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Eradication of Epstein–Barr virus by allogeneic bone marrow transplantation: Implications for sites of viral latency

(Epstein–Barr virus nuclear antigens/immunoblotting)

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ABSTRACT **Wild-type strains of Epstein–Barr virus (EBV) can be distinguished on the basis of variations in the molecular weight of virus-encoded, growth transformation-associated proteins. This approach was used to study the persistence of EBV in two seropositive recipients of allogeneic bone marrow transplants. The first patient received marrow from her EBV-seronegative brother, became EBV seronegative after grafting, and remained so for >1200 days. Subsequently, she became infected with a new EBV strain that differed from her pretransplant strain but was indistinguishable from the virus isolated from her husband. The second patient received marrow from his EBV-seropositive brother. This patient showed only a transient decrease in IgG antibodies to EBV capsid antigen. His pretransplant strain differed from the virus of his donor. On days 252 and 915 after transplantation, lymphoblastoid cell lines were grown from the peripheral blood of the patient and were found to carry exclusively the virus of the donor. These results suggest that the latently EBV-infected host cells reside in a cellular compartment that can be destroyed by graft-versus-host reactivity, irradiation, or cytotoxic drugs. Hemopoietic tissue is the most likely candidate.**

Herpes viruses persist in their hosts for many years after initial infection. Active infections with those viruses occur frequently after allogeneic bone marrow transplantation (BMT) (1). Active herpes simplex virus (HSV) and varicella-zoster virus (VZV) infections are usually due to reactivation of endogenous virus. Cytomegalovirus may also be reactivated, as shown by restriction analysis of viral DNA (2), but it may also be transmitted by blood products.

Active Epstein–Barr virus (EBV) infection has been reported in marrow graft recipients, but its frequency and spectrum of clinical manifestations are uncertain (3–6). The rare but fatal lymphoproliferative syndrome is the most clearcut EBV-associated disease after allogeneic BMT (7–11). Such lymphoproliferation may arise from donor (7, 8, 10, 11) or host (9, 10) cells, but the origin of the virus is unknown. Viral antigens or viral genomes have been detected in B lymphocytes (12) and epithelial cells of the oropharynx (13) and the salivary glands (14) in asymptomatic EBV carriers. Rickinson (15) has suggested that the oropharyngeal epithelium constitutes a permanent site of EBV latency. According to this hypothesis, EBV-carrying B cells have a relatively short survival, since they are destroyed by immune surveillance. They would be continuously replenished by new EBV-carrying B cells infected during their passage through the persistently infected oropharyngeal epithelium.

Seven virus-encoded proteins are known to be expressed in EBV-transformed lymphoblastoid cell lines (LCLs). Six of them are nuclear antigens (EBNA-1 to 6), and the seventh is the latent membrane protein (16). Immunoblotting studies have shown that most of the EBNA proteins vary in size among different viral isolates (17, 18). The size variation of EBNA-1 is due to differences in the length of the internal repeat sequence (19). We used the size variation of the EBNA proteins to study the persistence and transmission of EBV after allogeneic marrow grafting.

MATERIALS AND METHODS

Patients. Both patients received transplants at the Leiden University Hospital consisting of marrow from their HLA-A,B,C,DR-identical, mixed leukocyte culture-nonreactive siblings. The marrow grafts were not depleted of T lymphocytes. Prior to BMT, the patients received cytoreductive therapy consisting of cyclophosphamide (60 mg per kg per day for 2 days) and total body irradiation (8 Gy in one session, with lung shielding after 6 Gy). Methotrexate was given after BMT as prophylaxis for graft-versus-host disease (GVHD). During the peritransplant period, the patients were nursed in reversed isolation and their gastrointestinal tracts were partially decontaminated with antibiotics (20). Leukocyte-poor erythrocyte and platelet concentrates were administered after BMT to maintain the hemoglobin level above 5 mmol/liter and the platelet count above 10,000/mm^3, respectively.

Patient no. 1, a 26-year-old woman, received a transplant during the second remission of her acute lymphoblastic leukemia. On day 0 she received marrow from her brother. Her hematological reconstitution was uneventful. The post-transplant course was complicated by moderate (grade II) acute GVHD of the skin, which was treated with prednisolone but progressed to mild chronic GVHD for which no immunosuppressive therapy was given. Recurrences of HSV and VZV infections (at 1 week and 7 months after BMT, respectively) were treated with acyclovir. She is currently alive and well at >2200 days after BMT.

Patient no. 2, a 22-year-old man, underwent allogeneic BMT during the first remission of his acute myelogenous leukemia. On day 0 he received marrow from his brother. His hematological recovery was uneventful. He developed grade II acute GVHD affecting the skin and gut, which was treated successfully with high doses of methylprednisolone. A recurrence of HSV infection at day 7 was treated with acyclovir. He is currently alive and well at >2500 days after BMT.

Cell Cultures. Peripheral blood mononuclear cells (PBMC) were obtained from heparinized venous blood from the patients and their marrow donors prior to BMT and from the

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Abbreviations: BMT, bone marrow transplantation; EBV, Epstein–Barr virus; EBNA, EBV nuclear antigen; HSV, herpes simplex virus; VZV, varicella–zoster virus; GVHD, graft-versus-host disease; LCL, lymphoblastoid cell line; PBMC, peripheral blood mononuclear cells; VCA, viral capsid antigen; EA, early antigen complex.

patients at various times after BMT. At a later stage, PBMC were obtained from patient 1's husband and patient 2's donor. The PBMC were cryopreserved in liquid nitrogen. After thawing, suspensions of 6–12 million viable PBMC were seeded in 96-well flat-bottomed microtiter plates. Per well, 200,000 cells were cultured in 200 μl of RPMI-1640 medium containing glutamine, antibiotics, 10% fetal calf serum, and cyclosporin A (Sandoz, Basel) at 0.5 μg/ml at 37°C in a humid atmosphere containing 5% CO_2. The cultures were fed every 10 days with similar medium that did not contain cyclosporin A and were maintained for 60–80 days. Some days after initiation of the cultures, 100,000 cord blood mononuclear cells were added to each well to rescue EBV released from *in vivo*-infected cells (21). Growing cells were harvested from their wells and the resulting LCLs were expanded. EBV was also recovered from mouthwashes of patient 1, her husband, and patient 2's donor by using the cord blood cell transformation assay (22).

EBV Serology. IgM and IgG antibodies against the viral capsid antigen (VCA) (23) and IgG antibodies against the early antigen complex (EA) (24) were detected by means of indirect immunofluorescence. Antibodies against EBNA-1 and EBNA-2 were detected by anti-complement immunofluorescence using the monospecifically transfected target cells DG75-EBNA-1 and U698-EBNA-2, respectively (25).

Immunoblotting. Whole cell extracts were prepared for immunoblotting by resuspending LCLs in electrophoresis sample buffer, sonicating, and boiling (17). One million cells were loaded per lane for electrophoresis in gels containing 7.5% polyacrylamide and sodium dodecyl sulfate; a discontinuous buffer system was used. The proteins were transferred electrophoretically to nitrocellulose filters for 4 hr at 10 V/cm. After the filters had been stained with Ponceau red, the positions of the molecular weight markers were noted and the filters were destained. Subsequently, they were blocked by using phosphate-buffered saline containing 5% nonfat dry milk (PBSM) and incubated with serum diluted in PBSM for 16 hr at 4°C. Polyvalent serum from a patient with chronic lymphocytic leukemia (PG) (26) was used to detect EBNA-1, -2, -3, -4, and -6. In addition, a human serum was affinity-purified with the synthetic peptide 107 (27) to allow the selective detection of EBNA-1. After washing with PBSM, the filters were incubated with rabbit anti-human IgG conjugated with alkaline phosphatase (Sigma) for 90 min at room temperature. They were then washed in phosphate-buffered saline containing 0.5% Tween-20. Bound antibody was detected by using α-naphthyl phosphate as substrate and fast red salt to develop color.

RESULTS

The EBV serology of patients 1 and 2 and their marrow donors are set out in Table 1. Patient 1 was seropositive before she received marrow from her seronegative brother. After BMT, her VCA and EBNA antibodies decreased to undetectable levels, and she remained seronegative for >1200 days. On day 1421, IgM-class VCA antibodies were detected, followed by IgG antibodies to VCA (day 1440), EBNA-1 and -2 (day 1721), and EA (day 1820). LCLs were obtained from patient 1's PBMC obtained prior to BMT and on day 1820, but not from those obtained on day 118 and 774 and from her donor at day -9. EBV was also recovered from patient 1's mouthwash obtained on day 1881 and from her husband's PBMC and mouthwash obtained on days 1820 and 2103, respectively.

Immunoblots from those LCLs were probed with the polyspecific PG serum (Fig. 1) and the EBNA-1 specific anti-107 serum (Fig. 2). There was a clear difference in the molecular mass of EBNA-1 between patient 1's pretransplant LCL (95 kDa) on the one hand and her posttransplant PBMC-

Table 1. EBV serology of patients 1 and 2 and donors

Serum source	Day*	IgM VCA	IgG VCA	IgG EA	EBNA-1	EBNA-2
Donor no. 1	−51	<5	<5	<40	<5	<5
Patient no. 1	−2	<5	1000	<40	160	<5
	+60	<5	100	<40	80	<5
	+83	<5	100	<40	<5	<5
	+173	<5	10	nsp	<5	<5
	+216	<5	<10	nsp	<5	<5
	+774	<5	<10	<40	<5	<5
	+1421	160	<10	<40	<5	<5
	+1440	10	100	<40	<5	<5
	+1721	<5	1000	<40	20	10
	+1820	<5	1000	40	20	5
	+1894	<5	1000	40	20	5
	+2058	<5	1000	40	20	5
Donor no. 2	+4	<5	100	<40	40	10
Patient no. 2	−29	<5	1000	<40	160	40
	+32	<5	100	40	40	20
	+63	<5	100	<40	40	10
	+94	<5	100	<40	<5	<5
	+133	<5	100	<40	<5	<5
	+150	<5	10	<40	<5	20
	+175	<5	10	<40	<5	<5
	+224	<5	1000	<40	<5	20
	+252	<5	1000	40	<5	10
	+299	<5	1000	40	<5	20
	+376	<5	1000	160	<5	20
	+726	<5	1000	<40	80	40
	+915	<5	1000	320	<5	20
	+1860	<5	1000	160	5	40
	+2217	<5	1000	40	<5	20

nsp, Nonspecific staining.
*Relative to BMT.

and mouthwash-derived LCLs (80 kDa) on the other. In contrast, the two posttransplant LCLs and the LCLs derived from patient 1's husband contained EBNA-1 of similar size. Furthermore, probing with the PG serum showed that patient 1's pre- and posttransplant LCLs differed with regard to the sizes of the high molecular mass EBNAs (EBNA-3, -4, and -6), while her posttransplant LCLs and the LCLs from her husband were indistinguishable with regard to the sizes of these proteins. There was no informative difference in the size of EBNA-2 (85 kDa) between patient 1's pre- and posttransplant LCLs.

Patient 2 was seropositive before he received marrow from his seropositive brother (Table 1). After BMT, his EBNA-1 and EBNA-2 antibodies decreased to undetectable levels. IgG VCA antibodies decreased to a nadir of 10 on days 150 and 175. On day 224, IgG VCA antibodies had increased again to pretransplant levels. On this day, EBNA-2 antibodies were also detected. This was followed by EA antibodies on day 252 and EBNA-1 antibodies on day 726. IgM VCA antibodies were not detectable in any serum sample.

LCLs were obtained from patient 2's PBMC obtained 9 days prior to BMT and on days 252 and 915. Starting from the culture of the day 915 PBMC, 24 LCLs were established after separate harvesting of wells that contained growing cells. LCLs could not be established from PBMC obtained on days 63 and 150 and from the marrow donor at day 4. However, EBV could be recovered from the marrow donor's PBMC and mouthwash obtained on day 2401.

Probing of the immunoblots from these LCLs with the polyspecific PG serum (Figs. 1 and 3) and the EBNA-1

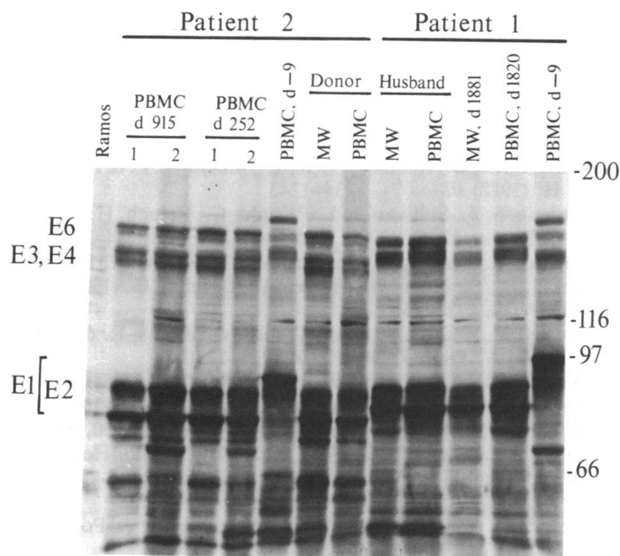

FIG. 1. Immunoblot probed with the polyspecific PG serum. Numbers on the right indicate molecular mass in kilodaltons (kDa). The molecular mass markers (Bio-Rad) were myosin (200 kDa), *Escherichia coli* β-galactosidase (116 kDa), rabbit muscle phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), and hen egg white ovalbumin (43 kDa). Ramos is an EBV-negative control line. EBNA-1, -2, -3, and -6 were assigned to their corresponding bands after probing with monospecific antisera (27, 28). The assignment of EBNA-4 was done by exclusion (28), since no EBNA-4 monospecific antiserum was available. Lanes 1 and 2 refer to different LCLs that were established after separate harvesting of wells containing growing cells. E, EBNA; d, day; MW, mouthwash.

specific anti-107 serum (Fig. 2) revealed a clear difference in molecular mass of EBNA-1 between patient 2's pretransplant LCL (90 kDa) and his donor's LCLs (77 kDa). The size of EBNA-2 in patient 2's pretransplant LCL was slightly larger (85 kDa) than in his donor's LCLs (83 kDa). The day 252 and 915 posttransplant LCLs of patient 2 expressed EBNA-1 and -2 similar in size to the donor's LCLs. The high molecular mass EBNAs (EBNA-3, -4, and -6) of patient 2's posttransplant LCLs were similar in size to his donor's LCLs but differed from those in the pretransplant LCL. All 24 day 915 LCLs expressed EBNA-1, -2, -3, -4, and -6 proteins similar in size to the donor's LCLs.

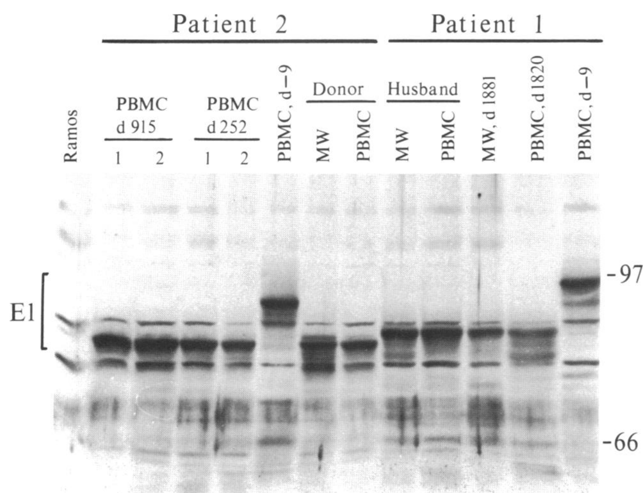

FIG. 2. Immunoblot probed with the EBNA-1-specific anti-107 serum. The anti-107 serum also detected two non-EBV-specific bands of 72 and 82 kDa, as evidenced by their reactivity with Ramos. See the legend to Fig. 1.

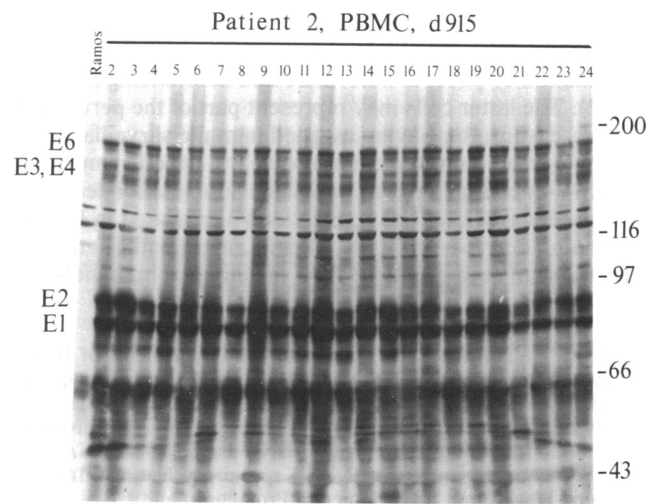

FIG. 3. Immunoblot probed with the polyspecific PG serum. The PG serum also detected non-EBV-specific bands of 60–65, 125, and 135 kDa, as evidenced by their reactivity with Ramos. Lanes 2–24 represent individual LCLs that were established after separate harvesting of wells containing growing cells. The lane 2 LCL is the same as the day 915 lane 2 LCL in Fig. 1. See the legend to Fig. 1.

DISCUSSION

The combined serological and immunoblotting studies show that the pretransplant EBV strains of both patients have been eradicated by the treatment and replaced by a different EBV strain. Patient 1 received marrow from a seronegative donor and became seronegative for more than 1200 days. Reinfection was accompanied by the appearance of IgM VCA antibodies in her serum. Patient 1's new strain was indistinguishable from the strain isolated from her husband. Patient 2 received marrow from a seropositive donor and showed only a transient decrease in IgG VCA antibody titers. On day 224, his IgG VCA antibody titer had already returned to the pretransplant level. The new strain was detected on day 252 and was similar to the one of his marrow donor.

Several features of the transplantation protocol and its complications may have contributed to the eradication of the pretransplant EBV strain. The 8-Gy total body irradiation is aimed to eradicate residual leukemic cells but kills most normal lymphocytes as well, including the B cells that carry latent EBV (12). Cytotoxic drugs may also be relevant. Cyclophosphamide was given for pretransplant cytoreductive therapy, and methotrexate was given as postgraft GVHD prophylaxis. Both patients have also been treated with acyclovir to control HSV and VZV infections. Acyclovir can inhibit EBV replication in mononucleosis patients, but it does not eradicate the virus (22). Graft-versus-host reactivity—both patients have developed grade II acute GVHD—may contribute to the elimination of residual host hemopoietic cells. In this context it is relevant to recall the study of Hill *et al.* (29), who showed a significantly increased incidence of mixed hematologic chimerism among patients with grades II–IV acute GVHD as compared to those with grades 0–I acute GVHD.

In addition to B lymphocytes, epithelial cells can also harbor EBV (13, 30). Irradiation, cytotoxic drugs, and GVHD may damage the oropharyngeal epithelium, but they do not cause widespread destruction. If the oropharyngeal epithelium were a permanent EBV reservoir, the pretransplant strain of the recipient would be expected to persist after BMT. Its eradication suggests that hemopoietic cells constitute a more likely site of permanent EBV latency. Large B-blasts of healthy EBV carriers can release virus *in vitro* and give rise to LCLs by infection of previously uninfected cells

(12). Small B lymphocytes can grow out *in vitro* directly, even under conditions in which virus release is inhibited by a combination of phosphonoformate and neutralizing antibodies (12). The latter cells may represent part of the permanent EBV reservoir after having escaped immune surveillance by the down-regulation of most of their EBV-encoded antigens. All EBV-encoded, growth transformation-associated antigens except EBNA-1 are actually down-regulated in Burkitt lymphoma-derived cell lines, which correspond phenotypically more to resting B cells than to activated immunoblasts (21).

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