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DECREASED EPSTEIN-BARR VIRUS-INDUCED TRANSFORMATION, AND ELEVATED 2-5A SYNTHETASE AND RNase L ACTIVITY IN PERIPHERAL BLOOD MONONUCLEAR CELLS FROM PATIENTS TREATED WITH RECOMBINANT INTERFERON ALFA 2b

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SUMMARY: Patients with cutaneous T-cell lymphoma (CTCL) were treated with recombinant alfa 2b interferon (rIFN alfa 2b) by intramuscular injection. Therapy-induced changes in Epstein-Barr virus (EBV) induced transformation of patient peripheral blood lymphocytes, 2′,5′ oligoadenylate (2-5A) synthetase levels and RNase L activation in peripheral blood mononuclear cells were monitored. Inhibition of EBV-induced transformation and elevation of 2-5A synthetase levels correlated with increased activation of RNase L, which provides evidence that intramuscular administration of rIFN alfa 2b induces a sustained anti-EBV state in CTCL patient peripheral blood mononuclear cells which can be detected in vitro.

Interferons comprise a class of heterogeneous proteins capable of conferring anti-viral properties upon treated cells. The in vitro effect of both cell-derived (1,2) and recombinant (3) interferons on Epstein-Barr virus (EBV)-induced transformation of human B lymphocytes has been examined. Both human leukocyte interferon (1,2) and fibroblast interferon (2) added to purified human lymphocytes prior to the addition of EBV prevents the outgrowth of immortalized human B cells. Recombinant α-, β-, and γ-interferons also have been shown to reduce EBV-induced transformation of human peripheral blood B cells when measured by DNA synthesis assays (3). Although recombinant α- and β-interferon effectively inhibit transformation when added between 24 h before and 24 h after EBV infection, recombinant γ-interferon inhibits B-cell responses from 24 h before and up to 3 days after virus infection (3).

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Henderson et al. (4) demonstrated that human leukocyte interferon as well as core 2′,5′ oligoadenylates mediate their anti-viral effects by inhibiting Epstein-Barr virus-directed nuclear antigen (EBNA) expression, an early event in EBV-induced transformation.

The capability of interferon to modulate 2-5A synthetase, a cellular enzyme associated with establishment of the antiviral state, has been examined both in vitro and in vivo (5-8). The addition of leukocyte interferon to cultured cells increases the 2-5A synthetase level (5). Mononuclear cells obtained from patients receiving leukocyte interferon also display increased 2-5A synthetase level (6,7). The significance of elevated 2-5A synthetase levels following interferon therapy in vivo has been correlated with the generation of an anti-vesicular stomatitis virus state in healthy volunteers receiving intramuscular injections of recombinant α-interferon (9). In order to further investigate the effectiveness of recombinant α-interferon to potentiate the anti-viral state in vivo, experiments were conducted to examine EBV-induced transformation of the peripheral blood lymphocytes (PBLs) obtained from cutaneous T-cell lymphoma (CTCL) patients treated with recombinant alfa 2b interferon (rIFN alfa 2b). 2-5A synthetase and activated RNase L levels were also monitored in peripheral blood mononuclear cells (PBMCs) and correlated with changes in EBV-induced transformation.

MATERIALS AND METHODS

Clinical Trial: A study (10) to determine the clinical efficacy of recombinant alfa 2b interferon (Intron A, Schering Corporation, Kenilworth, NJ) for treatment of patients with plaque-phase mycosis fungoides was conducted at the Skin and Cancer Hospital, Temple University Health Sciences Center, Philadelphia, PA. CTCL patients whose lesions responded locally to intralesional injections were treated with low-dose intramuscular (i.m.) rIFN alfa 2b (5×10^6 units) administered three times weekly for an initial 4-week interval. Approximately 3 months later, two of these patients received a second course of i.m. rIFN alfa 2b for an extended treatment interval, during which time blood samples were obtained for the studies presented here. Patient 5, a 43-year-old Caucasian male, classified as MFCG (11) stage IA(T1NxB0M0), received therapy for 12 weeks. Patient 2, a 68-year-old Caucasian male, classified as MFCG stage IIA(T2N1B0M0), received therapy for 16 weeks.

Virus: Supernatants containing EBV from the FF41-1 cell line (12) (provided by Dr. George Miller, Yale University, New Haven, CT) grown in RPMI 1640 medium with 5% fetal calf serum, were collected after 4 days incubation, filtered through 0.45-μm Nalgene filters (Nalge), and stored at -80°C for use in transformation studies.

Preparation of Peripheral Blood Mononuclear Cells: Whole venous blood from the mycosis fungoides patients and normal healthy adults was obtained by venipuncture at the Skin and Cancer Hospital, Temple University Health Sciences Center (Philadelphia, PA). Peripheral blood was drawn from the mycosis fungoides patients immediately before and at 24-, 48-, and 72-h intervals after interferon i.m. injections during the first month of treatment. Following Ficoll-Hypaque gradient separation, PBMCs were washed three times in Hank's balanced salt solution prior to use.

2-5A Synthetase-Specific Activity: The 2-5A synthetase activity was determined as described previously (13). Cell extracts were incubated with poly r(I)·r(C)-agarose and [α-^{32}P]ATP for 20 h at 30°C prior to addition to DEAE-cellulose columns. Nucleotides and 2-5A were displaced from DEAE-cellulose columns with 90 and 350 mM KCl solution, respectively. Specific activities in the 350 mM KCl fractions were determined. Enzyme activities for each extract were determined in triplicate and presented as the average picomoles adenosine triphosphate incorporated per µg of protein.

rRNA Cleavage Assay: The level of 2-5A-activated RNase (RNase L) in patient PBMC samples was detected in a cell-free system (14). RNase L-deficient L929 cell extracts were utilized as a source of intact ribosome. Cell extracts were incubated for 1 h at 30°C. Total RNA was extracted, denatured, and analyzed by electrophoresis on a 1.8% agarose gel (15). Following staining with ethidium bromide, RNA bands were visualized under UV light and photographed.

Epstein-Barr Virus-Induced Transformation Assay: The transformed centers assay (16) was utilized to determine susceptibility of PBLs to transformation by EBV. 1 x 10^6 cells per ml in RPMI 1640 with 20% heat-inactivated fetal calf serum plus antibiotics were infected with EBV at a multiplicity of infection of approximately 10^{-1} overnight prior to plating in 96-well microtiter dishes (Nunc) at 3-fold dilutions from 10^6 to 10^4 cells per well. Cells were refed every 4 days and plates were scored for foci of morphologically transformed lymphoblasts after 4 weeks. The Reed-Meunch formula for 50% endpoint titration was used to determine the cell density required to obtain transformation in 50% of the wells seeded (TD_{50}). Relative transformation was calculated as the ratio of the baseline mycosis fungoides patient TD_{50} to the TD_{50} obtained for each sample date.

RESULTS and DISCUSSION

The experiments described were designed to analyze the physiological response of PBMCs isolated from patients receiving rIFN alfa 2b. At 2, 4, and 11 days after the initiation of therapy, the levels of 2-5A synthetase, activated RNase L, and EBV-induced transformation were determined for blood samples drawn 48 h after the preceding i.m. injection of rIFN alfa 2b. The average level of 2-5A synthetase for the patients rose from a pretreatment value of ~7 to a value of >37 pmol ATP incorporated per µg protein (Fig. 1). The average 2-5A synthetase levels for the normal healthy donor peripheral blood samples were <4 pmol ATP incorporated per µg protein.

Further ramifications of rIFN alfa 2b therapy were evident by the levels of activated RNase L. An increase in activated RNase L was demonstrated by increased generation of specific cleavage products (Fig. 2, lanes 3-5). Samples analyzed prior to treatment (Fig. 2, lanes 1, 2; data not shown) revealed substantially less RNase L activity. 2-5A synthetase-specific activity was elevated above 40 for the 48-h post-injection time points on days 2, 4, and 11, while the baseline time point was 11. Thus it is plausible that increased intracellular levels of 2-5A synthetase were required to maintain the high level of activated RNase L observed over the course of therapy.

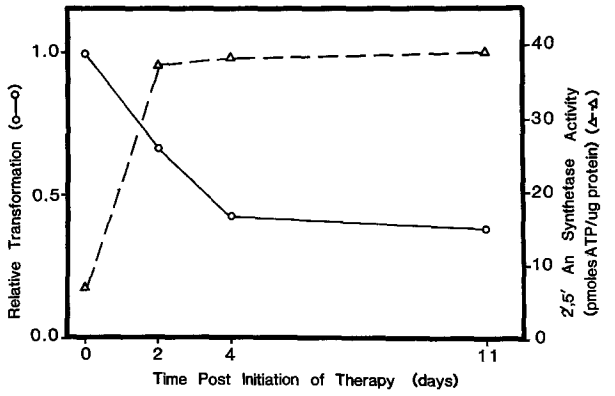

FIG. 1. Relative EBV-induced transformation (o——o) and 2-5A synthetase activity (Δ- - -Δ) of peripheral blood lymphocytes purified from peripheral blood drawn 48 h after intramuscular rIFN alfa 2b injection. Data represents average of results for two patients assayed over the course of 11 days of therapy.

A direct correlation between the anti-EBV state and increased levels of 2-5A synthetase and activated RNase L was observed during the first 11 days of therapy (Figs. 1, 2). It is possible that the EBNA mRNA may have been a substrate target for RNase L thus inhibiting transformation. 2-5A synthetase

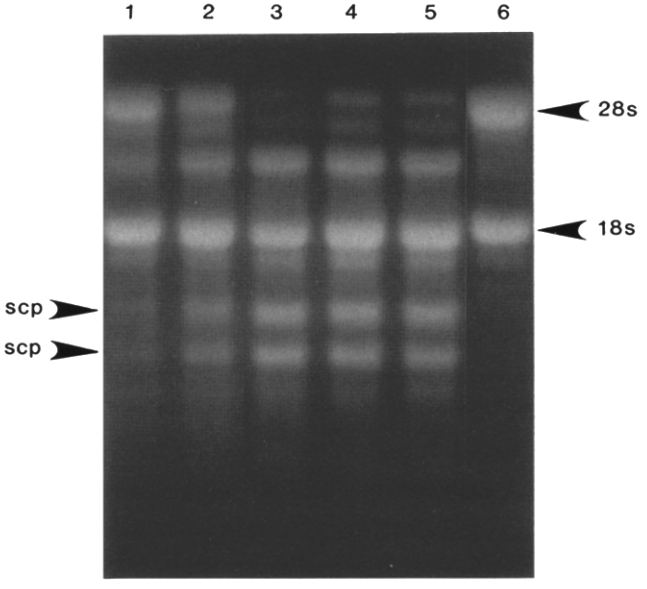

FIG. 2. rRNA cleavage assay. Mouse ribosomes incubated with extracts of peripheral blood mononuclear cells from untreated healthy control 2 (lane 1), patient 2 days 0, 2, 4, and 11 after initiation of rIFN alfa 2b therapy (lanes 2-5, respectively), and mock incubation (lane 6). Activated RNase L-specific cleavage products separated by agarose gel electrophoresis and visualized by ethidium bromide staining. The RNase L-specific cleavage products (scp) are indicated.

levels were maximally elevated 24 h post-injection. Consequently, RNase L activity was maximal 48 h post-injection, at which time the most potent anti-EBV state was established.

Pharmacokinetic studies of α-interferon administration by the i.m. route indicate that maximal serum interferon levels are achievable at approximately 6-8 h after a single injection, with residual levels remaining detectable at 24 h (17,18). It has also been shown that 2-5A synthetase levels peak at 24 h before returning to pretreatment levels 96 h following interferon administration (7). The 2-5A synthetase levels of the patients in the present study were also elevated to the greatest extent 24 h after the initiation of therapy (56 pmol ATP incorporated per μg protein on average). By 48 h after injection, 2-5A synthetase levels had diminished slightly, however, activated RNase L levels were higher in patient PBMCs. Concomitant with this rise in activity was an inhibition of the ability of EBV to transform the B lymphocytes of this population.

Since unfractionated PBMCs were utilized in the EBV transformation assays described here, it is not possible to distinguish between the biochemical and cellular responses to interferon therapy in regard to their individual contribution to the manifestation of the antiviral state. Interferon augmented cellular immunity, including activated natural killer and cytotoxic T cells, are known to play a key role in the control of EBV infection during infectious mononucleosis in the immunocompetent host (19). Thus interferon-augmented cellular immunity may have also contributed to the inhibition of EBV-induced transformation observed here. Additional work is required to clearly delineate which effects of interferon therapy are responsible for establishment of the anti-EBV state.

In a study by Levin and Hahn (20), patients with viral disease were treated with leukocyte interferon. Therapy resulted in the induction of an antiviral state in patient PBMCs which correlated with clinical improvement and control of viral infection (20). HTLV-I (21), HTLV-II (22), and HTLV-V (23) have been etiologically associated with CTCL. Thus, monitoring the antiviral status of CTCL patient PBMCs over the course of rIFN alfa 2b therapy potentially provides a method to evaluate a relevant clinical parameter. In the present study, elevated 2-5A synthetase and RNase L activity was associated with inhibition of EBV-induced transformation. Clinically, a slight overall improvement in patient 2 was observed, whereas no significant change was observed in patient 5 (10). Investigations are currently in progress to more precisely correlate the clinical efficacy of rIFN alfa 2b with changes in the patient PBMC 2-5A synthetase and RNase L levels and anti-EBV state.

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