Human plasma accelerates immortalization of B lymphocytes by Epstein–Barr virus

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Received 22 July 2007; revision accepted 3 September 2007

Abstract. *Objective:* Serum and plasma contain species-specific factors that modulate cell population growth and function, and that are required for proliferation of most cell cultures. Foetal calf serum (FCS) is the most common source of these growth factors. We studied the effect of human plasma (HP) on the immortalization process of B lymphocytes by Epstein–Barr virus (EBV) was studied. *Materials and methods:* The effect of HP as compared to FCS was done through assessment of cell proliferation. *Results:* It was found that HP (autologous and non-autologous plasma) is more effective than FCS in generating lymphoblastoid cell lines, regardless of EBV status of the donors: 65% of HP-supplemented cultures developed into lymphoblastoid cell lines by 7–14 culture days, as compared to 16% of cultures with FCS. In addition, 6% of HP-supplemented cultures did not achieve becoming lymphoblastoid cell lines by day 35 in comparison to 94% of cultures with FCS. The higher proliferative effect of HP was not altered by heat inactivation or filtration. HP maintained its proliferative activity at 4 °C over 8 months, thus indicating that HP contains a stable growth factor(s), which accelerates B-lymphocyte immortalization. *Conclusion:* The results support other studies that recommend the use of autologous plasma for tissue culture, mainly in the case of autologous transplantation. Furthermore, the use of HP allows preparation of lymphoblastoid cell lines from a small amount of peripheral blood in a shorter period of time and with a higher rate of success.

INTRODUCTION

Epstein–Barr virus (EBV) is a herpes virus that infects B lymphocytes, and uniquely induces their immortalization (Henle *et al.* 1967). Immortalization of human B lymphocytes by EBV provides unlimited amount of cells and DNA that are needed for a wide range of genetic, immunological and biochemical studies. The ability to establish B-lymphocyte cell lines in a relatively short time and from relatively small amounts of blood would significantly improve our ability to perform these studies.

Serum and plasma contain species-specific factors that modulate cell growth and function, and are required for most cell cultures. In most culture procedures, including lymphoblastoid cell line preparation, culture medium is supplemented with foetal calf serum (FCS) as the most

common source of growth factors (Freshney 1994). However, considering clinical applications, FCS has a potential risk of viral or prion-related disease transmission and foreign protein contamination. Due to this risk investigators have recently begun replacing FCS with autologous human serum (HS) or human plasma (HP) obtained from peripheral blood. It has been found that HP and HS support stem cell expansion and differentiation (Koller *et al.* 1998; Kuzentsov *et al.* 2000; McAlinden & Wilson 2000; Hankey *et al.* 2001; Anselme *et al.* 2002; Yamamoto *et al.* 2003; Ryang *et al.* 2004; Kobayashi *et al.* 2005). Serum and plasma contain species-specific factors that modulate cell growth and function (Maurer 1992), plasma being richer than serum in growth factors secreted by platelets (Piche & Graves 1989; Hwang *et al.* 1992; Slater *et al.* 1995; Lucarelli *et al.* 2003).

In the present study, the effect of HP on B-lymphocyte immortalization, as compared to FCS was analysed. The effect of HP (autologous and non-autologous) versus FCS with respect to time interval needed for immortalization of B lymphocytes was compared.

MATERIALS AND METHODS

Mononuclear (MN) cells from 3 to 5 mL of peripheral blood of 70 healthy volunteers (34 women and 36 men, mean age 24 years) were separated using histopaque according to the standard method. Plasma was collected from the upper fraction and was centrifuged. MN cells were collected from the intermediate fraction and washed twice with RPMI-1640 medium. Cells were immortalized by infection with EBV, using the same amount of filtered supernatant from chronically EBV-infected B95-8 cells, as previously described (Sugden & Mark 1977). Cells ($1-3 \times 10^5$ cells/mL) were cultured in RPMI-1640 medium (Bet-Haemek, Israel), supplemented with either 10% FCS (various FCS batches were used during the study), with 10% autologous plasma (AP), 10% filtered (0.2 μ m) AP, heated inactivated AP (56 °C, 30 min) or non-autologous plasma (filtered pooled plasma), respectively. Plasma was stored at 4 °C for 8 months. Mock infected cells were cultured with 10% human plasma (HP) (autologous or non-autologous) for testing the effect of HP only, on B lymphocytes. The cells were counted at time intervals of 0, 7, 10 and 14 days after infection and then every 3–4 days until the culture achieved a cell concentration of 10^6 cells/mL, at which the first passage was performed. This was completed using HP or FCS, respectively, but subsequent passages were supplemented with FCS. After 3–4 passages, cells were kept frozen at –70 °C for 2–4 months and then were thawed and cultured for 3–4 passages.

RESULTS

Table 1 summarizes the length of time needed for lymphoblastoid cultures to reach a cell concentration of 10^6 cells/mL (first passage) for each culture growth condition: FCS, autologous plasma (AP), filtered non-autologous human plasma (FHP) and heat-inactivated human plasma (HHP).

The same proliferative effect was found, regardless of donor gender and EBV status, whether the culture grew in the presence of AP or FHP. In addition, there was no difference in time elapsed from culture establishment to the first passage between the parallel cultures, grown either with FHP or with HHP. Figure 1 illustrates cumulative percentages of cell lines established

Table 1. Number of lymphoblastoid cell lines (% cell lines) established in each interval of culture with different culture supplements

Days in culture	Type of culture supplement			
	FCS (%)	AP (%)	FHP (%)	HHP (%)
≤ 7	4 (6)	6 (9)	3 (12)	3 (27)
8–10	3 (4)	12 (16)	5 (20)	3 (27)
11–14	4 (6)	28 (40)	9 (36)	5 (46)
15–18	6 (9)	13 (19)	2 (8)	0 (0)
19–28	1 (1)	7 (10)	0 (0)	0 (0)
29–35	3 (4)	0 (0)	0 (0)	0 (0)
≥ 35	49 (70)	4 (6)	6 (25)	0 (0)
Total	70	70	25	11

AP, autologous plasma; FCS, foetal calf serum; FHP, filtered human plasma; HHP, heated human plasma.

Figure 1. Percentages of cell lines achieved at ≤ 7–14, 15–28 and ≥ 29 days in culture with each supplement. Numbers above the bars represent the composite percentage.

at three arbitrarily designated intervals: 7–14 days, 15–28 days and more than 28 days. Sixty-five per cent of the lymphoblastoid cultures grown with AP or FHP reached a cell concentration of 10^6 cells/mL by 7–14 days in culture, while only 16% of cultures grown with FCS reached their first passage by 7–14 days. Ninety per cent of the cultures grown with HP reached their first passage 1–3 weeks earlier than those grown with FCS. Ten per cent of cultures reached the first passage at the same time, regardless of whether they were grown with FCS or with HP. Seventy per cent of cultures supplemented with FCS and only 6% of the cultures supplemented with HP showed late occurrence of immortalization (35 days). In order to address the question whether EBV donor status affected the immortalization process, cells were cultured with HP but with no EBV infection. All the control cultures (mock-infected cultures) remained alive for 2 weeks only with no expansion.

There was correlation between cell concentration and the time interval needed to establish lymphoblastoid cell lines. It took longer to establish lymphoblastoid cell lines when there was cell concentration of less than 2×10^4 cells/mL (3–5 weeks) and less time when cell concentration was above 5×10^5 cells/mL (1–3 weeks). HP supplement always significantly promoted cell immortalization (six experiments).

In order to determine whether the effect of HP was due to an increased amount of EBV adsorbed to B cells, or whether HP enhances proliferative activity, mononuclear cells were infected with EBV and after 2 h of EBV adsorption, the cells were washed twice to elute non-adsorbed viruses, after which medium supplemented with either FCS or filtered HP was added to the cultured cells. There were no differences in HP time to immortalization of B lymphocytes comparing the washed and the non-washed cultures (three experiments).

To further clarify whether the supporting activity of human serum on lymphoblastoid production is that of a proliferation inducer, we thawed seven lymphoblastoid cell lines, which were 6 years old and had been kept at -70°C . They were cultured in medium with either FCS or HP. All seven cell lines grew well with HP, while only three out of the seven cell lines with FCS thrived. The proliferative effect of 5% HP compared to that of 10% HP was studied in 21 cultures. About 50% (11 out of 21) of the cultures supplemented with 5% HP showed less cell proliferation than that of 10% HP and a similar effect to that was observed in the parallel cultures supplemented with 10% FCS. All cell lines established in this study were frozen and thawed 2–3 months later. All were grown in FCS successfully for 3–4 passages.

DISCUSSION

Present results indicate that HP (autologous as well as non-autologous) is more effective than FCS for the development of lymphoblastoid cell lines regardless of EBV status of the donors, and the effect was more pronounced at low cell numbers (less than 10^4 cells/mL). Use of HP rather than FCS enables preparation of lymphoblastoid cell lines from a small amount of peripheral blood (minimum 1.5 mL, 10^4 MN cells/mL), in a shorter period of time and with a higher rate of success. Cultures with HP reached their first passage 1–3 weeks earlier than cultures with FCS in about 90% of the preparations; among them 75% reached the first passage by 7–14 days. Only 30% of the cultures with FCS developed to lymphoblastoid cell lines by 35 days, as compared to 94% of those supplemented with HP. The low success rate in achieving cell lines using FCS was most probably due to low cell concentration ($1\text{--}3 \times 10^5$ MN cells/mL) used in this study compared to the standard protocol (10–15 mL blood sample, $1\text{--}5 \times 10^6$ MN cells/mL). In our experience (250 cell lines), using 10–15 mL of blood with FCS results in 95% success of generating lymphoblastoid cell lines usually in 14–28 days. Thus, cell concentration plays a role in success rate in the establishment of lymphoblastoid cell lines derived from peripheral blood.

Variability in HP effect expressed by difference in time to the first passage is most likely due to differences in composition and quantity of growth factor(s) in each donor's plasma, as well as differences in the intrinsic potential of each donor's B lymphocytes to proliferate or to immortalize. The growth factor(s) remain active whether kept at 4°C for at least 8 months, heated or filtered. The exact mechanism of growth and expansion activity of HP is unclear. However, three main findings have been published that support the assumption that HP contains factor(s), which mainly enhance EBV proliferation activity (immortalization) rather than playing a role as EBV adsorption enhancers.

1 EBV latent membrane protein 1 (LMP1) presence is essential for B-cell immortalization, as it is the only EBV protein that has transforming potential (Wang *et al.* 1985; Baichwal & Sugden 1988; Moorthy & Thorley-Lawson 1993). LMP1 stimulates B-cell proliferation through the JAK/STAT signalling pathway (Gires *et al.* 1999) and induces expression of B-cell activation markers such as CD23, CD40, cell adhesion molecules ICAM1, LAF1 and LFA3 (Wang *et al.* 1990) and EGFR (Miller *et al.* 1997).

- 2 The proliferation process of B lymphocytes during immortalization is promoted by autocrine and paracrine factors, such as IL-6 (Tosato *et al.* 1990), 3B6 Il-1 (Rimsky *et al.* 1986; Wakasugi *et al.* 1987) and soluble CD23 (Kinter & Sugden 1981; Yukawa *et al.* 1987). They most probably accumulate during culturing and act as growth factors for EBV-immortalized B cells and promote lymphoblastoid generation (Blazar *et al.* 1983; Gordon *et al.* 1984a,b; Tosato *et al.* 1988a,b). The prolonged time needed at low cell concentrations (less than 2×10^4 cells/mL) to establish lymphoblastoid cell lines could be due to the low level of autocrine and paracrine factors in those cultures.
- 3 Stem cell culture in medium with HP results in enhanced proliferation (Koller *et al.* 1998; Kuzentsov *et al.* 2000; McAlinden & Wilson 2000; Hankey *et al.* 2001; Anselme *et al.* 2002; Yamamoto *et al.* 2003; Ryang *et al.* 2004; Kobayashi *et al.* 2005). HP enriched with platelets contains growth factors, such as PDGF, TGF- β and EGF that enhance stem cell proliferation (Hwang *et al.* 1992; Piche & Graves 1989; Slater *et al.* 1995; Lucarelli *et al.* 2003). It is likely that factor(s) in HP stimulate a signalling pathway process (such as JAK/STAT), thus promoting B-cell immortalization by EBV.

The last assumption is further strengthened by two observations that were made in this study: (i) HP also enhances growth of thawed lymphoblastoid cell lines, as compared to FCS; and (ii) cultures that were washed after EBV infection followed by HP or FCS supplemented medium, showed the same enhanced proliferation effect as non-washed cultures. Because there was no difference between the effects of autologous and non-autologous plasma, the possibility of self-specific factors in autologous versus non-autologous plasma, is ruled out. The data apparently point to a common factor that exists in plasma of the general human population. HP alone maintained mock-infected lymphocytes alive but showed no proliferative effect on them, while stem cells (mesenchymal, adipose stromal cells, osteoblasts) cultured with HP or HS, expanded and differentiated (Koller *et al.* 1998; Kuzentsov *et al.* 2000; McAlinden & Wilson 2000; Hankey *et al.* 2001; Anselme *et al.* 2002; Yamamoto *et al.* 2003; Ryang *et al.* 2004; Kobayashi *et al.* 2005). This points to the possibility that B lymphocytes, which are already differentiated, need first LMP1 stimulation for the signalling pathway, and only then are they further stimulated by growth factors from HP.

In conclusion, the present experiments demonstrate that substitution of FCS by either autologous or non-autologous filtered HP in the culture medium of B lymphocytes being immortalized by EBV, enhances cell expansion and improves establishment of lymphoblastoid cell lines. Using HP rather than FCS, lymphoblastoid cell lines can be generated from small amounts of peripheral blood (~1.5 mL) in a shorter time and with a higher rate of success than by other standard methods. This can be achieved without special effort beyond the use of the same blood sample for the B lymphocytes and for the plasma. The results also support other studies showing the potential of HP as a non-risky source of growth factors for human tissue culture, mainly in cases of autologous transplantations. Knowledge of the exact growth factor(s) in HP that mediate these effects and the mechanism through which they act, might open new insights into the understanding of the cell cycle and the interaction between cells and cell immortalization by EBV.

ACKNOWLEDGEMENTS

I would like to thank Monica Neuhaus, BSc, for her technical assistance and Juan Chemke, MD, for his editorial assistance.

REFERENCES

- Anselme K, Broux O, Noel B, Bouxin B, Bascoulegue G, Dudermel AF, Bianchi F, Jeanfils J, Hardouin P (2002) *In vitro* control of human bone marrow stromal cells for bone tissue engineering. *Tissue Eng.* **8**, 941–953.
- Baichwal VR, Sugden B (1988) Transformation of Balb 3T3 cells by the BNLF-1 gene of Epstein-Barr virus. *Oncogene* **2**, 461–467.
- Blazar B, Sutton A, Strome M (1983) Self stimulating growth factor production by B cell line derived from burkitt's lymphoma and other lines transformed *in vitro* by Epstein-Barr virus. *Cancer Res.* **43**, 4562–4568.
- Freshney R (1994) *Culture of Animal Cells: A Manual of Basic Technique*, pp. 90–91. New York: Wiley-Liss.
- Gires O, Kohlhuber F, Kilger E, Baumann M, Kieser A, Kaiser C, Zeidler R, Scheffer B, Ueffing M, Hammerschmidt W (1999) Latent membrane protein 1 of Epstein-Barr virus interacts with JAK3 and activates STAT proteins. *EMBO J.* **18**, 3064–3073.
- Gordon J, Ley SC, Melamed MD, Amam P, Huges-Jones NC (1984a) Soluble factor requirement for autostimulatory growth of B lymphoblasts immortalized by Epstein-Barr virus. *J. Exp. Med.* **159**, 1554–1559.
- Gordon J, Ley SC, Melamed MD, English LS, Huges-Jones NC (1984b) Immortalized B lymphocytes produce B cell growth factor. *Nature (London)* **310**, 145–148.
- Hankey DP, McCabe RE, Doherty MJ, Nolan PC, McAlinden MG, Nelson J, Wilson DJ (2001) Enhancement of human osteoblast proliferation and phenotypic expression when cultured in human serum. *Acta Orthop. Scand.* **72**, 395–403.
- Henle W, Diebe V, Kohn G, Zur Hausen H, Henle G (1967) Herpes-type virus and chromosome marker in normal leukocytes after growth with Burkitt cells. *Science* **157**, 1064–1065.
- Hwang DL, Latus LJ, Lev-Ran A (1992) Effects of platelet-contained growth factors (PDGF, EGF, IGF-I, and TGF-beta) on DNA synthesis in porcine aortic smooth muscle cells in culture. *Exp. Cell Res.* **200**, 358–360.
- Kinter C, Sugden B (1981) Identification of antigenic determinants unique to the surfaces of cells transformed by Epstein-Barr virus. *Nature (London)* **294**, 458–460.
- Kobayashi T, Watanabe H, Yanagawa T, Tsutsumi S, Kayakabe M, Shinozaki T, Higuchi H, Takagishi K (2005) Motility and growth of human bone-marrow mesenchymal stem cells during *ex vivo* expansion in autologous serum. *J. Bone Joint Surg. Br.* **87**, 1426–1433.
- Koller MR, Maher RG, Manchel I, Oxender M, Smith AK (1998) Alternatives animal sera for human bone marrow cell expansion: human serum and serum-free media. *J. Hematother.* **7**, 413–423.
- Kuznetsov SA, Mankani MH, Robey PG (2000) Effect of human serum on human bone marrow stromal cells: *ex vivo* expansion and *in vivo* bone formation. *Transplantation* **70**, 1780–1787.
- Lucarelli E, Beccheroni A, Donati D, Sangriogi L, Cenacchi A, Del Vento AM, Meotti C, Bertojia AZ, Giardino R, Fornasari PM, Mercuri M, Picci P (2003) Platelet-derived growth factors enhance proliferation of human stromal stem cells. *Biomaterials* **24**, 3095–3100.
- Maurer HR (1992) Towards chemically defined serum-free media for mammalian cell culture. In: Fersheny RI, ed. *Animal Cell Culture-A Practical Approach*, 2nd edn, pp. 15–46. Oxford, UK: I.R.L. Press.
- McAlinden MG, Wilson DG (2000) Comparison of cancellous bone-marrow derived cell proliferation in autologous human and fetal bovine serum. *Cell Transplant.* **9**, 445–451.
- Miller WE, Mosialos G, Kieff E, Rabb-Traub N (1997) Epstein-Barr virus LMP1 induction of epidermal growth factor receptor is mediated through a TRAF signaling pathway distinct from NF-kB activation. *J. Virol.* **71**, 581–596.
- Moorthy RK, Thorley-Lawson DA (1993) All three domains of the Epstein-Barer virus-encoded latent membrane protein LMP1 are required for transformation of rat1 fibroblasts. *J. Virol.* **67**, 1638–1646.
- Piche JE, Graves DT (1989) Study of the growth factors requirements of human bone derived cells: a comparison with human fibroblasts. *Bone* **10**, 131–138.
- Rimsky L, Wakasugi H, Ferrera P, Robin P, Capdevielle J, Turz T, Fradelizi D, Bertoglio J (1986) Purification to homogeneity and NH2 terminal amino acid sequence of a novel interleukin 1 species derived from a human B cell line. *J. Immunol.* **136**, 3304–3310.
- Ryang HL, Byung CK, Iksoo C, Hanna K, Hee SC, Keun TS, Yong CB, Jin SJ (2004) Characterization and expression analysis of mesenchymal stem cells from human bone marrow and adipose tissue. *Cell. Physiol. Biochem.* **14**, 311–324.
- Slater M, Patava J, Kingham K, Meason RS (1995) Involvement of platelets in stimulating osteogenic activity. *J. Orthop. Res.* **13**, 655–663.
- Sugden B & Mark W (1977) Clonal transformation of adult human leukocytes by Epstein-Barr virus. *J. Virol.* **23**, 503–508.
- Tosato G, Gerrard TL, Goldman NG, Pike SE (1988a) Stimulation of EBV-activated human B cells by monocyte and monocyte products. Role of INF-beta2/B cell stimulatory factor 2/IL-6. *J. Immunol.* **140**, 4329–4336.

- Tosato G, Seamon KB, Goldman ND, Sehgal PB, May LT, Washington GC, Jones KD, Pike SE (1988b) Identification of monocyte-derived human B cell factor as interferon beta2 (BSF-2, IL-6). *Science* **239**, 502–504.
- Tosato G, Tanner J, Jones KD, Ravel M, Pike SE (1990) Identification of interleukin-6 as autocrine growth factor for Epstein-Barr Virus-immortalized B cell. *J. Virol.* **64**, 3033–3041.
- Wakasugi L, Rimsky H, Mahe Y, Kamel AM, Fradelizi D, Turz T, Bertoglio J (1987) Epstein-Barr virus containing B cell line produces interleukin 1 that is use as a growth factor. *Proc. Natl. Acad. Sci. USA* **84**, 804–808.
- Wang D, Liebowitz D, Kieff E (1985) An EBV membrane protein expressed in immortalized lymphocytes transforms established rodent cells. *Cell* **43**, 831–840.
- Wang F, Gregory C, Sample C, Rowe M, Leibowitz D, Murray R, Rickinson K, Kieff E (1990) Epstein-Barr virus latent membrane protein (LMP1) and nuclear protein 2 and 3C are effectors of phenotypic changes in B lymphocytes: EBNA-2 and LMP1 cooperatively induce CD23. *J. Virol.* **64**, 2309–2318.
- Yamamoto N, Isobe M, Negishi A, Yoshimasu H, Shimokawa H, Ohya K, Amagasa T, Kasugai S (2003) Effect of autologous serum on osteoblastic differentiation in human bone marrow cells. *J. Med. Dent. Sci.* **50**, 63–69.
- Yukawa K, Kuritani H, Owaki H, Yamasaki K, Yokota A, Nakamura H, Barsumian EL, Hardy RRR, Suemura M, Kishimoto T (1987) A B cell-specific differentiation antigen, CD23, is a receptor for IgE (Fc & R) on lymphocytes. *J. Immunol.* **138**, 2576–2580.