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Dithiocarbamates and Viral IL-10 Collaborate in the Immortalization and Evasion of Immune Response in EBV-infected Human B Lymphocytes

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Abstract

Epstein-Barr Virus (EBV) is implicated in the development of a number of human malignancies including several subtypes of non-Hodgkin lymphoma (NHL) [1]. Lymphoproliferative disease and NHL occurring in severely immunosuppressed individuals almost all involve EBV and have been extensively studied and modeled in vitro. EBV has also been causally associated with some cases of NHL occurring in otherwise immunocompetent individuals. However, a direct role for EBV in the pathogenesis of neoplasms developing in the presence of an otherwise competent immune system has not been established. We investigated potential interactions between dithiocarbamates (DTC), an important class of thiono-sulfur compounds, and EBV leading to immortalization of human B lymphocytes and evasion of cell-mediated immune response in culture. Primary lymphocyte cultures employing wild-type and recombinant EBV mutants were used to assess the respective roles of DTC and viral genes in lymphocyte transformation and survival. Pretreatment of EBV-infected human B lymphocytes with DTC directly enhanced transformation in the absence of T cells (5 nM) and independently increased survival of transformed cells in the presence of competent autologous T cells (10 nM). Both DTC-induced transformation and immortalization of EBV-infected B lymphocytes were dependent on the expression of viral IL-10. These results provide a biological basis for studying collaborations between chemical and virus that alter lymphocyte biology, and provide a rationale for further molecular epidemiology studies to better understand the potential influence of these interactions on the development of NHL and perhaps other viral-associated malignancies.

Keywords

dithiocarbamates; Epstein-Barr virus; IL-10; immune evasion; B lymphocytes

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1. Introduction

Epstein-Barr virus is a γ-herpes virus that infects and transforms B lymphocytes both in culture and in vivo, maintaining a lifelong latent infection in humans that is controlled by cytotoxic T lymphocytes. EBV is virtually always involved in the pathogenesis of lymphoproliferative disease (LPD) and immunoblastic B cell lymphomas associated with severe immunosuppression, for which EBV-induced transformation of B lymphocytes in culture in the absence of competent T cells is recognized to be the in vitro counterpart [2;3]. Early events in the infection and transformation of B lymphocytes by EBV are not completely understood, although EBV-induced transformation is thought to involve activation of signaling pathways in normal B lymphocyte proliferation including the up-regulation of Bcl-2. [4–8]. EBV is also implicated in the development of a subset of sporadic malignancies occurring in otherwise immunocompetent individuals, including: nasopharyngeal carcinoma, leiomyosarcoma, gastric carcinoma, Hodgkin disease (HD), and several subtypes of NHL including those of B, T and NK cell origin [1;9]. However, the precise role of EBV in the development and evolution of these neoplasms is unknown and there are no satisfactory paradigms to explain the direct role for EBV in the pathogenesis of neoplasms developing in the presence of an otherwise competent immune system.

The EBV genome encodes for 94 proteins, only about 10 of which are expressed in immortalized B cells, and with the possible exception of a single nuclear protein, EBV nuclear antigen-2 (EBNA2), there is no general agreement that expression of any particular gene is essential for B lymphocyte transformation [10]. Newly infected B lymphocytes express a set of viral genes under the control of EBNA2 [11–13]. However, in latent infected B lymphocytes, EBV gene expression is under the control of EBNA1 and may be restricted to only a single latent membrane protein, LMP2 [14]. The EBV BamHI C Fragment open reading frame (BCRF1) encodes for a viral homologue of IL-10 (vIL-10) that is reported by most but not all groups to be differentially expressed late in the EBV lytic cycle and occasionally in EBV-transformed or immortalized B lymphocytes [15–18]. The BCRF1 protein can be detected 9–15 hr post infection, rapidly decreases thereafter [19] but is not usually expressed in latent EBV-infected B cells or most EBV-transformed tumor cell lines [20]. Human IL-10 (hIL-10) is a pleiotropic cytokine that is effective in the suppression of primary immune response and the development of immunological tolerance. hIL-10 directly stimulates proliferation of EBV-infected B cells [21;22], prevents spontaneous death of germinal center B cells [23], induces peripheral anergy in T lymphocytes, increases expression of MHC Class II and co-stimulatory molecules on B lymphocytes and antigen presenting cells and mediates evasion of local immune surveillance in EBV-associated neoplastic diseases [18;21;24;25]. With the exception of the N-terminal region of the peptide, vIL-10 shares considerable structural homology with its human counterpart [16]. However, vIL-10 is far less effective in stimulating MHC Class II expression on B lymphocytes and inhibiting IL-2 production by activated CD4+ cells [26; 27]. Although antisense nucleotides for vIL-10 mRNA are reported to block B lymphocyte transformation by EBV [18], studies utilizing EBV recombinants indicate that the BCRF1 gene does not play an obligatory role in maintaining latent EBV infection or inducing lymphocyte transformation, nor does it appear necessary for the continued growth of transformed cells in vivo or in vitro [28].

Dithiocarbamates (DTC) are an important class of low molecular weight thiono-sulfur compounds with diverse biological activity that have been used in agriculture and the clinic to control fungi and bacteria and as accelerating agents in rubber and polymer manufacturing. DTC are employed worldwide as fungicides in agriculture, for the control of mold in a variety of products, including paint, leather, paper and fabrics, and are trace constituents of residues found in fruits, vegetables and other food products [29]. Epidemiology studies have reported generally inconsistent results for an association between occupational exposure to

dithiocarbamates or their breakdown products and lymphoid neoplasms [30–33]. However, to date, no studies have evaluated specific multifactorial associations, including influences such as microbial infection, drug or chemical exposure, on the development of NHL.

The biochemistry of DTC is remarkably complex. They exert a variety of striking molecular changes in cell systems, the significance of which is not fully understood. These include the ability to: 1) oxidize protein thiols and inhibit hydroxyradical formation, 2) inhibit nuclear factor-kappa B (NF-κB) activation via mechanism(s) that notably do not involve either oxidation-reduction dependent modification of the NF-κB protein or interference with NF-κB-DNA binding, 3) induce AP-1-dependent cell differentiation and gene expression via *de novo* transcription of *c-fos* and *c-jun* [34] and 4) result in profound changes in some cells via a mechanism that involves alterations in the intracellular transport of copper ions [35–40]. The sensitivity and concentration-response between individual cell types differ widely but are remarkably consistent among various substituted DTC for which the dimethyldithiocarbamate (DMDTC) is a prototype.

Herein, we tested the premise that exposure of a virus-infected cell to a biologically reactive molecule can alter cell survival and transformation under conditions that independently do not result in immunosuppression. We used in vitro cellular, molecular and recombinant approaches to analyze the effects of DTC on naïve and EBV-infected peripheral blood mononuclear cells (PBMC), isolated B lymphocytes and reconstituted mixed autologous B and T lymphocytes. We found that treatment of EBV-infected PBMC or purified B lymphocytes with DMDTC results in a marked increase in transformation and immortalization of lymphocyte clones apparently via independent processes that are both dependent on vIL-10. These experiments also demonstrate that EBV-transformed clones from cells initially treated with DMDTC continue to be capable of inducing unresponsiveness and inhibiting reactivation of autologous T lymphocytes in the absence of chemical.

2. Materials and Methods

2.1 Production of EBV stock

The EBV producing cell line, B95-8 (American Tissue Culture Collection, Rockville, MD), was cultured in complete media consisting of RPMI 1640 containing 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 10% v/v heat inactivated fetal bovine serum (FBS) (Gibco/BRL, Grand Island, NY). Cells were maintained at a density of 1×10^6 cells/ml in a 37°C incubator with a humid atmosphere of 5% CO_2. For the production of viral stocks, cell were kept at a starting density of 5×10^5 cells/ml and left for 14 days without the replacement of media. Supernatants from cultures were subsequently isolated and filtered through a 0.45 μm pore filter and 1 ml aliquots were stored in liquid N_2.

2.2 Cell isolation and purification

Mononuclear cells used for the EBV infections were isolated from heparinized blood from healthy donors obtained with informed consent. Peripheral blood mononuclear cells (PBMC) were isolated by separation on a Ficoll gradient and washed twice with phosphate buffered saline containing 1% bovine serum albumin (PBS/BSA). Flow cytometric analysis of preparations of freshly isolated PBMC indicated that the B:T lymphocyte ratio ranged from 1:2.0 to 1:2.3. B and T lymphocytes were purified from the ficoll isolated mononuclear cells by incubating with an anti-CD19 (for B cells) or an anti-CD3 (for T cells) microbead conjugated antibody (Miltenyi Biotec, Auburn, CA) and passed through a magnetic separator column (Miltenyi Biotec). Percent purity of CD19 or CD3 positive cells was measured by flow cytometry (Epics 752, Coulter Electronics, Hialeah, FL) using anti-CD19 (HIB19, Pharmingen

San Diego, CA) or anti-CD3 (UCHT1, Pharmingen) monoclonal antibody (mAb) and determined to be greater than 95%.

2.3 EBV infection and dithiocarbamate exposure

Mononuclear cells or B lymphocytes (1×10^7) were suspended in 1 ml of complete RPMI 1640 media and 1 ml of EBV stock was added. Cultures were incubated at 37°C for 2 hours in 5% CO_2 with occasional agitation. Cells were then pelleted and resuspended in complete media at a density of 1×10^6 cells/ml and then treated with various concentrations of DMDTC, DEDTC or PBS (Sigma, St. Louis, MO). For IL-10 neutralization experiments, 1 μg/ml goat anti-human IL-10 antibody (AF-217-NA, R&D Systems, Minneapolis, MN) was added.

2.4 Transformation and immortalization of EBV-infected cells

EBV-infected and/or DTC-exposed cells were plated using complete media into 96 well plates in triplicate at 1×10^4 cells/well or 1×10^3 cells/well for B lymphocytes and mononuclear cells, respectively. Plates were incubated at 37°C, 5% CO_2 and half of the media changed weekly. Wells were counted for transformed clones by light microscopy after 4 weeks of culture. For the determination of immortalized clones, 12 positive wells were randomly selected, re-plated under limiting dilution conditions, subcultured for an additional 4 weeks and scored. Continual passage and subcloning of these selected clones beyond the additional 4 weeks were also performed to insure that these LCLs were truly immortal.

2.5 ELISPOT assays for determination of IL-10 producing cells

EBV-infected and DTC-exposed cultures were plated at 1×10^4 cells/well onto 96-well Multiscreen HA nitrocellulose plates (MAHAS4510, Millipore, Bedford, MA) previously coated overnight with sterile PBS containing JES3-9D7 mAb (1 μg/ml) to capture both hIL-10 and vIL-10 producing cells. After overnight incubation, plates were washed with sterile PBS at room temperature and blocked by incubation with complete RPMI for 2 hr at 37°C. Plates were then washed with PBS containing 0.05% Tween 20 to remove unbound cells. vIL-10 producing cells were detected with a biotinylated JES3-6B11 mAb, and total hIL-10 + vIL-10 producing cells were detected using a biotinylated JES3-12G8 mAb after overnight incubation at 4°C (Pharmingen). Cells were visualized using a peroxidase-labeled Streptavidin-HRP secondary reagent (Sigma). Cells secreting cytokine were enumerated following the color reaction using light microscopy by the appearance of spots. The IL-2 ELISPOT assay used the same procedure as above using an anti-IL-2 capture and detector antibody (Pharmingen).

2.6 T cell add-back assay

B lymphocytes were purified and infected with EBV as previously described. Donor matched CD3+ T cells were purified and added in increasing concentrations to the EBV infected B lymphocyte culture wells. Mixed cultures were either assayed shortly after the addition of T cells by ELISPOT or propagated for an additional 3 weeks and then assessed for transformation and subsequent immortalization as previously described.

2.7 Recombinant vIL-10 EBV cultures

Lymphoblastoid cell lines containing recombinant wild type or vIL-10-deleted EBV were obtained from S. Swaminathan [28] and maintained in complete RPMI (RPMI 1640 supplemented with 10% FBS, 100 IU/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine). Lytic virus infection was induced by the addition of 20 ng/ml 12-tetradecanoyl phorbol acetate (TPA), cells were incubated for 3 days, washed, irradiated with 90Gy via a cyclotron and plated with purified B lymphocytes in 96-well plates at a ratio of 5×10^5 irradiated cells to 1×10^4 B cells (150 μl/well). Co-cultivations were carried out in either

complete RPMI media or media containing various concentrations of DMDTC. Transformed colonies were scored after 4–6 weeks.

2.8 Reverse transcription-PCR (RT-PCR)

Total RNA from primary B lymphocytes was isolated after 12, 24, 48 and 72 hours post infection. RNA preparations were treated with RNase-free DNase (Roche Diagnostics, Indianapolis, IN) for 30 minutes at 37°C. After inactivation of the enzyme, 1 μg RNA was reverse transcribed (+RT; Superscript Plus; Gibco/BRL) with random hexamers for 60 minutes at 37°C. Reverse transcription was omitted for control purposes where indicated (−RT). PCR with one tenth of the volume (2 μL) was performed in buffer containing 1.5 mmol/L $MgCl_2$, 100 pmol of each primer, 0.2 mmol/L final concentration of each dNTP, and 0.5 μL Ampli-Taq-polymerase (Fisher Scientific, Pittsburgh, PA) at a final volume of 50 μL in a 9600 GeneAmp thermal cycler (Perkin Elmer, Norwalk, CT). The following PCR primers (IDT, Coraville, IA) were used:

BCRF1: 5′-TGGAGCGAAGGTTAGTGGTCAC-3′ and 5′-ATGGTCTTTGGCTTCAGGGTCC-3′

LMP1: 5′-GTGATTCTGACGAAGCCAGAG-3′ and 5′-CGTGGGGCGCCCCAGGCACCA-3′

EBNA2: 5′-AGAGGAGGTGGTAAGCGGTTC-3′ and 5′-TGACGGGTTTCCAAGACTATCC-3′

β-actin: 5′-CG TGGGCCGCCCTAGGCACCA-3′ and 5′-TTGGCCTTAGGGTTCAGGGGGG-3′.

Amplified bands were analyzed by electrophoresis through a 1.5% agarose gel and ethidium bromide stain.

2.9 Flow cytometric analysis

EBV-infected B cells (2×10^6) were isolated at 24, 48 and 72 hours post infection and subjected to flow cytometry analysis. Cells were washed in PBS/BSA and incubated with FITC conjugated antibodies for EBNA2 (clone PE2, Dako, Carpinteria, CA), BZLF1 (clone BZ1, Dako) and viral capsid antigen p120 (VCA) (clone 2G2, Biogenesis, Kingston, NH) and analyzed using an Epics 752 (Coulter Electronics). A total of 10,000 cells were counted per individual analysis. For FCM analysis of EBNA2, incubation of anti-EBNA2 antibody was carried out in the presence 0.1% Tween 20. For FCM analysis of accessory molecule expression, human B lymphocytes were isolated at daily intervals over 1 week of culture and mean fluorescence intensity measured using anti-B-7.1 (CD80) [clone BB1], anti-B-7.2 (CD86) [clone 2331(FUN-1)], and HLA-DR [clone G46-6(L243)] (Pharmingen).

3. Results

3.1 Increased proliferation and immortalization of EBV-infected B lymphocytes by DMDTC

Growth and transformation of B lymphocytes by EBV in vitro have long been used as a surrogate for the study of immunoblastic transformation in vivo [41–44]. Therefore, we measured the influence of DMDTC on transformation of EBV-infected lymphocytes in cultures of human PBMC and purified B lymphocytes. PBMC (containing both B and T lymphocytes) or purified B cells were infected with EBV, treated with DMDTC or phosphate buffered saline (PBS) and the outgrowth of colonies compared after 4 weeks. Pretreatment with DMDTC resulted in a significant dose-dependent increase in the transformation of B lymphoblasts in PBMC containing autologous T lymphocytes and in purified B lymphocytes at concentrations as low as 10 and 5 nM, respectively (Figure 1). To determine the effects of DMDTC on

lymphocyte immortalization we assessed the cloning efficiency of these colonies by subculturing cells under conditions of limiting dilution for an additional 4 weeks and again scoring colony formation. Immortalization of EBV-transformed lymphocytes was enhanced 16 fold in PBMC and over 40 fold in purified B lymphocytes. These results demonstrated that DMDTC induced a marked increase in immunoblastic transformation in EBV-infected B cells and suggested that while treatment of EBV-infected cells with DMDTC increased lymphocyte proliferation it is arguably more potent in inducing immortalization.

3.2 The kinetics of viral gene expression in primary EBV-infected B lymphocytes

To evaluate differences in early EBV gene expression that might correlate with DMDTC-induced lymphocyte transformation, we inoculated primary B lymphocytes with supernatants from EBV-producing B95-8 cells, treated the cultures 2 hr later with DMDTC or PBS alone and then measured viral gene- and protein- expression using flow cytometry and RT-PCR techniques (Figure 2). Gene products associated with both lytic (BZLF1, vIL-10) and latent (EBNA2) EBV infection were present 12 hr post infection with a latent pattern of gene expression consistent with lymphocyte transformation (EBNA2, LMP-1) evident by 48 hr. Increases in viral capsid antigen (VCA) were not observed at any time point (Figure 2: A1, B1), indicating that treatment-related differences in the number of viral-transformed cells were not due to an increased rate of virion production or lymphocyte infection. Biphasic expression of BCRF1 (24–72 hr) was consistently observed in untreated cells infected with EBV, and treatment with DMDTC always resulted in expression of vIL-10 at 48 hr post infection. However, DMDTC pretreatment did not result in observable early changes in the kinetics of expression of other EBV genes typically associated with either transformation or lytic virion replication (Figure 2: A2, B2). These findings suggest that pretreatment with DMDTC altered expression of vIL-10 in the absence of apparent changes in latent EBV gene expression or viral replication.

3.3 The role of vIL-10 in DMDTC-induced transformation of EBV-infected B lymphocytes

We performed a series of experiments to evaluate the role of vIL-10 in DMDTC-induced transformation of EBV-infected cells. First, we determined the effects of DMDTC on vIL-10 producing cells using an ELISPOT assay (Figure 3A). Treatment of EBV-infected B cells with DMDTC resulted in a significant increase in vIL-10 producing cells that was not observed following DMDTC treatment of uninfected B lymphocytes. We also ascertained that addition of neutralizing anti-IL-10 antibody functionally abrogated DMDTC-enhanced growth transformation (Figure 3B). We then repeated the transformation experiments described in Figure 1 using recombinant virus techniques. Freshly isolated purified B lymphocytes were infected with strains of EBV containing either a 3.3 kb deletion between bp 9535 -12870 that spans the entire BCRF1 open reading frame or a recombinant wild-type (WT) virus [28] (Figure 3C). Cultures treated with and without DMDTC (1nM–100nM) were maintained for 4–6 weeks and scored for transformation. No differences in the rate of B lymphocyte transformation were observed in untreated cultures infected with either recombinant EBV strain. In contrast, a marked increase in B cell transformation was observed in DMDTC treated cultures infected with WT EBV but not the BCRF1-deletion mutant. These results demonstrated that the vIL-10 gene was essential for DMDTC-enhanced transformation of EBV-infected B lymphocytes.

3.4 DMDTC pretreatment of EBV-infected B cells enhanced their ability to inactivate autologous T cells

Our experiments indicated that DMDTC treatment directly enhanced transformation of EBV-infected B cells via a mechanism that was mediated via vIL-10. Treatment-related increases in B lymphocyte immortalization occur in the presence of T cells at concentrations of DMDTC that are as much as 500 times lower than those that directly inactivate human T lymphocytes

[37]. However, vIL-10 alone is also known to abrogate the inhibitory capacity of T cells targeting EBV-induced B cell transformation [21]. Therefore we performed experiments to determine whether DMDTC pretreatment of B lymphocytes indirectly resulted in the inactivation of autologous T cells. EBV infected purified human B lymphocytes were cultured under limiting dilution conditions with DMDTC for 7 days, washed, and then reconstituted with increasing numbers of autologous T lymphocytes previously unexposed to chemical. The addition of autologous T cells at any titer ≥ 1:1 effectively prevented the outgrowth of EBV-infected B cells in untreated cultures (Figure 4). In contrast, pretreatment of B cells with DMDTC resulted in proliferation and outgrowth of B cell clones even in reconstituted cultures containing high ratios of T to B lymphocytes. Consistent with previous reports that different substituted DTC share a common chemistry and biological effects, we repeated these experiments using diethyldithiocarbamate (DEDTC) and obtained identical results (data not shown).

A number of studies provide evidence that peripheral T cell tolerance or anergy can be overcome by addition of exogenous IL-2 which temporarily restores CD4 cell responsiveness [21;45]. We investigated the influence of exogenous IL-2 on the dynamics of CD4 cell activation and EBV-infected B cell transformation in DMDTC-treated and control PBMC. PBMC were infected with EBV, treated with 100 nM DMDTC or PBS, cultured for 14 days and then supplemented with rhIL-2 or PBS. We confirmed in initial experiments that purified untreated T and B lymphocytes do not secrete IL-10 or IL-2 under the culture conditions used in these experiments (data not shown). Therefore, we used the number of IL-2-producing and vIL-10-producing cells, as determined by ELISPOT assays, as indicators of the numbers of activated CD4 cells and EBV-infected B cells, respectively. DMDTC pretreatment of EBV-infected cells resulted in a marked increase in vIL-10-producing cells relative to untreated cultures of EBV-infected cells (Figure 5A). Addition of exogenous IL-2 on day 14 completely ablated vIL-10-producing cells in untreated cultures and also resulted in a delay and attenuation of vIL-10-producing cell proliferation in DMDTC-pretreated cultures. The addition of exogenous IL-2 markedly increased the number of IL-2-producing cells in EBV infected cultures. However, DMDTC-pretreatment did not affect the number or kinetics of these cells (Figure 5B). Peripheral tolerance induced by vIL-10 has been shown to involve direct paralysis of B7-1-mediated co-stimulation and not down regulation of MHC or B7 expression [46]. Using flow cytometry, we determined that DMDTC pretreatment did not alter expression of HLA-DR, B-7.1 or B-7.2 in EBV-infected B cells over 7 days in culture prior to the addition of T cells (data not shown). These findings are consistent with the previously described mechanism for vIL-10 induction of local tolerance [25] and demonstrate that the continued presence of DMDTC is not necessary for EBV-transformed B lymphocyte evasion of cell mediated immune response.

4. Discussion

Despite intensive investigation, a unified understanding of the biology of lymphocyte transformation by EBV remains elusive, and controversies persist with respect to the contribution of individual viral genes in mediating B lymphocyte transformation or immune evasion. vIL-10 has been shown to enhance B cell survival both by directly stimulating growth and differentiation as well as facilitating immune evasion by EBV-infected transformed B cells in vivo. Nevertheless, some previous studies have suggested that vIL-10 is not an absolute requirement for lymphocyte immortalization or survival [20]. Our experiments demonstrate that treatment of EBV-infected cells with DTC results in a marked increase in both B lymphocyte transformation and immortalization. Consistent with the previously demonstrated dual roles for IL-10 in enhancing B lymphocyte proliferation and promoting T cell anergy, our experiments indicate that vIL-10 plays an obligatory role in mediating DTC-induced increases in transformation as well as attenuation of T cell inhibition of EBV-infected B lymphocytes.

These findings suggest that transient exposure of B lymphocytes to DTC early in the cycle of EBV infection can permanently alter the regulation of vIL-10 expression, suggesting that B lymphocyte transformation and survival may be subject to modulation by environmental or therapeutic agents.

However, the molecular basis for these effects remains unclear. Several laboratories including ours have reported that DTC directly suppresses cell-mediated immune response and T lymphocyte activation [33;35;37;47]. DTC are historically considered not to be mutagenic, although Soloneski et al recently reported increases in chromosome aberrations in cells treated with relatively high concentrations of ethylene bis(dithiocarbamate) [48;49]. These effects occur at concentrations of DTC that are 200–500 times higher than those demonstrated to enhance transformation or immortalization of EBV-infected B lymphocytes in these experiments. Independently, we have not observed increases in structural chromosome aberrations in banded chromosome analysis of immortalized human B lymphocyte clones produced in our studies (data not shown).

The mechanism(s) whereby DTC specifically alter the regulation and expression of vIL-10 remain obscure, and very little is specifically known about the regulation of vIL-10 in infected B cells. DTC are generally regarded as prototype inhibitors of transcriptional activation of NF-κB [50], and are implicated in both pro-oxidant radical scavenging and copper-dependent intracellular oxidation of protein thiols [35;51]. Transcription of hIL-10 is controlled by the constitutively expressed transcription factors, Sp1 and Sp3 [52], the induction of which have been demonstrated to be sensitive to oxidation of critical protein thiols [53;54]. Alternatively, the half-life of hIL-10 mRNA appears to be predominantly determined by post-transcriptional signals [55]. Homeostatic regulation of the subcellular distribution of copper is highly orchestrated [56], and alterations in intracellular copper concentration have been demonstrated to alter post-translational gene expression via multiple mechanisms [57;58]. Taken together it is attractive to hypothesize that DTC influence cell-specific gene expression of vIL-10 via copper-dependent direct oxidation of protein thiols.

IL-10 has been implicated in the development of immune tolerance for a variety of human tumors and LPD that are presumably mediated via cell-specific suppression of cell-mediated immune function, i.e. defective cytotoxic T-cell response, induction of antigen-specific anergy and T lymphocyte unresponsiveness [45;59–63]. Exploitation of cellular or viral IL-10 homologues by other intracellular pathogens appears to be a common mechanism of immune evasion [64], and a number of vIL-10 homologues have been identified including those encoded by EBV, equine herpes virus- type 2, and cytomegalovirus [64].

Over the past 20 years the incidence of sporadic B cell NHL steadily increased with almost a doubling of the incidence between 1980 and 2000 by about 3–4% per year worldwide [65]. In the past few years the incidence of NHL has apparently plateaued [31]. An etiological role for EBV in the pathogenesis of a subset of human cancers in non-immunosuppressed individuals, including cases of sporadic NHL and HD, has been repeatedly suggested [66–68], and it has been widely speculated that dietary, iatrogenic or environmental factors may influence viral carcinogenesis [9]. However, no direct biologically plausible mechanism has emerged to explain potential viral-chemical interactions in otherwise immunocompetent individuals in which EBV-infected B cells are normally held in check by T lymphocyte immunosurveillance [59]. The results of our studies reveal that exposure of human B lymphocytes to DTC at very low concentrations produces enhanced production of vIL-10 that is capable of inducing EBV-mediated B lymphocyte proliferation and immortalization in the presence of otherwise competent T cells. NHL is widely appreciated to be a complex and multifactorial disease. Numerous epidemiology studies have variously implicated a wide range of possible etiological associations between infectious agents, occupation, dietary factors, the environment and

previous underlying diseases. However, consistent statistically significant results have been observed for only a relatively small subset of these [31]. Our results suggest the potential for collaboration between chemical and at least one Herpes virus, EBV, in the immortalization and immune evasion of human cells. These results provide a point of departure for further molecular epidemiology studies to better understand the biological basis of chemical-biological interactions and their potential influence on the development of NHL and perhaps other viral-associated malignancies.

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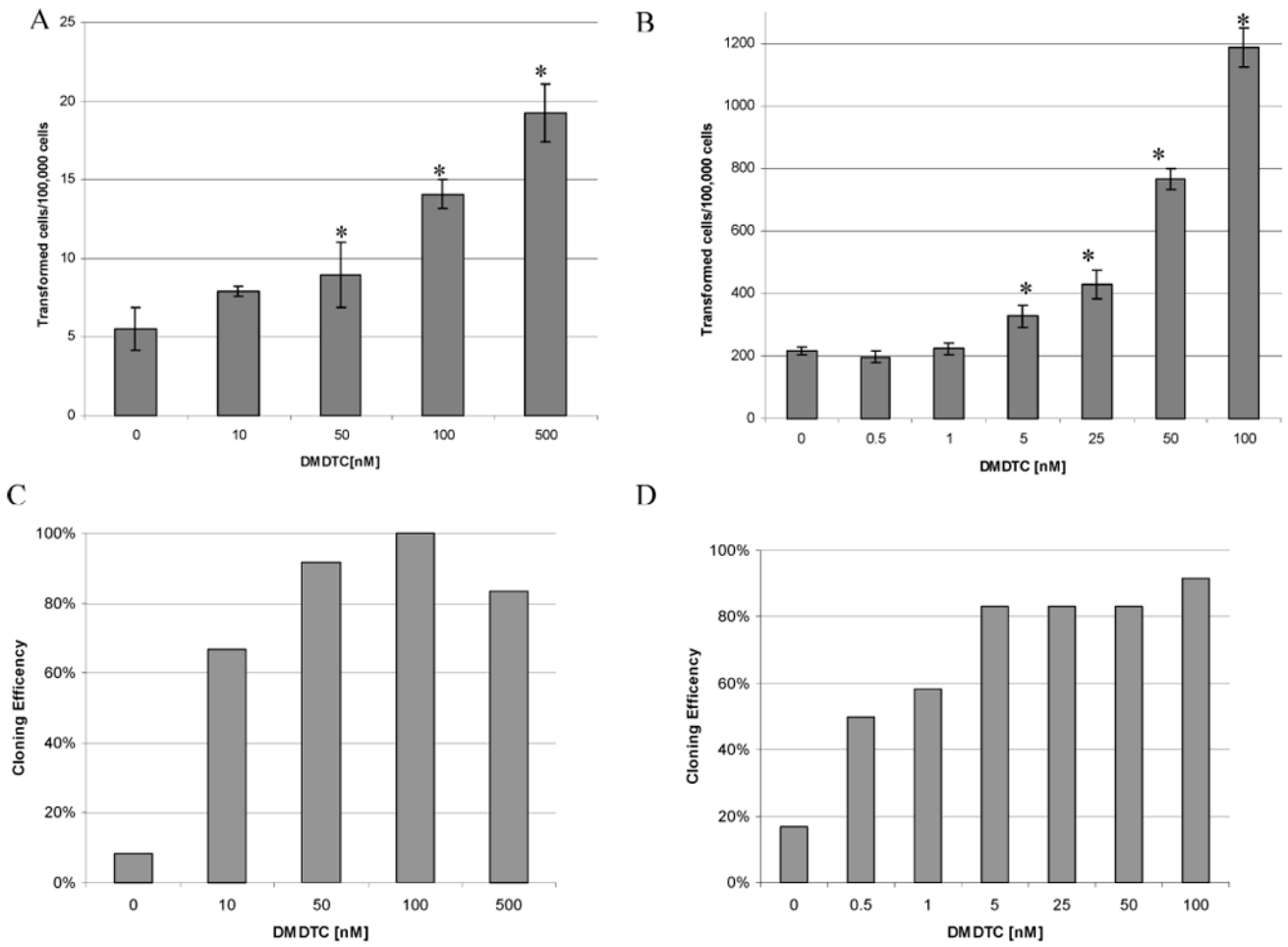

Figure 1.

Transformation and immortalization of EBV-infected B lymphocytes treated with DMDTC. Human Ficoll-purified PBMC (A) or isolated B lymphocytes (B) were infected with B95-8 EBV supernatants, treated with DMDTC or buffer, cultured for 4 weeks and colonies enumerated. Cells from resulting colonies were then subcultured under limiting dilution conditions for another 4 weeks and cloning efficiency expressed as the percent of immortalized colonies (%) for PBMC (C) or purified B lymphocytes (D). Results represent the average number of colonies per treatment group from triplicate cultures +/− SEM. Asterisks indicate significant difference from buffer-treated cultures using Student t-test ($p \leq 0.05$). Data is representative of 1 out of 6 independent experiments performed using PBMC, and 1 out of 8 independent experiments using isolated B lymphocytes. Cells for each experiment were obtained from different donors (n=14).

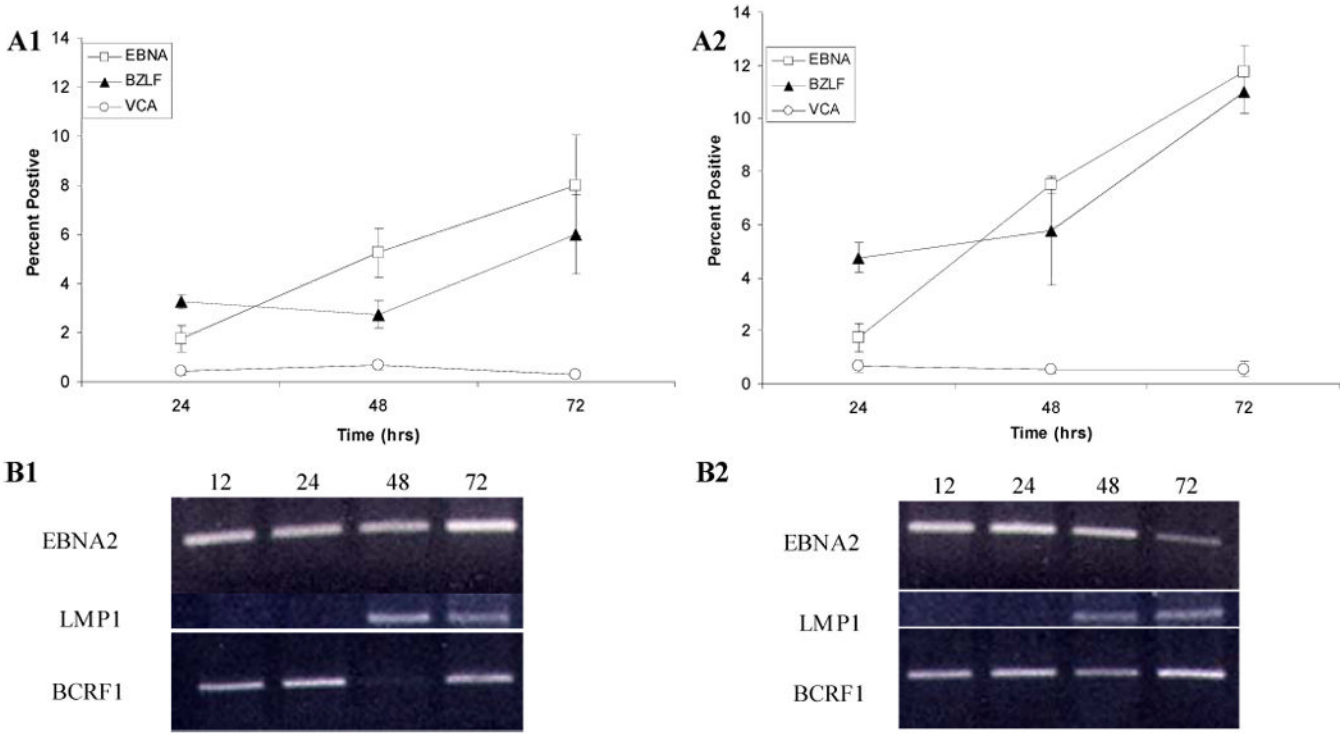

Figure 2.

Kinetics of gene expression in EBV-infected primary B lymphocytes. B cells were harvested at intervals as indicated (hours post-infection) and analyzed by flow cytometry or RT-PCR techniques. (A) Flow cytometric analysis of 2×10^6 EBV-infected B cells treated with PBS (A1) or 100 nM DMDTC (A2) using antibodies against: EBNA2, BZLF-1 and VCA (viral capsid antigen p120). Values represent Mean + S.E.M. (n=4). (B) RT-PCR analysis of RNA extracts from 1×10^6 EBV-infected B cells treated with PBS (B1) or 100 nM DMDTC (B2). Representative agarose gel (n=6) of PCR products for EBNA2, LMP1 and BCRF1 is shown. Primers for the specific transcripts and PCR conditions are indicated in Materials and Methods.

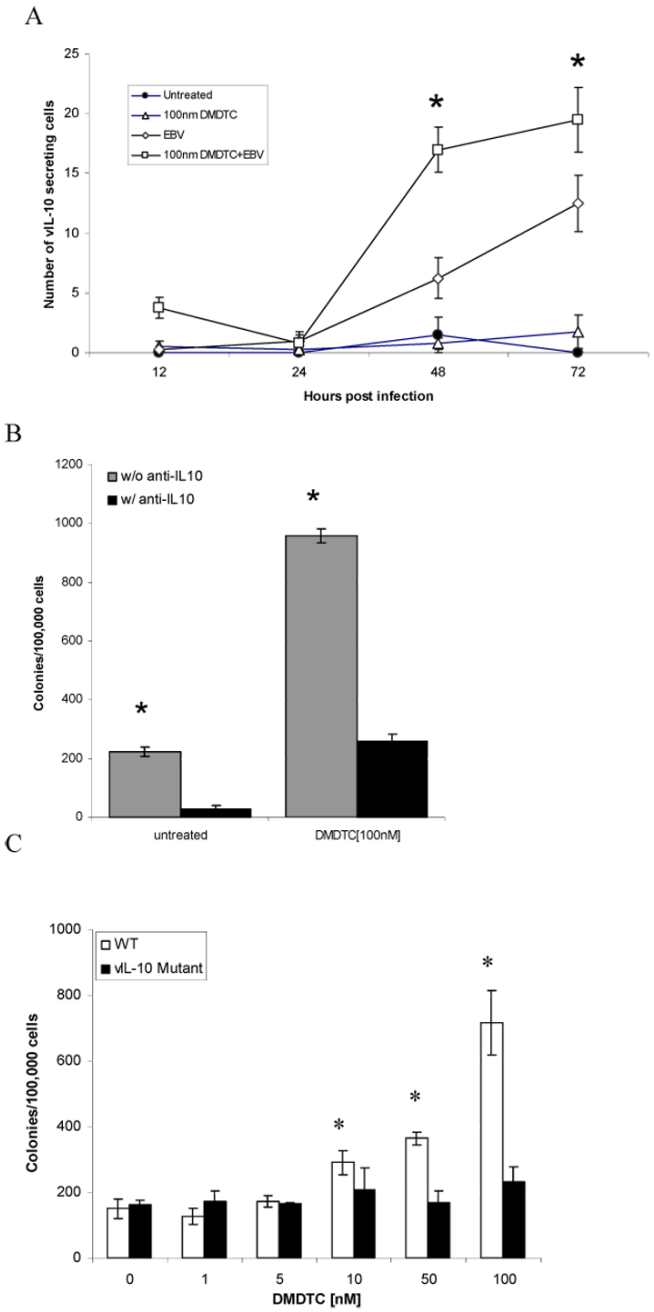

Figure 3.

The role of vIL-10 in DMDTC enhanced B cell transformation. (A) The number of vIL-10 secreting cells was determined at 24, 48 and 72 hr in EBV- infected or uninfected B lymphocytes treated with PBS or 100 nM DMDTC. Asterisks indicate significant difference between DMDTC-treated and untreated EBV-infected cultures using Student t-test ($p < 0.05$). (B) Transformation of EBV-infected and/or DMDTC-pretreated B lymphocytes was evaluated in the presence and absence of anti-vIL10 antibodies. Colonies were scored after 4 weeks in culture. Asterisks indicate significant difference with and without addition of neutralizing anti-IL-10 antibodies using Student t-test ($p < 0.05$). (C) Effect of DMDTC treatment on purified B lymphocytes co-cultivated with irradiated lymphoblastoid cell lines containing BCRF1-

deleted or WT EBV recombinant strains. Colonies were scored after 6 weeks in culture. Error bars represent +/− SEM (n=3). Asterisks indicate significant difference from buffer-treated cultures using Student t-test ($p < 0.05$).

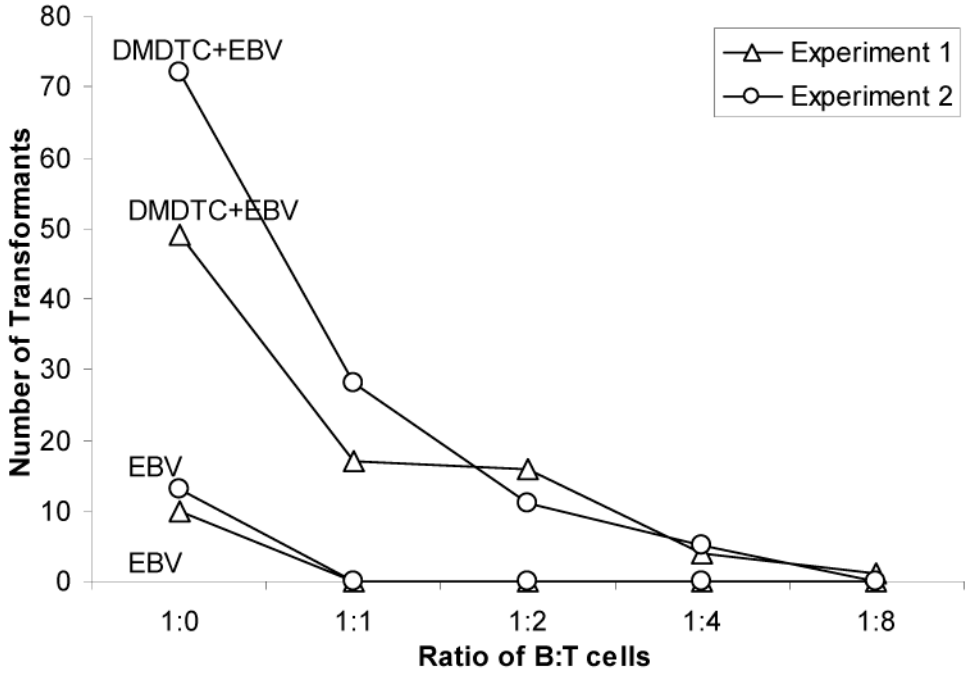

Figure 4.

Influence of DMDTC pretreatment on EBV-infected B lymphocyte immortalization in the presence of T cells. EBV-infected purified B cells were cultured for one week at a density of 500 cells per well in the presence of PBS or 100 nM DMDTC. Cells were washed, varying numbers of autologous purified T cells added, cultured for an additional 4–6 weeks and scored for colonies. Symbols indicate the number of transformants per well from duplicate experiments. The ratio of B:T cells: 500:0 (1), 500:500 (1:1), 500:1000 (1:2), 500:2000 (1:4), 500:4000 (1:8).

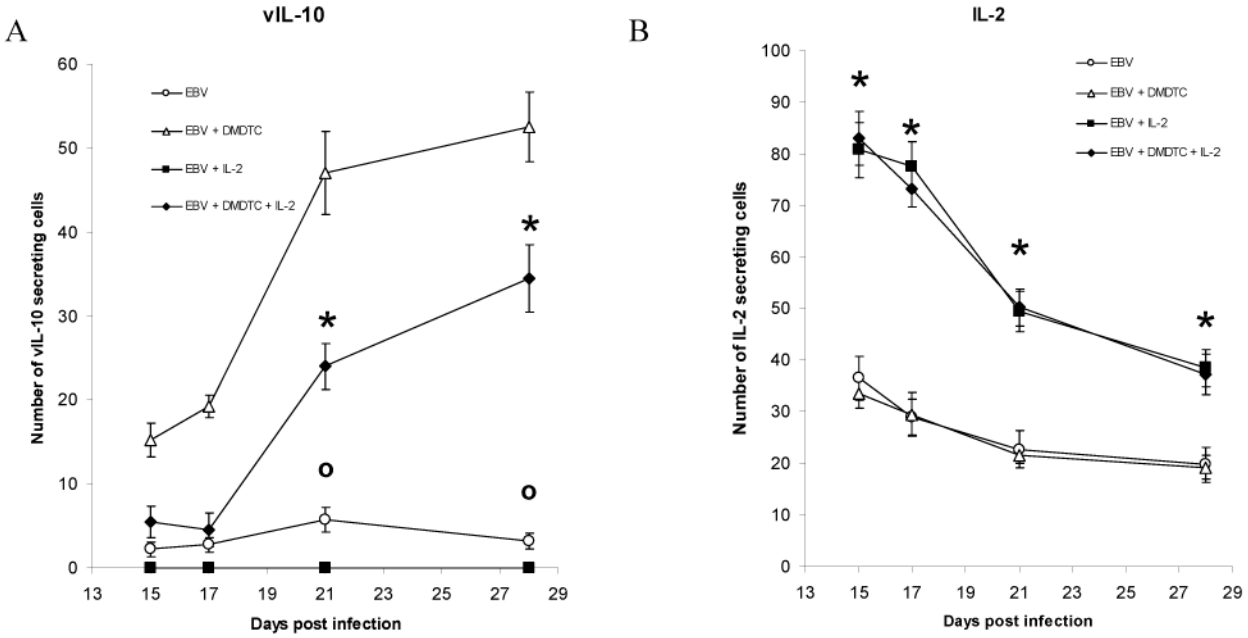

Figure 5.

Exogenous IL-2 partially restores T lymphocyte response against DMDTC-treated/EBV-infected B lymphocytes. Ficoll-purified PBMC were infected with EBV, treated with 100 nM DMDTC, cultured for 14 days and then supplemented with PBS or 200 U rhIL-2. (A) vIL-10-producing B cells. Asterisks indicate significant difference compared with DMDTC pretreated cells without IL-2 supplementation using Student t test ($p < 0.01$). Circle indicates significant difference compared with EBV infected cultures without DMDTC pretreatment or IL-2 supplementation using Student t test ($p < 0.01$); and (B) IL-2-producing T cells were enumerated by ELISPOT at 15, 17, 21 and 28 days post-infection. Data are expressed as the mean +/− SEM for secreting cells (n=4). Asterisks indicate significant difference compared with identical cultures without IL-2 supplementation using Student t test ($p < 0.01$).