Effect of Mycophenolic Acid on Epstein-Barr Virus Infection of Human B Lymphocytes

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Mycophenolic acid (MPA) noncompetitively inhibits IMP dehydrogenase, the rate-controlling enzyme for de novo biosynthesis of GMP, thus depleting cellular pools of GTP and dGTP (14, 26). Because lymphoid cells cannot use the salvage pathway for purine synthesis (3), depletion of dGTP inhibits DNA replication (6, 21). MPA was shown to inhibit proliferation of B and T lymphocytes in vitro and in vivo (2, 7–9). While various human T-cell, B-cell, and promonocytic cell lines are highly sensitive to the cytostatic effects of MPA (2, 7), nonlymphoid cells, such as the K562 erythroleukemia line, are less susceptible (7).

MPA and its ester derivative, mycophenolate mofetil, have significant immunosuppressive activity in animal transplant models (8, 9, 15–17) and are undergoing investigation in human organ transplantation, a setting in which Epstein-Barr virus (EBV) can produce lymphoproliferative disease (5, 11, 23, 27). Since EBV infects, growth transforms, and replicates in B lymphocytes (for a review, see reference 12), MPA might have significant effects on EBV infection or EBV-induced cell proliferation. The objective of the experiments described here was to evaluate the effects of MPA on EBV initiation of primary B-lymphocyte growth transformation, on the growth of EBV-transformed B lymphocytes, and on EBV lytic-cycle protein expression.

Effect of MPA on initiation of transformation by EBV. To test the effect of MPA on initiation of EBV-induced cell growth transformation, fresh cord blood mononuclear cells, separated by density centrifugation on Ficoll-Hypaque gradients, were infected with EBV (B95-8 strain) for 1 h at 37°C and plated at 106/ml in RPMI 1640 medium containing 10% fetal bovine serum and various serial dilutions of MPA. On day 3 of culture, the cells were pulsed overnight with [3H]thymidine (1 µCi/ml; DuPont, NEN Research Products), harvested with a Skatron apparatus, and assayed for [3H]thymidine incorporation, measured as counts per minute, with a Betaplate scintillation counter (Pharmacia LKB Biotechnology Inc., Piscataway, N.J.). By 3 days postinfection, large cell clumps were evident only in cultures exposed to MPA concentrations of less than 1.0 µM, a concentration which inhibited thymidine uptake in both EBV-infected and uninfected cells (Fig. 1; see below).

Indirect immunofluorescence microscopy was performed 4 days after primary B-lymphocyte infection, with monoclonal antibodies PE2 and CS1-4 (27) (donated by L. Young and A. Rickinson, Birmingham, England), which are reactive to EBV nuclear antigen 2 (EBNA-2) and latent membrane protein 1 (LMP-1), respectively. A goat anti-mouse secondary antibody conjugated to fluorescein isothiocyanate (Jackson ImmunoResearch Laboratories Inc., West Grove, Pa.) was added in a secondary step. The results in Table 1 indicate that 2 to 5% and 1 to 2% of the cells expressed EBNA-2 and LMP-1, respectively, following a single round of cell division. This expression of EBNA-2 and LMP-1 was not affected by an MPA concentration of 0.1 or 0.01 µM. However, 1.0 µM MPA inhibited, but did not completely block, EBNA-2 and LMP-1 expression, thereby reducing the number of EBNA-2- and LMP-1-expressing cells to 1% or less. At concentrations higher than 1.0 µM MPA, no further reduction in EBNA-2 and LMP-1 expression was noted, indicating that even high concentrations of MPA could not completely block EBNA-2 and LMP-1 expression. A 50% reduction of EBNA-2 and LMP-1 fluorescence might be expected because of a nonspecific antiproliferative effect of MPA (see below), since the first round of cell division after EBV infection of B lymphocytes in vitro is between 36 and 72 h postinfection (1, 19, 25). Since both EBNA-2 and LMP-1 are putative mediators of EBV-induced B-cell transformation (12), the inability of MPA to inhibit their expression completely might indicate that MPA cannot prevent initiation of the transforming process.

MPA concentrations of 1.0 µM and above inhibited the outgrowth of EBV-transformed cells (Fig. 1). However, even this was likely due to an antiproliferative effect of MPA rather than an effect on EBV initiation or maintenance of transformation, since reversibility studies showed that treatment with 2.5 µM MPA from the time of infection to 5 days

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postinfection resulted in almost no effect on EBV-induced cell growth. Five of six cultures grew as well as untreated controls. These cultures eventually gave rise to fully transformed lymphoblastoid cell lines (Table 2). It is noteworthy that recent clinical trials demonstrated that levels of MPA in plasma exceeding 1 μM can be maintained without development of nephrotoxicity, hepatotoxicity, or other serious side effects and with unambiguous clinical benefit (22).

**Antiproliferative effects of MPA on EBV-transformed B lymphocytes.** MPA at concentrations of 1.0 μM or higher had no acute toxic effects, as measured by trypan blue exclusion, on EBV-infected B lymphocytes after 48 h of treatment (Table 2). However, when EBV-transformed B lymphocytes were treated with MPA, cell counts showed that active proliferation was drastically inhibited in cultures exposed to MPA concentrations of 1.0 μM and higher (Table 2). At a concentration of 0.1 or 0.01 μM MPA, the cell number increased for 3 days almost as rapidly as in untreated cultures, with a doubling time of approximately 36 h.

**Effect of MPA on lytic EBV protein expression.** The effect of MPA on lytic EBV infection was assayed with a latently infected Burkitt lymphoma cell line, Akata, which can be induced to be permissive of EBV replication. Lytic replication is at less than 1% in Akata Burkitt lymphoma cells and increases to over 50% after cross-linking of surface immunoglobulin (24). Akata cells were induced to express EBV lytic-cycle proteins by addition of 1.5% anti-human immunoglobulin (Cappel, Organon Teknika, West Chester, Pa.) to the culture medium for 48 h. During this period, the cells were also treated with various serial dilutions of MPA. Polycrystalline gel electrophoresis and blotting were performed as previously described (4, 13). Cell extracts corresponding to 3 × 10^5 cells per lane were loaded onto the stacker portion of a 6 to 14% polyacrylamide gradient gel. Lytic-cycle protein expression was measured by immunoblot with EBV-immune serum with high-titer reactivity to lytic-cycle proteins. This serum detected early lytic-cycle proteins (early-antigen complex diffuse component), as well as late-cycle proteins, including gp350 and its spliced product, gp220. ^125I-labelled protein A (0.1 μCi/ml; Amersham) was added in a secondary step to bind the primary antibody. Reactive bands were revealed by overnight exposure to Kodak X-Omat film. Surprisingly, even concentrations of MPA which blocked cell proliferation had no effect on early or late lytic-cycle EBV infection, including concentrations of 2.5 and 5.0 μM (Fig. 2). Live-cell immunofluorescence microscopy with monoclonal antibody 72A-1 (obtained from the American Type Culture Collection, Rockville, Md.) failed to show an effect of MPA on the number of gp350/220-positive cells or on the level of gp350/220 expression (data not shown).

The lack of effect of MPA on EBV latent- or lytic-gene expression is surprising, particularly since lytic-gene expression is dependent on ongoing viral DNA synthesis and is abrogated by inhibitors of viral DNA synthesis. This indicates that MPA does not inhibit viral DNA replication and only affects DNA synthesis in uninfected or latently infected cells. Herpesviruses, including EBV, have among their early lytic-cycle genes a number of enzymes involved in DNA synthesis (for a review, see reference 20). Among these are ribonucleotide reductase and a deoxyribonucleotide kinase, which is usually referred to as a thymidine kinase but has broad substrate reactivity that includes guanosine. These enzymes apparently relieve the inhibitory effect of MPA on de novo GTP synthesis from inosine by salvaging

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**TABLE 1. Effect of MPA on EBNA-2 and LMP-1 expression in lymphocytes acutely infected with EBV**

<table>
<thead>
<tr>
<th>MPA concn (μM)</th>
<th>% of cells* expressing:</th>
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<tbody>
<tr>
<td></td>
<td>EBNA-2 b</td>
</tr>
<tr>
<td>0</td>
<td>2-5</td>
</tr>
<tr>
<td>0.01</td>
<td>2-5</td>
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<tr>
<td>0.1</td>
<td>2-5</td>
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<tr>
<td>1.0</td>
<td>≤1</td>
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<tr>
<td>2.5</td>
<td>≤1</td>
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<tr>
<td>5.0</td>
<td>≤1</td>
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</tbody>
</table>

* Each value represents the range of three experiments. Six high-power fields (400-fold magnification), each containing approx 50 cells, were counted.

b Determined by immune microscopy using monoclonal antibody PE2 on fixed cells harvested at 4 days postinfection.

c Determined by immune microscopy using monoclonal antibody CS-1-4 on fixed cells harvested at 4 days postinfection.
GTP from cellular pools, thereby enabling viral DNA synthesis to be unaffected by even high levels of MPA.

Because MPA does not inhibit EBV replication, any virus released in vivo could potentially continue to infect B cells, with resulting expression of EBNA-2 and LMP-1. These infected cells should not proliferate as long as therapeutic levels of MPA are present. Human renal allografts have been maintained in good functional condition for several years by using low doses of cyclosporine (thus reducing nephrotoxicity), mycophenolate mofetil, and low doses of prednisone (22). In human liver allograft recipients, it has been possible to withdraw cyclosporine and maintain the grafts in good functional condition with mycophenolate mofetil and low-dose prednisone (10). Cyclosporine does not inhibit the outgrowth of EBV-transformed B cells but suppresses the T-cell-mediated surveillance that normally inhibits that outgrowth (18). In contrast, MPA, in clinically attainable concentrations, inhibits the proliferation of EBV-transformed B cells. It is therefore reasonable to suggest that long-term maintenance therapy with mycophenolate mofetil, with or without cyclosporine, might reduce the risk of development of EBV-related lymphoproliferative disease.

REFERENCES