Physiologic Concentrations of Normal Human Plasma Lipoproteins Inhibit the Immortalization of Peripheral B Lymphocytes by the Epstein-Barr Virus

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ABSTRACT Epstein-Barr virus (EBV)-induced immortalization of adult human B lymphocytes is suppressed by physiologic concentrations of human plasma lipoproteins. Several inhibitory mechanisms appear to be operative. First, low density lipoproteins (LDL) directly reduce the ability of EBV to transform human B cells. Second, LDL as well as intermediate and very low density lipoproteins modulate early inductive events rendering the B cell refractory to transforming signals from EBV. Third, LDL also selectively inhibit an EBV-inducible step that occurs within 24 h after transformation. Finally, very low density lipoproteins can abrogate the ongoing, cellular proliferation of EBV-transformed, established B cell lines. The plasma lipoproteins may therefore prevent the emergence of EBV-transformed malignant B cell clones in vivo. Conceivably, on this basis, environmental and genetic influences on plasma lipoprotein concentrations may affect the global distribution of Burkitt's lymphoma, a lymphoid malignancy putatively caused by EBV.

INTRODUCTION

Human bone marrow-derived (B) lymphocytes are susceptible to infection by the Epstein-Barr virus (EBV)[1] both in vitro and in vivo (1). *De novo* infection of B cells in vitro regularly produces transformed cell lines which fulfill the criteria for malignant transformation by oncogenic viruses (2) (e.g., lymphoblastoid morphology, continuous unrestricted growth, induction of fatal metastasizing lymphoma in immunosuppressed animals, etc.). In vivo infection of humans most commonly produces a self-limited lymphoproliferative disease (infectious mononucleosis) (3) and is frequently associated with two highly aggressive malignancies: i.e., African Burkitt's lymphoma (4) and nasopharyngeal carcinoma (5). The oncogenic potential of EBV is further established by its ability to induce fatal metastasizing lymphomas in primates either infected in vivo or injected with autologous B cells previously infected in vitro by EBV (2).

Despite the worldwide distribution of EBV and the near universality of infection (6), most infected humans escape EBV-induced malignancy even though a proportion of their B cells become latently infected for life and are capable of spontaneous transformation in vitro (7). Apparently, control mechanisms exist that inhibit the acquisition and/or the expression of the potential of EBV-transformed B cells to proliferate indefinitely in vivo. Others have reported the existence of cellular mechanisms that operate either to suppress the proliferation of EBV-infected B cells (8, 9) or to kill them (10). Because of the known ability of normal human plasma lipoproteins to inhibit the proliferative response of mitogen- and antigen-stimulated peripheral blood B and T cells (11–16), we have investigated their effect on EBV-induced B cell transformation.

METHODS

EBV production

Transforming virus was obtained from the supernate of the EBV-producing B95-8 marmoset lymphoblastoid cell line (8) carried in a humidified atmosphere of 5% CO_2 in air at 37°C in RPMI 1640 (Gibco Laboratories, Grand Island Biological

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Abbreviations used in this paper: B cell, bone marrow-derived lymphocyte; EBNA, Epstein Barr virus nuclear antigen; EBV, Epstein Barr virus; HDL, high density lipoprotein; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; PBM, peripheral blood mononuclear cells; PHA, phytohemagglutinin; VLDL, very low density lipoprotein.

Co., Grand Island, N. Y.) containing 10% fetal calf serum, 50 μg/ml streptomycin, 100 U/ml penicillin, and 2 mM glutamine (complete medium). The cells were fed by diluting to 0.2×10^6 cells/ml every 3–4 d. For maximum virus production the cells were maintained without manipulation for 12 d from the last feeding before harvesting. After centrifugation (3,000 rpm, room temperature, PR-6000, International Equipment Co., Needham Heights, Mass.), the clear supernate was filtered through a 0.45-μm filter and stored in aliquots at −70°C. Vials of virus-containing supernate were thawed immediately before each experiment.

Peripheral blood mononuclear cell isolation

Several healthy EBV-seropositive adults and a single EBV-seronegative donor provided the lymphocytes for most of the studies reported herein. Peripheral blood mononuclear cells (PBM) were isolated from heparinized venous blood (Vacutainer, Becton, Dickinson & Co., Oxnard, Calif.) by sedimentation onto a barrier of Ficoll-Hypaque (rho = 1.077 g/ml) according to the method of Boyum (17) as previously described (11). The cells collected at the interface were suspended in complete media described above. Absolute yield of mononuclear cells was >75% with 95% purity and viability and consisted of 85–90% lymphocytes and 10–15% monocytes as determined by the peroxidase reaction.

B lymphocyte enrichment

B lymphocytes were enriched from the PBM population by negative selection. Following 24-h cultivation in tissue culture plates (Falcon Labware, Div. Becton, Dickinson) to remove adherent cells, PBM were mixed at a ratio of 1:50 with sheep erythrocytes that had been treated with neuraminidase as previously described (18). The mixture was incubated at 37°C for 10 min, centrifuged at 200 *g* for 5 min, and incubated at room temperature for 1 h at which time it was gently resuspended and sedimented onto a barrier of Ficoll-Hypaque as described above. The cells remaining at the interface were washed three times in complete media and the percentage of rosette-forming cells remaining was determined. The purification process was repeated until <5% of the interface cells formed rosettes with neuraminidase-treated sheep erythrocytes. Such cells were designated "B cells" and contained ~10% monocytes.

Lymphocyte culture

EBV and mitogen stimulation. PBM and B cells were cultured at 0.5×10^6/ml in complete medium (200 μl) in the wells of flat-bottomed sterile tissue culture plates (Microtest II; Falcon Labware) or, where indicated, in 25 cm^2 tissue culture flasks in 2-ml vol (Falcon Labware). The cells were usually cultivated for 24 h in the presence of either 50 μl (wells) or 500 μl (flasks) of purified lipoproteins before the addition of an equal volume of EBV although the time of addition of lipoproteins relative to EBV was varied in selected experiments as indicated. For descriptive purposes, the time of addition of EBV will be taken as "time zero" in this manuscript. For comparison purposes in selected experiments lymphocytes cultured in the microtiter system were stimulated by the mitogen phytohemagglutinin (PHA-m, Gibco Laboratories) at a previously determined optimal concentration of 1 μl/well (11).

[^{3}H]Thymidine uptake

The DNA synthetic capacity of EBV-infected and PHA-stimulated lymphocytes (measured as [^{3}H]thymidine uptake) and the effect thereupon of purified plasma lipoprotein fractions was assessed in quadruplicate cultures in the microtiter system described above. EBV-infected cultures were routinely harvested every 3 d for 1 mo after infection. PHA-stimulated cells were harvested 3 d after addition of the mitogen. All cultures were pulsed with 0.5 μCi of [^{3}H]-thymidine (2.0 Ci/mM) (New England Nuclear, Boston, Mass.) for 18 h before harvesting onto glass fiber filters using a multiple automated sample harvester (MASH II, Microbiological Associates, Walkersville, Md.) with saline washes. The dried filters were counted for ^{3}H-beta emission in 3.0 ml of toluene containing Omnifluor (New England Nuclear) as previously described (11). An abbreviated form of this assay system for EBV-induced transformation has been described (8).

Outgrowth assay

Outgrowth of EBV-transformed lymphocytes was assessed visually by phase contrast microscopy immediately before harvest (see above) and scored on a scale of one to four as described by Thorley-Lawson et al. (8). A score of one represents only dead cells; two, living but not transformed cells and clumps; three, transformed cell clumps and large transformed single cells; four, the well overrun by transformed and proliferating cells.

EBV nuclear antigen (EBNA) assay

EBV infected cells were examined for the expression of EBNA by indirect, complement-fixation immunofluorescence. Air-dried cell preparations were fixed in acetone-methanol (1:1, vol/vol) for 2 min at room temperature. The fixed smears were incubated with 50 μl of a 1:20 dilution of heat-inactivated standard EBV-seropositive serum from a healthy donor for 30 min at room temperature. The slides were rinsed three times with 0.01 M sodium phosphate, 0.15 M NaCl, pH 7.2 (phosphate-buffered saline), and incubated for 30 min with 50 μl of a 1:10 dilution of EBV-seronegative human serum as a complement source. The slides were again washed and incubated for 30 min with a 1/20 dilution of fluorescein-conjugated goat antihuman C3 antiserum purchased from Meloy Laboratories (Springfield, Va.). After three final washes the slides were coverslipped with 1% Tris-HCl, 90% glycerol, pH 9.5, and examined with a Zeiss RA microscope (Carl Zeiss, Inc., New York) equipped with an HB 200 mercury arc lamp, a 490-nm interference type excitation filter, a NA 1.2–1.4 dark-field condenser, and a 530-nm barrier filter. Cells containing the characteristic intranuclear fluorescence of EBNA; 19, 20) were considered positive. The EBV-infected continuous B cell line (Wil-2) and uninfected normal PBM served, respectively, as positive and negative controls for the assay.

Cell lines

Continuous cell lines were established from selected EBV-transformed B cell cultures. Cell lines were maintained in complete media and were split periodically to 0.2×10^6/ml at intervals dependent on the doubling time of the particular cell line. Wil-2 cells, an established EBV-transformed cell line, were also carried as above and split twice a week. In separate experiments, the effect of purified plasma lipoproteins on the proliferation of Wil-2 cells was assessed by daily viable cell counts in a hemocytometer in the presence of trypan blue.

Plasma lipoprotein isolation and characterization

Fresh human plasma, anticoagulated with EDTA, was obtained from normal healthy donors by plasmapheresis and adjusted to 0.1% EDTA (vol/vol). The lipoproteins were separated by sequential ultracentrifugation of the plasma at progressively increasing densities of 1.006, 1.019, 1.063, and 1.21 g/ml using solid KBr for density adjustment (21). Lipoprotein-depleted autologous serum was prepared by a single ultracentrifugation at 1.21 g/ml. The isolations initially used a 45-Ti rotor (Beckman Instruments, Inc., Fullerton, Calif.) at 42,000 rpm and, finally, a 60-Ti rotor at 58,000 rpm for 16–40 h. at 4°C. The lipoprotein fractions were dialyzed thoroughly against lipoprotein buffer (0.15 M NaCl, 0.15 mM EDTA, 0.0005% alpha-tocapherol, pH 7.4), and filter sterilized (0.45-μm filters, Millipore Corp., Bedford, Mass.). The lipoprotein fractions included the chylomicrons ($d < 0.095$ g/ml), very low density lipoproteins (VLDL; d 0.095 – 1.006 g/ml), intermediate density lipoprotein (IDL; $d = 1.006 - 1.019$ g/ml), low density lipoprotein (LDL; $d = 1.019 - 1.063$ g/ml), and high density lipoprotein (HDL; $d = 1.063 - 1.21$ g/ml). All preparations were analyzed by lipoprotein electrophoresis in 1% agarose (22) and by double diffusion in gel with rabbit antisera to human albumin and IgG, and if free of contaminating proteins, used and stored sterile at 4°C for no >20 d. HDL infranate, representing all residual nonlipoprotein serum proteins was dialyzed and sterilized as above before and after precipitation of immunoglobulins with 50% ammonium sulfate as described (18). All preparations were analyzed for protein by a modification (23) of the method of Lowry using a bovine albumin standard.

Effect of EDTA on EBV-induced B cell transformation

In early experiments, we found that the lipoprotein buffer control cultures inhibited EBV-induced B cell thymidine uptake. Studies were therefore designed to evaluate the effect of individual buffer constituents on the response. Among the constituents, only EDTA was found to be inhibitory. Final EDTA concentrations as low as 0.01, 0.1, and 1 mM in culture inhibited [^{3}H]thymidine uptake by 26.1, 43.5, and 95.7%, respectively. Based on these observations, all subsequent experiments were performed with lipoproteins dialyzed immediately before use into lipoprotein buffer devoid of EDTA to remove residual-free EDTA. In a control experiment, lipoproteins purified in buffer entirely devoid of EDTA throughout the isolation procedure possessed similar biologic activity (data not shown).

EBV serology

Sera were examined for antibodies to EBNA as described above and to viral capsid antigen using reagents purchased from Litton Bionetics, Inc. (Kensington, Md.).

RESULTS

EBV-induced DNA synthesis in adult peripheral blood B lymphocytes. Before assessing the effects of plasma lipoproteins on EBV-induced lymphocyte transformation, a series of studies was performed to characterize the salient features of the experimental system.

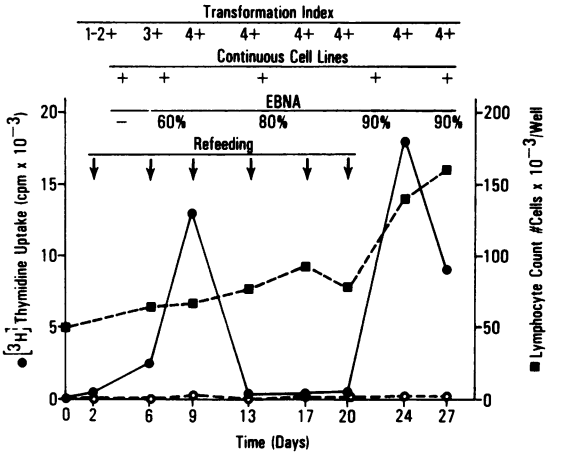

FIGURE 1 EBV-induced B-lymphocyte transformation. B cells were cultured at $5 \times 10^5/\mu$l in 250 μl cultures in the presence of 20% (vol/vol) undiluted B95-8 supernate (EBV). At the times indicated the cultures were assessed for [^{3}H]-thymidine uptake, viable cell number, evidence of transformation, and immortalization and expression of EBNA. Each arrow represents the removal of 150 μl of culture fluid and replacement with 150 μl of fresh complete media. Control cultures receiving no EBV demonstrated no [^{3}H]-thymidine uptake (○), morphological evidence of transformation or EBNA. [^{3}H]thymidine uptake; ■, lymphocyte count.

Peripheral blood B cell-enriched populations isolated from 12 healthy adults (11 seropositive, 1 seronegative) were cultured in the presence of 20% (vol/vol) undiluted B95-8 supernate (EBV) for varying lengths of time and harvested 18 h after the addition of [^{3}H]thymidine. In every instance a characteristic time-action curve was observed regardless of serological status of the donor. The B cell-enriched population was uniformly more responsive to EBV than unfractionated peripheral blood B lymphocytes at all time points tested as described by Thorley-Lawson et al. (8) (data not shown). A consistent biphasic response characterized by an initial wave of DNA synthesis that reached a peak at between 8 and 12 d after infection, followed by a trough and a second wave ~1 wk later was consistently observed (Fig. 1).

Because this trough phenomenon has not been previously described, we examined it further to determine whether the first peak represented viral transformation rather than merely mitogenic stimulation. We wished to study the effects of the lipoproteins on the former phenomenon since their effects on mitogenesis have already been described (11–16). We therefore examined EBV-infected B cell cultures over a 4-wk period for evidence of viral genome expression and immortalization (8).

Using the morphologic criteria for outgrowth and immortalization described by Thorley-Lawson et al. (8), we determined the transformation index for infected B

cell cultures immediately before harvest. Morphologic evidence of transformation was seen as early as 2 d postinfection, was unquestionable during the first peak, and persisted throughout the trough in DNA synthetic activity and for the duration of the second peak (Fig. 1). At the time of harvest for [^{3}H]thymidine uptake replicate wells were also harvested for the purpose of establishing continuous B cell lines. As seen in Fig. 1, continuous cell lines were established from cultures harvested before and during the first peak as well as throughout the trough and second peak of [^{3}H]thymidine uptake. Several of the cell lines derived from cells harvested throughout each phase of the response described above have been maintained in our laboratory for over 1 yr. Finally, confirmation of EBV infection by identification of intranuclear EBNA was obtained. Using indirect complement fixing immunofluorescence, EBNA was detectable as early as day 6, at the beginning of the first wave of [^{3}H]thymidine uptake and continued uninterrupted through the trough and second wave. It is likely that these events begin before day 6 since Takada and Osato (24) have reported the onset of [^{3}H]thymidine uptake and EBNA expression within 36 h after infection using more sensitive assay systems.

The first peak therefore appears to reflect early cellular events related to virus-induced immortalization. In contrast, the trough probably represents an in vitro artefact related to cell crowding, the depletion of essential growth factors, or the production of soluble inhibitors of DNA-synthesis by the EBV-transformed B cells. The removal of 150 μl of culture media followed by replenishment with fresh media at the times indicated in Fig. 1 had no noticeable effect on the profile of the response, suggesting that the trough was not due to growth factor depletion. Subsequent experiments not reported here suggested that both cell crowding and the release of soluble inhibitors may contribute to the observed biphasic nature of [^{3}H]thymidine uptake in these long-term cultures.

Effect of plasma lipoproteins on EBV-induced DNA synthesis. Against this background, experiments were performed to determine whether physiologic concentrations of normal human plasma lipoproteins could modulate EBV-induced B cell transformation. Because of donor B cell variability in the timing of the two peaks and the trough, each experiment was designed to permit twice weekly harvesting of cells subjected to each variable under investigation for a period of 4 wk. Since it was established that this first peak represented EBV-induced B cell transformation, results were selected for presentation based on the time of the peak of the first phase in control cultures. This usually occurred between days 5 and 15 after EBV infection. However, in all instances tested the effects of the lipoproteins on the early phase were mimicked by similar effects on the second wave of DNA synthesis as well. In addition, only the magnitude of the response was affected by the lipoproteins, whereas the timing of the peaks and trough were unchanged.

All of the major density classes of plasma lipoproteins inhibited EBV-induced B lymphocyte transformation in a dose-dependent fashion. As seen in Fig. 2, inhibitory activity was inversely related to buoyant density when expressed on the basis of lipoprotein protein per 250-μl culture. 50% inhibition of EBV-induced B cell transformation occurred at lipoprotein concentrations of 0.64 μg/ml (chylomicrons); 8.4 μg/ml (VLDL); 30 μg/ml (LDL); and >2,440 μg/ml (HDL). For reference, the normal range of fasting plasma concentrations of the various lipoprotein classes is: 0, 69–173, 726–836, and 1,315–1,600 μg lipoprotein protein per ml of plasma for the chlylomicrons, VLDL, LDL, and HDL, respectively.

Varying concentrations of each of the major lipoprotein classes also inhibited the PHA-induced [^{3}H]-thymidine uptake of whole PBM cultures. In contrast to the EBV cultures harvested at the peak of the first phase of DNA synthesis, PHA cultures were harvested 72 h after the addition of mitogen. However, both systems were susceptible to lipoprotein-mediated inhibition (Fig. 2), and the lipoproteins followed the same rank order of potency. In every instance, except HDL, the B cell response to EBV was more easily inhibitable than the PBM cell response to PHA. It is noteworthy that all of the lipoprotein preparations were devoid of IgG, and specifically negative for antibody to EB viral capsid antigen. As previously reported by us (11, 12), the HDL infranate (lipoprotein-depleted serum) did not inhibit the mitogen response. Additionally, whole HDL infranate from EBV-seronegative donors and immunoglobulin-depleted HDL infranate from EBV-seroposi-

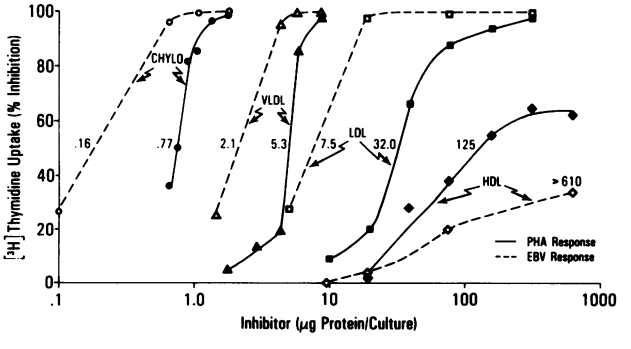

FIGURE 2 Effect of plasma lipoproteins on EBV- and PHA-induced [^{3}H]thymidine uptake. Lipoproteins (50 μl) were added to 2 × 10^5 B cell and to 2 × 10^5 PBM cultures 24 h before the addition of EBV (20% vol/vol undiluted B95-8) and PHA, respectively. Results were expressed as percent inhibition relative to buffer controls. For each lipoprotein class, the numbers indicate the protein concentration per 250 μl culture which inhibited thymidine uptake by 50%.

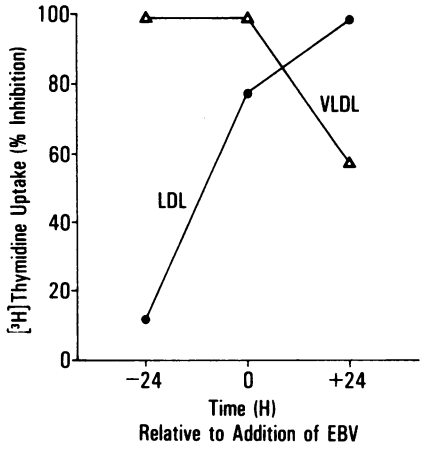

FIGURE 3 Evidence suggesting different inhibitory mechanisms for VLDL and LDL. The lipoproteins (5 μg protein/culture) were added to 2 × 10^5 B cells at the times indicated relative to the addition of EBV (20% vol/vol undiluted B95-8). Results expressed as percent inhibition relative to simultaneous buffer controls for each time point.

tive donors did not affect the EBV response (data not shown). We, therefore, concluded that the lipoprotein effect was due neither to preparation artefact nor to contaminating antibody.

In each and every instance, the lipoproteins inhibited the late EBV response to the same degree as the first wave of DNA synthesis. Furthermore, unlike the spontaneously occurring trough in the DNA synthetic response to EBV during which the cells remained fully transformed morphologically, the lipoprotein-suppressed DNA synthetic response was uniformly accompanied by a proportionate reduction or total suppression of the emergence of morphologically transformed cells and of continous cell lines.

Temporal aspects of lipoprotein-mediated suppression of EBV-induced B cell transformation. To determine whether all lipoprotein classes were inhibitory on the basis of identical or dissimilar mechanisms, we assessed the temporal requirements for optimal suppression. Varying amounts of VLDL and LDL were added to B cell cultures at various times relative to the addition of EBV. At high concentrations (20 μg/culture), both lipoproteins were totally inhibitory regardless of the time of addition. At sufficiently low concentrations (<1 μg/culture) both classes were noninhibitory. At intermediate concentrations, as shown in Fig. 3, VLDL were maximally inhibitory when added before or simultaneously with EBV, whereas LDL were maximally inhibitory when added after EBV, suggesting multiple inhibitory mechanisms.

Identification of the inhibitable target of the bioregulatory lipoproteins: B cell vs. EBV. In these experiments, EBV and B cell suspensions were separately preincubated either with buffer or with a previously determined optimally inhibitory concentration of lipoproteins for 24 h at 37°C at which time the buffer and lipoproteins were removed by washing (two cycles at 100,00 *g* for 60 min in 50 vol of fresh media for EBV and three cycles of 500 *g* for 20 min in 50 vol of fresh media for B cells). The lipoprotein-exposed EBV was then added to fresh B cells while fresh EBV was added to the lipoprotein-exposed B cells, and the DNA synthetic response was measured. Results were expressed as percent inhibition of DNA synthesis produced by lipoproteins relative to buffer controls and were compared with the response observed when EBV and B cells were cultivated together in the continuous presence of lipoproteins or buffer for the duration of the experiment. Maximum inhibition was observed when EBV and B cells were simultaneously incubated with each of the lipoprotein classes studied (Table I). Similarly, each of the lipoprotein classes was maximally inhibitory when preincubated individually with just B cells suggesting that the B cell is the major target of lipoprotein-mediated suppression. EBV appeared resistant to neutralization by VLDL and IDL; however, the DNA synthetic response of fresh B cells to LDL-preincubated EBV was reduced to ~25% of control levels. Thus, while the "transformability" of the B cell appears to be the exclusive target of VLDL and IDL, the transforming capacity of EBV can also be directly reduced by LDL, albeit at higher concentrations than are needed to inhibit the preincubated B cell response to a similar degree.

Effect of lipoproteins on the proliferation of an established EBV-transformed cell line (Wil-2). The preceding experiments defined the susceptibility of the inductive phase of B cell transformation to modulation by plasma lipoproteins. We also assessed the effect of plasma lipoproteins on the growth rate of an established EBV-transformed cell line (Fig. 4). Optimally suppressive concentrations of VLDL, IDL, and LDL

TABLE I
EBV-induced Human B Lymphocyte Transformation. Identification of the Inhibitable Target of the Bioregulatory Lipoproteins: B Cell vs. EBV

Lipoprotein preincubated with:	Lipoprotein classes*		
	VLDL	IDL	LDL
	% Inhibition		
EBV	2.9	5.4	77.8
B cells	98.9	96.8	97.1
EBV + B cells	99.9	95.3	98.9

* VLDL, 20 μg/ml; IDL, 30 μg/ml; LDL, 50 μg/ml. Results expressed as percent inhibition of EBV-induced [^{3}H]thymidine uptake relative to media control for each incubation condition.

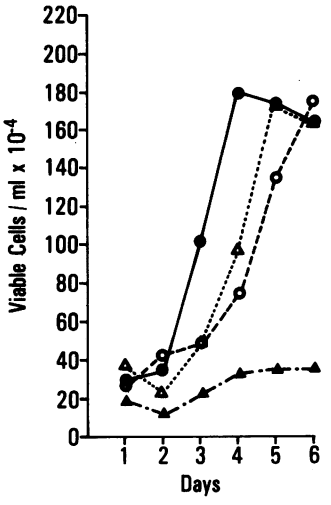

FIGURE 4 Effect of lipoproteins on the proliferation of an established EBV-transformed cell line (WIL-2). Lipoprotein concentrations were selected on the basis of previously determined maximum inhibition of EBV-induced B cell [^{3}H]-thymidine uptake for the particular lipoprotein preparation employed and were used at 10, 50, and 100 μg/culture for VLDL, IDL, and LDL, respectively. Lipoproteins (50 μl) were added to freshly split 200-μl cultures of WIL-2 cells at an initial concentration of 2 × 10^5/ml. ●, control; ▲, VLDL; ○, LDL; △, IDL.

for B cell EBV transformation were added to freshly split cultures of Wil-2 cells, and viable cell counts performed daily. All three lipoprotein classes produced a lag or delay in the initiation of growth when compared with buffer controls. However, absolute suppression of proliferation was achieved only with VLDL.

DISCUSSION

EBV is considered a prime candidate human tumor virus (25–27). EBV is ubiquitous and infection is virtually universal, occurring relatively early in life (1). Following recovery, latently infected B cells, which have oncogenic potential in vitro, persist for life (28). Nonetheless, EBV-associated malignancy is rare on a worldwide basis and is also relatively restricted geographically. Fairly universal host resistance mechanisms that prevent the unrestrained proliferation of EBV-infected B cells must therefore exist and local environmental or genetic cofactors may be responsible for the unopposed expression of the oncogenic potential of EBV in Central Africa and Asia. However, the nature of these resistance factors is unclear.

The present study clearly establishes a possible role for plasma lipoproteins as physiologic antiproliferative agents capable of suppressing EBV-induced B cell transformation. Physiologic concentrations of chylomicrons, VLDL, IDL, and LDL prevent the induction of a proliferative B cell response to EBV in vitro as measured by [^{3}H]thymidine uptake. This includes both early and late phases of DNA synthesis as well as morphologic evidence of transformation. Although the active moiety within each lipoprotein class responsible for inhibition of EBV-induced B cell transformation is not known, the inverse relationship between density and specific inhibitory activity is sufficiently similar (Fig. 2) to that already described for mitogen-induced PBM stimulation (15) to suggest that they may be identical. Considerable effort is currently underway in our laboratories to identify the active moieties in these systems.

The precise mechanism responsible for the inhibitory effect of lipoproteins in EBV-induced B cell proliferation is not clear; however, some insight is provided by several of the experiments. First of all, at least in the case of VLDL, IDL, and LDL, the lipoproteins appear to exert their effect primarily on the B cell (Table I). Thus, for the most part, viral neutralization does not appear to be nearly as important as modulation of the B cell's responsiveness to the EBV proliferative signal. This is entirely compatible with the in vivo situation in which EBV is clearly capable of infecting B cells despite the presence of high concentrations of lipoproteins. However, not all B cells are initially infected in vivo, suggesting the existence of factors that might reduce the infectivity titer of a virus inoculum or otherwise reduce the efficiency of cell to cell transmission of EBV before the appearance of specific antiviral antibody. The observed partial reduction in the transforming capacity of EBV after preincubation with LDL (Table I) is compatible with this hypothesis.

Despite the apparent qualitative similarity of the B cell effect of the different lipoprotein classes, they appear to exert their suppressive influence via somewhat different mechanisms. When suboptimal concentrations of the lipoproteins are added to the cultures at varying times relative to the addition of EBV, different inhibition profiles are observed (Fig. 3). VLDL are most inhibitory when they are delivered to the B cell together with or before EBV, observations compatible with the hypothesis that VLDL render the B cell refractory to the delivery or receipt of the EBV signal. In contrast, LDL are most inhibitory when delivered slightly after EBV suggesting that at low concentrations, LDL are only transiently active and that EBV induces an LDL-inhibitable step in the transformation process, which functions as a secondary safeguard in the event that other inhibitory mechanisms fail to abort the process.

Once EBV transformation has occurred as in the Wil-2 B cell line, LDL and IDL are only minimally if at all effective as antiproliferative agents, whereas VLDL effectively suppress cell division (Fig. 4), providing yet another level of protection. Because

VLDL metabolism is exquisitely diet dependent and plasma VLDL levels are highest in industrialized societies, which consume large quantities of refined carbohydrates and have the lowest incidence of Burkitt's lymphoma, it is conceivable that this mechanism might not be fully operative in more primitive regions such as those areas where Burkitt's lymphoma is prevalent. Thus, diet may be one of the cofactors postulated as a necessary condition for the development of EBV-induced malignancy. Alternatively, liver function disorders induced by hepatic parasitic infections may generate abnormalities of lipoprotein metabolism that might reduce the antiproliferative effect of plasma lipoproteins in these patients.

Thus, the plasma lipoproteins appear to offer several levels of protection against EBV-induced malignant transformation. First, LDL appear to directly reduce the ability of EBV to transform human B cells. Second, VLDL, IDL, and LDL all affect early inductive events rendering the B cell refractory to mitogenic EBV signals. Third, LDL appear to also inhibit a distinct EBV-inducible step that occurs slightly later in the transformation process. Finally, if transformation occurs despite these several inhibitory mechanisms, VLDL is capable of suppressing ongoing EBV-driven cellular proliferation. Because the plasma concentration and composition of the various lipoprotein classes are, to varying degrees, dependent on diet, it is possible that the uneven worldwide distribution of Burkitt's lymphoma may be partially explained on a dietary basis. Experiments designed to test this hypothesis are currently underway.

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