaxis for *Pneumocystis carinii* infection was provided as reported (7). Groups of 7–9-wk-old mice of both sexes were inoculated intraperitoneally with 60–100 × 10^6 unfractionated PBMC, monocyte-depleted PBMC (PBMC-MΦ), CD4- or CD8-depleted PMBC, or 30–60 × 10^6 purified B lymphocytes. The effective number of injected B cells was calculated by cytofluorographic analysis of the enriched non-T cell populations with anti-CD19 mAb. In a set of experiments, T and B cells were separated as above, and injected together (60 × 10^6 T plus 10 × 10^6 B cells) into the same mice.

Before injection, and every 10 d thereafter, the mice were bled from the retroorbital plexus to monitor human Ig. Serum or plasma samples were stored at −20°C until use. The animals were observed every other day for signs of illness (ruffled fur, respiratory distress, inactivity, weight loss, and palpable abdominal masses). When they became sick, they were killed by excess ethyl ether anesthesia, and autopsied. Interesting tumor masses and tissues were divided into three samples: one was immediately frozen in liquid nitrogen for DNA studies; one was fixed in paraformaldehyde/saline for histopathologic and immunohistochemical studies; and the third was minced to obtain single cell suspensions for immunophenotypic analysis. Follow-up was completed after 36 wk. Survivors were killed and autopsied, and tissue samples were routinely collected. In one set of experiments, cyclosporin A (CsA; kindly provided by G. Corbetta, Sandoz Italia, Milan, Italy; 50 μg/g i.p. daily) treatment was initiated the day before unfractionated PBMC injection, and continued for 30 d.

Human Ig Assays. Human Ig contents in mouse plasma or serum samples were assessed by solid-phase RIA as reported elsewhere (10, 11). Briefly, goat anti-human Ig antibody (courtesy of S. Siervo, H. S. Raffaele, Milan, Italy; 5 μg/ml in carbonate/bicarbonate buffer, pH 9.6) was coupled to 96-well flexible polyvinyl plates (Falcon, Grenoble, France), and the wells were saturated with 3% BSA (Sigma Chemical Co.) in PBS. After three washes, 50 μl of serial sample dilutions were added in triplicate to the wells. The plates were left to stand for 3 h at room temperature, washed, and then incubated with ^{125}I-labeled goat anti-human Ig $F(ab)_2$ (Amersham Corp., Arlington Heights, IL; sp act 19–74 TBq/mM) for 4 h at room temperature. Finally, the plates were washed thoroughly, and individual wells counted in an LS 1801 gamma-counter (Packard, Grove Hills, IL). Serum Ig contents were evaluated against a reference curve obtained by doubling dilutions of a standard human serum containing a known amount of Ig. Standard curves in these RIA were linear between ∼10 and 500 ng/ml. When necessary, samples were further diluted so that Ig levels would fall into this range.

Statistical Analysis. Data were managed using the Mann-Whitney test.

Results

Tumors developed in 31 of 38 PBMC-injected animals (Table 1), and usually consisted of multiple masses at the hepatic hilum, lower splanchnic region, and within the mesenteric tissue. Supradiaphragmatic lesions (usually perithymic) were rare. Histologic and genotypic analysis disclosed that these tumors were oligoclonal expansions of B cell origin most likely sustained by the few EBV-infected B cell precursors present

Table 1. *Tumor Development in SCID Mice Injected Intraperitoneally with Different PBMC Subpopulations from EBV^+ Donors*

Injected population	Mice with tumors (percent)*	Latency (wk)‡
PBMC (38)	31 (81.6)	8.3 ± 2.5 (6–17)
PBMC-MΦ (7)	5 (71.4)	7.2 ± 1.1 (6–8)
B (21)	0	–
T + B (12)	9 (75.0)	9.4 ± 3.0 (5–14)
CD8-depleted (8)	8 (100)	7.8 ± 3.0 (4–14)
CD4-depleted (4)	3 (75.0)	18.3 ± 6.4 (11–23)§
PBMC + CsA (10)‖	2 (20.0)	12,16

* The number of animals developing tumor, as judged by autoptic, histologic, and genotypic evidence in a 36-wk follow-up is reported.
‡ Mean ± SD (range in parentheses).
§ p = 0.03, according to Mann-Whitney test, compared with CD8-depleted PBMC.
‖ PBMC-injected mice were treated with CsA (50 μg/g/d) as detailed in Materials and Methods.

in the inoculum, even though the recruitment of newly infected B cells could not be excluded (data not shown). Their histologic, immunophenotypic, and genotypic features were completely similar to those described by other workers (5–7).

Tumor development was accompanied by high levels of human Ig in mouse serum, that were measurable early after PBMC injection (Fig. 1). However, at a given follow-up time, there was considerable variation, with up to fivefold differences in Ig values among mice injected with PBMC from the same donor (data not shown). In line with a previous report (7), tumor progression in most animals was associated with the appearance of a distinct oligoclonal IgG pattern in serum (not shown). Preliminary investigations into the specificity of these Ig seem to indicate that a small fraction is directed against mouse antigens (A. Veronesi, et al., manuscript in preparation).

Removal of monocytes did not change the tumor development rate (Table 1, PMBC-MΦ); moreover, compared with unfractionated PBMC, human Ig production in mouse serum was enhanced (Fig. 1). This latter finding was not surprising, as PBMC treatment with Leu-O-ME to remove accessory cell function also selectively ablates NK cells (11, 13), which are potent modulators of B cell activity (14, 15).

On the other hand, 21 mice inoculated with purified B cells showed no tumors over a 36-wk follow-up (Table 1), and plasma human Ig levels never exceeded 10 μg/ml (Fig. 1). These findings were unexpected, as we thought that removal of residual T cell control would indeed favor the expansion of latently infected B cells. Moreover, compared with unfractionated PBMC, much higher numbers of EBV-infected precursors were likely present in the injected B cell population.

That EBV-infected precursor damage or loss during the B cell purification procedure might be responsible for this failure is unlikely, because tumors developed after injection of a mixture of purified T and B cells into the same animal (Table 1). These data clearly indicated that the presence of T cells within the injected population was necessary for tumor development. As shown in Table 1, both $CD4^+$ and $CD8^+$ subpopulations sufficed in providing the putative help for B cell expansion. However, tumor appearance was significantly delayed (Table 1), and serum human Ig levels were much lower in mice injected with CD4-depleted PBMC, compared with those inoculated with CD8-depleted PBMC (130.2 ± 93.0 μg vs. $11{,}060 \pm 4{,}403$ μg 40 d after injection, respectively).

To gain an insight into the relevance of the T cell component in lymphomagenesis, PBMC-injected mice were also administered agents aimed at preventing T cell activation. CsA treatment strongly reduced human Ig release in mouse serum (Fig. 1), and accordingly affected tumor development rate as well as latency. Compared with untreated controls, only 2 of 10 CsA-treated, PBMC-injected mice developed tumors, and with a significant delay (Table 1).

Discussion

This study confirms and extends previous observations in human PBMC-injected SCID mice (5–7), and shows that B cell expansion and tumor development are absolutely dependent on the presence of functional T lymphocytes in the inoculum. Thus, it seems reasonable to conclude that T cell–derived factor(s) probably play a major role in sustaining B cell progression to tumor in the human PBMC-injected SCID mouse model. After PBMC transfer into SCID mice, it is probable that T cells are activated and eventually produce cytokines (16, 17), which in turn stimulate proliferation and

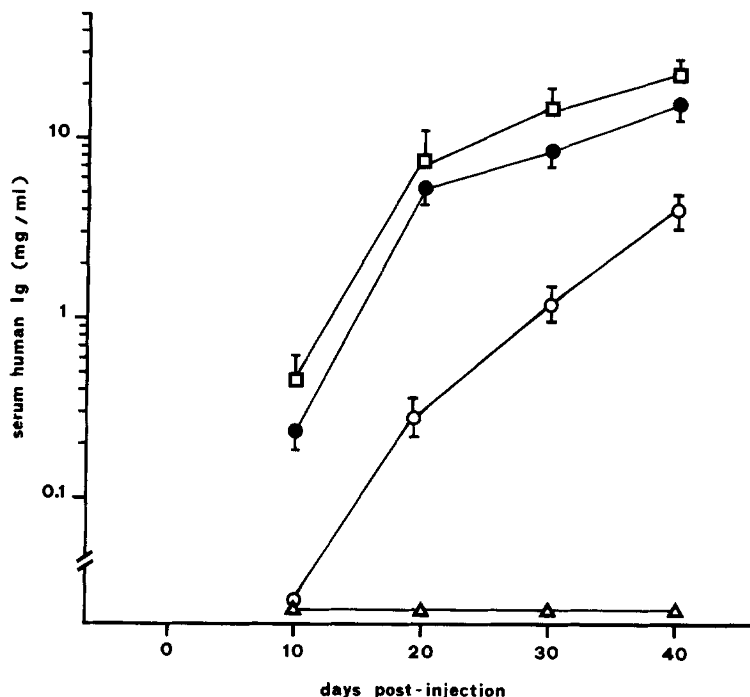

Figure 1. Kinetics of human Ig production in serum of SCID mice injected with different PBMC subpopulations. SCID mice were injected intraperitoneally with unfractionated PBMC (●), monocyte-depleted PBMC (□), and purified B cells (△). In a set of experiments, PBMC-injected mice (○) were also injected with CsA as detailed in Materials and Methods. Results represent mean values (± 1 SD) obtained in 32, 7, 21, and 10 animals, respectively.

differentiation of B cells, including EBV-infected lymphocytes. In the absence of T cell–derived factors, the destiny of the injected B cells is unclear. In this regard, it is worth recalling that NK activity in SCID mice is maintained or even increased (18). Thus, in the absence of T cell–derived promoting factors, it is possible that the very few EBV-infected precursors present in the inoculum (estimated between $\simeq$ 10 and 30 in 100 × 10^6 unfractionated PBMC [19]) are rapidly eliminated by the host's natural immunity effector mechanisms.

It is also noteworthy that both CD4- and CD8-depleted PBMC injections were associated with lymphoproliferative disease. It is now clear that $CD8^+$ lymphocytes as well can produce cytokines, albeit to a lesser extent (20). This different behavior probably underlies the significant delay in tumor onset observed in SCID mice inoculated with CD4-depleted PBMC, compared with animals injected with unfractionated or CD8-depleted PBMC.

Data obtained with CsA were most surprising, since CsA, as well as other immunosuppressive regimens, is associated with an increase in B cell lymphoma frequency (21). Moreover, the presence of CsA in culture promotes an efficient in vitro generation of EBV^+ lymphoblastoid cell lines by preventing T cell control of infected B cells. In addition to this effect on T cells, CsA also modifies the function of other cytotoxic populations, such as NK cells, by potentiating their generation from bone marrow precursors (22). Thus, this activity could possibly explain in part the decreased tumor rate in PBMC-injected, CsA-treated animals. However, it is also very likely that the decrease in the amount of human Ig in the serum of CsA-treated mice could be due to impaired cytokine release by transferred T cells (23). Further investigation into this complex network of interactions is necessary, as CsA is also known to alter B lymphocytes, and render them resistant to immune cytolysis (24).

The relevance of our findings to lymphomagenesis in human immunodeficiency disorders is thought-provoking. Non-Hodgkin's lymphomas, mostly EBV^+ immunoblastic lymphomas with no evidence of *c-myc* deregulation, are a major concern in organ transplant recipients undergoing immunosuppressive therapy to prevent graft rejection (22), as well as in HIV-infected patients (25, 26). The early events that promote B cell deregulation are mostly unclear. EBV infection, in conjunction with a deficient T cell control of infected cells, could constitute a proper trigger. On the other hand, chronic antigenic stimulation, which in these patients is obviously provided by foreign HLA molecules or antigens derived from opportunistic pathogens, might also play a role. Indeed, lymphoma development is a common finding even in immunocompetent animals undergoing continuous administration of foreign antigens (27, 28).

The relative contribution of each of these triggering factors to lymphomagenesis is not readily individuated. The present findings indicate that complete removal of T cell control is not sufficient for EBV-infected B cell expansion and progression to tumor in an immunodeficient host. Indeed, the importance of a strong T cell stimulatory component for B cell tumor development clearly emerges in the SCID mouse model. In a different setting, our data might also provide a reasonable explanation for the extremely high lymphoma risk in patients undergoing immunosuppression through maneuvers that also entail possible T cell stimulation, such as anti-CD3 mAb treatment (29). It is hoped that further work in this field will open new avenues of understanding and treatment of the B cell malignancies occurring in the immunosuppressed patient.

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Address correspondence to Dr. Alberto Amadori, Institute of Oncology, Via Gattamelata 64, I-35128, Padova, Italy.

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