EPSTEIN-BARR VIRUS-POSITIVE RECIPIENT TYPE B-CELLS SURVIVE IN A “COMPLETE CHIMERA” AFTER ALLOGENEIC BONE-MARROW TRANSPLANTATION

Heinrich H. Gerhardt1,5, Johann Mittermüller2, Anand Raghavachar3, Helga Schmetzer1, Christof Clemm1, Hans-J. Kohl1, Claus C. Bartram4 and Hans Wolf4

1Med. Klinik III, Klinikum Großhadern, Munich University; 2Department of Transfusion Medicine, Ulm University; 3Department of Pediatrics II, Ulm University; and 4Max v. Pettenkofer-Inst., Munich University, Munich, FRG.

Latency of Epstein-Barr virus infection may be generated by surviving immortalized B cells or by continuous re-infection. EBV-positive B-cell tumors have been found following bone-marrow transplantation (BMT) and were of donor type in the few cases investigated. We established a B-cell line from the bone marrow of a patient in complete remission following allogeneic BMT for aplastic anemia 18 months post-grafting. Differences in sex and isoenzymes allowed an exact determination of chimerism in our case. While the patient showed persistent complete chimerism of all cell lineages, cells grown in culture were of recipient type, whereas cells grown in vivo, additional patho-physiological steps like severe graft versus host disease or T-cell suppression are obviously required because the patient was still free of lymphoma 3 years post-grafting.

Epstein-Barr virus infection can lead in vivo to immortalized B-cell clones which are suppressed by T-cell-mediated immunity (Rickinson et al., 1981). It is an open question whether the persistence of such B cells in vivo is the result of continuous re-infection by virus shedding from saliva (Wolf et al., 1984; Yao et al., 1985a) or of survival of low numbers of EBV-transformed B cells of the recipient survived for long periods in this patient. For the development of secondary B-cell neoplasms in vivo, additional patho-physiological steps like severe graft versus host disease or T-cell suppression are obviously required because the patient was still free of lymphoma 3 years post-grafting.

Allologic BMT offers an opportunity to study this problem since either sex differences between donor and recipient or genetic polymorphism of cellular isoenzymes such as AcP or PGM-1 may make it possible, in most cases, to determine whether the cells in BMT patients are of donor or recipient origin (Mittermüller et al., 1986). More than 90% of European adults are infected with EBV (Yao et al., 1985a). It is an open question whether EBV-immortalized B-cell clones of the recipient persist or whether they are eliminated either by BMT conditioning or by immunological mechanisms. We report findings which suggest that EBV-transformed clones of recipient origin can indeed persist after BMT even if the patient has become a “complete chimera” according to the most sensitive criteria that are presently available.

PATIENT AND METHODS

Patient

A 43-year-old woman presented with severe tricytopenia following nitrefazol (anti-obesity drug) complicated by bleeding episodes and septic complications in June 1984. A diagnosis of severe aplastic anemia was confirmed by bone-marrow biopsy and in August 1984 the patient was prepared for allogeneic BMT by cyclophosphamide i.v. (40 mg/kg body weight days -5 to -2) plus total nodal irradiation (7 Gy, day -1). She received 4 X 10⁹ nucleated cells per kg body weight from her HLA-identical MLC-unreactive brother and recovered promptly with a leucocyte count of > 1 X 10⁹/1 on day 17. Post-grafting immunosuppression consisted of Cyclosporin A i.v. 5 mg/kg body weight for 5 days followed by 3 mg/kg until day 34 and subsequently orally until day 250 (9 months). No steroids were given at any time. At the time of writing, she is in full clinical remission (40 months).

Cell preparation

The patient’s bone marrow cells were aspirated with preservative-free heparin after informed consent had been obtained. For culture and cytogenetic studies the cells were centrifuged over Ficoll (density 1.077 g/l). For enzyme analyses the polymorphonuclear cells were isolated from the pellet of Ficoll separations by lysis of the contaminating erythrocytes with ammonium chloride. Monocytes were recovered from the upper layers of the buffy coat after mild sedimentation. T cells and monocytes were isolated in the pellet of Ficoll-gradients by a direct immune-rosetting technique (Wilhelm et al., 1986) using erythrocytes coated with pan-T-antibodies (CD2, Rieber et al., 1986) or with anti-monocyte antibodies. T-depleted lymphocytes were recovered from the interface.

Morphological studies

Smears were prepared by standard methods employing May-Grünwald-Giemsa stain, periodic acid Schiff reaction and myeloperoxidase.

Immunological phenotyping

Surface markers were identified with an enzyme-immunoasaysay on poly-l-lysine coated adhesive slides (Morich et al., 1983) employing polyvalent alkaline phosphatase (Sigma)-conjugated goat-anti-mouse immunoglobulin. The color reaction was performed with 0.1% naphthol-AS-MX-phosphate (Sigma).

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plus 2% dimethylformamide (Merck), 0.1% fast red (Serva, Heidelberg, FRG) and 0.024% levamisole in 0.1 molar Tris-buffer. Each slide contained a control without primary antibody. The MAbs used were VIM-D5 and VIM-2 (CD15) which are directed against myeloid differentiation antigens, kindly provided by Dr. W. Knapp, Vienna, B-1 and B-2 (CD20, CD19) which are found on B cells and their progenitors, anti-human IgG, -IgM, anti-kappa and anti-lambda light chain antibodies as well as J5 (anti-common acute lymphoblastic leukemia antibody, characteristic for immature lymphoid cells) (CD10), which were purchased from Coulter, Hialeah, FL, OKT-3 (CD3) and OKT-9 (which detects the transferrin receptor) from Ortho, Raritan, NJ. The CD-classification has been reviewed recently (Foon and Todd, 1986).

Cultures
Suspension cultures were initiated in 30-ml flasks with 2.5 \times 10^6 MNC in 5 ml Iscove-modified Dulbecco's medium supplemented with 10% fetal calf serum, 10% horse serum (both by HyClone, Logan, UT) and 5% of a supernatant of cell line, OKT-9 which detects the transferrin receptor from Ortho, Raritan, NJ. The CD-classification has been reviewed recently (Foon and Todd, 1986).

Cytochromes
Karyotypic analyses were performed according to standard Giesma-banding techniques (Seabright, 1971).

Enzyme analysis
After washing the cells with isotonic saline, freezing and thawing and ultrasonic treatment, the debris-free supernatant was analyzed. Unprocessed hair roots were typed after pre-incubation with Triton X 100 for 20 min (Mittermüller et al., 1984). PGM-1 isoenzyme analysis was performed by ultra-thin isoelectric focusing on polyacrylamide gels (size 240 \times 120 \times 0.24 mm, pH 5-6.5) (Mittermüller et al., 1985). AcP isoenzymes were determined by ultra-thin-layer isoelectric focusing in polyacrylamide gels (Burdet and Whitehead, 1977) with modifications (size 240 \times 120 \times 0.24 mm, pH 3.5–9.5).

Western blots
EBNA was demonstrated by Western blot analysis (Motz et al., 1986). Briefly, cell lysates were separated by SDS-polyacrylamide gel electrophoresis, transferred onto nitrocellulose filters and incubated overnight with a 1:50 diluted serum pool. Bound antibodies were detected by peroxidase-conjugated anti-human-IgG antibodies (Ortho), stained with 0.1% H2O2 and 0.5 mg/ml diaminobenzidine.

DNA analysis
DNA was extracted from cultured cells by standard techniques (Maniatis et al., 1982). Fifteen micrograms of DNA were digested with appropriate restriction enzymes (Boehringer, Mannheim, FRG), electrophoresed on a 0.7% agarose gel, blotted and hybridized (Raghavachar et al., 1986). To demonstrate Ig-gene rearrangements, Hind III digests were hybridized to a 1.3-kb Eco RI Cp probe which detects a 17-kb germline band (Bakhshi et al., 1983) and from Bam HI digests to a c-kappa probe which detects a 12-kb germline band (Bakhshi et al., 1983). Both probes were kindly provided by Dr. P. Leder. After hybridization, the filters were washed under stringent conditions and exposed to XAR-5 film (Kodak) using Dupont Lightning-Plus intensifying screens for up to 24 hr at ~70°C.

RESULTS

Cell line
Suspension cultures of the patient's bone-marrow cells were set up 18 months post-grafting. The possibility that the culture supernatant of the 5637 cell line which was used for initial stimulation contained EBV was ruled out by previous experiments in which human cord-blood cells failed to grow continuously with this stimulator. The patient's cells decreased in number during the first 6 weeks of culture but thereafter increased continuously to about 10^8 MNC per flask per week. The cells within the cultures were morphologically undifferentiated. They were negative for peroxidase and a minority of about 2% was positive for periodic acid Schiff reaction (data not shown).

Immune phenotyping
Table I shows that the cells expressed B markers (CD19, CD20, CD10) with surface IgM and kappa light chains, but no T-cell or myeloid markers.

EBV analysis
The cells were positive for EBNA and VCA at the surface. Moreover, in a Western blot analysis of cell lysates, EBNA was clearly shown as a typical band (gel not shown). This finding supported the view that the cells in culture were immortalized by EBV.

DNA analysis
Surface marker analysis suggested that the cells were monoclonal. This was confirmed by Southern blot analysis of DNA extracted from the cell line, Fig. 1). Hybridization to \( \mu \)-and kappa-specific probes clearly demonstrated a monoclonal biallelic rearrangement of both genes with no residual germ-line bands.

Cytogenetic analysis
In direct preparations of bone-marrow cells taken from the patient 11 and 19 months post-grafting, 25 and 27 metaphases were analyzed, respectively, all of which were male (Fig. 2a). Surprisingly, cells of the cultured line all had a female karyotype (Fig. 2b). The cell line, therefore, must have been derived from the recipients' B cells. This finding prompted a more detailed determination of the chimeric state using isoenzyme analyses.

Acid phosphatase typing (Fig. 3)
Figure 3 shows the results of AcP typing for all the different purified cell preparations. The recipient was heterozygous BA.

| Table I – Phenotype of BMT-Chimera-Derived Cell Line |
|---|---|---|---|---|---|---|---|
| B-1 (CD20) | B-2 (CD19) | anti-IgM | IgG | IgD | kappa | lambda |
| % positive | 90 | 50 | 30 | 0 | 0 | 70 | 0 |
| J5 (CD10) | OKT-9 | VIM-D5 (CD15) | VIM-2 | OKT-1 (CD14) | My4 | My7 |
| % positive | 50 | 50 | 0 | 0 | 0 | 0 | 0 |
for AcP, showing the 3 main bands in isoelectric focusing of her hair root cells. The donor had the phenotype B comprising the 2 main bands on the gel. Following BMT all the different cell populations—erythrocytes, platelets, polymorphonuclear leukocytes, T lymphocytes, T-depleted lymphocytes, monocytes, total sedimented bone-marrow cells as well as Ficoll-isolated MNC—showed the B-type bands only (Fig. 3). In contrast, the cultured cells had 3 bands, thus demonstrating the presence of the recipient type A. However, a sub-population of type-B cells could not be excluded. Therefore, corresponding investigations were made by phosphoglucomutase-1 isoenzymes.

Phosphoglucomutase-1 typing (Fig. 4)

Four alleles of PGM-1 exist (a1 to a4). The patient was homozygous a1 as demonstrated by analysis of her hair root cells (erythrocytes contaminated with a2 and a3 bands via transfusions, Fig. 4). The donor cells were heterozygous a1/a3. Following BMT, all cell lineages showed the heterozygous bands a1/a3, implying presence of donor-type cells. In contrast, the cultured cells showed the a1 band only, thus proving unequivocally that the cell line was of recipient origin.

DISCUSSION

Most adult individuals show immunity to EBV. The level of virus-infected B cells in the blood has been shown to correspond to the level of EBV-shedding from the throat of healthy seropositive individuals (Wolf et al., 1984; Yao et al., 1985a). This, together with similar observations in immunosuppressed patients, supports the hypothesis that the EBV carrier state represents a chronic infection rather than a reactivation of “latent” EBV-immortalized clones (Yao et al., 1985b; Modrow et al., 1987). Allogeneic BMT offers an excellent opportunity to study these problems for several reasons, e.g., (a) allogeneic BMT chimeras often have markers which make it possible to determine whether the cells originate from donor or host; (b) they are, temporarily, heavily immunosuppressed and might therefore be more susceptible to reactivation of EBV-induced diseases.

However, the conditioning regimen of BMT might not only destroy the patient’s own hemopoietic cells but also eliminate B cells which have been immortalized by EBV. In fact, the development of secondary EBV-positive B-cell lymphomas has been only rarely observed and all cases have been of donor origin, whenever this was investigated (Schubach et al., 1982; Martin et al., 1984).

In our case, both the recipient and the donor were seropositive for EBV but did not exhibit any clinical or serological signs of reactivated infection. Nevertheless, an EBV-positive B-cell line could be grown 18 months post-grafting when the patient was off any immunosuppressive therapy and with no signs of GvHD. Differences in sex as well as in AcP and PGM-1 isoenzymes between donor and recipient enabled us to investigate the chimeric state of the transplanted patient in detail. Cytogenetic analysis may fail to identify 5–10% of different cells, but the isoenzyme methodology is independent of cell proliferation and can detect a proportion of only 1% foreign cells (Mittermüller et al., 1986). It has been repeatedly shown, by both methods, that our patient was a complete
The contribution of the 5637-conditioned medium to the outgrowth of the cell line is unclear. Since factors produced by the cell line do not support growth of lymphoid cells (Myers et al., 1984), these might have acted—if influencing growth of the cell line at all—via stimulation of accessory cells.

Our findings suggest that recipient-derived EBV-immortalized B cells persist after allogeneic BMT in patients who appear to be “complete chimeras” even when the most sensitive methods presently available are used. One may speculate that such cells could give rise to secondary B-cell lymphomas under certain circumstances. However, our patient did not develop lymphoma within the 3 years following grafting.

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