Development of Suppressor T Lymphocytes for Epstein-Barr Virus-Induced B-Lymphocyte Outgrowth During Acute Infectious Mononucleosis: Assessment by Two Quantitative Systems

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A system of $^3$H-thymidine incorporation by lymphocytes in culture for 3 wk has been utilized for quantitative assessment of the ability of T lymphocytes to inhibit outgrowth of autologous Epstein-Barr virus (EBV) transformed B lymphocytes. Lymphocytes from EBV-seronegative individuals lack the ability to suppress outgrowth of autologous EBV-transformed B lymphocytes. This capability appears during the course of primary EBV infection. The ability of T lymphocytes from EBV-seropositive subjects or convalescent IM patients to inhibit B-lymphocyte outgrowth is not HLA restricted. Thus, T lymphocytes capable of inhibition of in vitro EBV-induced B-cell outgrowth emerge during the acute stage of IM and may represent an important control mechanism of EBV-induced B-lymphocyte proliferation in vivo. The system provides a highly sensitive quantitative means for in vitro assessment of cell-mediated immunity to EBV.

INFECTIOUS mononucleosis has been termed a self-limiting lymphoproliferative syndrome. Epstein-Barr virus (EBV) transformed B lymphocytes, capable of continuous in vitro proliferation, may be recovered from the peripheral blood of patients with infectious mononucleosis (IM). A gradual fall in the number of circulating EBV-infected lymphocytes occurs during the first 3 mo after onset of IM. Following recovery from primary EBV infection, the virus remains within the host in latent form and may be recovered intermittently from oropharyngeal washings and from peripheral blood lymphocytes. The immune response that prevents further expansion of the EBV-transformed B-lymphocyte population is complex, but may be divided into humoral and cell-mediated immune components. Signs and symptoms of IM often progress despite the early appearance of antibodies specific for several EBV-related antigenic determinants. The lack of correlation between the appearance of humoral immunity and recovery from IM, and between antibody titers and viral expression in other clinical states, has been one of the factors that has led to investigations of cell-mediated immune functions, which may play a role in control of EBV.

Several cell-mediated immune functions, including blastogenesis and lymphokine elaboration in response to EBV or EBV-derived antigens, have been measured during and after IM. In general, the responses have been comparatively modest and begin considerably after the peak of the clinical illness. The timing and prominence of the atypical lymphocyte proliferation during IM have lead to the hypothesis that the atypical lymphocyte is responsible for termination of proliferation of the EBV-transformed B-lymphocyte population. Direct evidence in support of this hypothesis, however, has been lacking. Support for an active role for the T lymphocyte in recovery from IM has been provided by studies demonstrating cytotoxicity of T lymphocytes during EBV and cytomegalovirus (CMV) induced IM for EBV-containing continuous cell lines. Additionally, T lymphocytes from EBV-seropositive individuals have been shown to delay outgrowth of autologous EBV-infected B lymphocytes. T lymphocytes from patients with IM inhibit outgrowth of cocultured EBV-transformed human fetal cord blood lymphocytes. Each of the latter two studies utilized the rather subjective technique of visual estimation for assessment of the outgrowth suppressive effects of the T lymphocyte. Neither study demonstrated the emergence of this capability during the course of IM, nor attempted to determine what role the atypical lymphocyte might be playing in this function. The present study was designed in an effort to determine in a quantitative system the temporal relationship between clinical events, the atypical lymphocytosis, and the emergence of the capability of T lymphocytes to inhibit in vitro outgrowth of autologous EBV-transformed B lymphocytes during the course of EBV-induced IM.

MATERIALS AND METHODS

Human Subjects

Patients aged 14-22 yr with typical heterophile-positive IM seen and examined at the University of Maryland Health Center and at the National Institutes of Health (NIH) were included in the study. Diagnosis was based on heterophile positivity, the presence of atypical lymphocytes in the peripheral blood, and a compatible
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clinical syndrome. The interval between appearance of the first symptom recognized by the patient as part of the illness studied and phlebotomy was taken as the duration of illness. Controls aged 18-28 yr included healthy University of Maryland students, Health Center personnel, and NIH laboratory personnel.

Hematologic Data

White blood cell counts were performed on a Coulter Model FN cell counter (Coulter Electronics, Hialeah, Fla.). Differential cell counts were performed on Wright's stained preparatk. of peripheral blood using standard morphological criteria.17

EBV Serology

Antibody titers to viral capsid antigen (VCA) were determined by indirect immunofluorescence with fluorescein-conjugated goat anti-human IgG (Meloy, Springfield, Va.) and acetone-fixed P3HR1 cells (kindly provided by Dr. Paul Gerber, Bureau of Biologics, FDA).18 The reciprocal of highest dilution of serum exhibiting fluorescence was taken to be the anti-VCA titer. Individuals whose serum exhibited no immunofluorescence at a titer of 8 were considered EBV-seronegative.

Virus Stock

B95-8 cells (kindly provided by Dr. Philip Pizzo, National Cancer Institute, NIH) were grown in a 6-liter spinner flask at 34°C in RPMI-1640 with glutamine, 10% heat-inactivated fetal calf serum (HEM Research, Rockville, Md.), gentamicin (10 μg/ml) (Schering, Kenilworth, N.J.). Eight to 10 days after a final feeding, the supernatant was clarified by centrifugation at 200 g for 15 min at 4°C, filtered twice through a 0.45-M filter, and stored in 2-mI aliquots at -70°C until use. Supernatants prepared in this fashion contained 10^2-10^3 50% transforming doses/ml when assayed in a microtiter system with fetal cord blood lymphocytes.

Cell Preparation

Mononuclear cells were separated from heparinized peripheral blood by Ficoll-Hypaque density centrifugation.19 T-cell-enriched (TCE) and T-cell-depleted (TCD) fractions were prepared by a second Ficoll-Hypaque centrifugation following rosette formation with sheep erythrocytes (SRBC) as previously described.20 Prepared in this fashion, the TCE fraction contained 99% ± 1% cells rosetting with SRBC, and 2% ± 0.5% cells bearing surface immunoglobulin (sIg), as determined by immunofluorescent staining with fluorescein-conjugated goat F(ab), immunoglobulin (lg) prepared against polyvalent human Ig (Cappel Laboratories, Inc., Cochranville, Pa.) The TCD fraction contained 4% ± 1% cells rosetting with SRBC.

Cell Cultures

Cells were cultured in 0.225 ml RPMI-1640 with glutamine, 20% heat-inactivated fetal calf serum, and gentamicin (10 μg/ml) in covered flat-bottomed microtiter plates (Linbro, Hamden, Conn.), at 34°C in 5% CO2 and 100% humidity. Cell cultures were fed at 1 wk, then twice weekly with 0.1 ml fresh medium after removal of an equal volume of supernatant following centrifugation of culture plates at 80 g for 5 min.

Assessment of Cell Culture for EBV Activity by Visual Estimation of Outgrowth

Triplicate cultures were evaluated 6, 10, 13, and 20 days after initiation for evidence of outgrowth by visual estimation using the following scoring system: 0, small numbers of scattered lymphocytes; 1 +, nearly confluent lymphocytes without clusters of blasts; 2+, scattered small clusters of lymphocytes with or without blasts; 3+, 5-10 clusters/well of ~50-100 lymphocytes; 4+, numerous large clusters of lymphocytes covering ±25% of the surface of the well.

Assessment of Cell Cultures for EBV Activity by 3H-Thymidine Incorporation

Triplicate wells were pulsed for 4 hr with 0.4 μCi 3H-thymidine (specific activity 28 μCi/m mole thymidine, New England Nuclear, Boston, Mass.) and harvested on fiberglass discs using a multiple cell cultures harvesting unit (Skatron, Norway). 3H-thymidine was assayed by liquid scintillation counting (Beckman Instruments, Inc., Irvine, Calif.).

Statistical Methods

Data were analyzed by the Student's t test.
RESULTS

EBV-Seropositive Individuals

When $2 \times 10^4$ TCD lymphocytes were cultured without added EBV, no outgrowth was apparent after 3 wk in culture when assessed either by $^3$H-thymidine incorporation (Fig. 1A) or by visual estimation (Fig. 1B). In contrast, when TCD lymphocytes were cultured with 25 $\mu$ of EBV stock, outgrowth was apparent by day 10 when assessed by visual estimation. By day 7 in culture, $^3$H-thymidine incorporation was threefold greater than that of cultures without added virus and continued to increase over the ensuing 2 wk in culture. By day 21, EBV-transformed TCD lymphocytes incorporated 25-fold more $^3$H-thymidine than TCD lymphocytes cultured without added EBV ($p < 0.001$). Significant B-cell outgrowth was also apparent on day 21 in culture when assessed by visual estimation ($p < 0.001$).

When $5 \times 10^4$ autologous TCE lymphocytes were added to each well, an autologous mixed lymphocyte response (MLR) was observed. $^3$H-thymidine incorporation was brisk on day 7 in culture, both with and without added EBV. $^3$H-thymidine peaked during days 7–10 in cultures, both with and without added EBV, but fell by day 21 to levels comparable to those observed in culture of TCD without added EBV ($p > 0.2$). Assessment of the inhibition of B-lymphocyte outgrowth by visual estimation correlated well with the results obtained by the $^3$H-thymidine incorporation method (Fig. 1B).

EBV-Seronegative Individuals

TCD lymphocytes from EBV-seronegative individuals exhibited similar outgrowth and $^3$H-thymidine incorporation patterns to those exhibited by TCD lymphocytes from seropositive donors (Fig. 2A). $^3$H-thymidine incorporation patterns of cultures containing autologous TCE lymphocytes and added EBV were, however, quite different in the case of EBV-seronegative donors from those observed with seropositive donors. Despite addition of TCE lymphocytes to cultures of EBV-transformed B lymphocytes, $^3$H-thymidine incorporation on day 21 in culture proceeded at a rate equal to that observed in the cultures of EBV-transformed TCD lymphocytes alone (Fig. 2A). Lack of outgrowth suppression by autologous TCE lymphocytes was also evident by visual estimation of B-cell outgrowth (Fig. 2B). The outgrowth index, calculated by dividing the $^3$H-thymidine incorporation at day 21 by cultures of EBV-transformed TCD lymphocytes with added TCE lymphocytes by $^3$H-thymidine incorporation by cultures of EBV-transformed TCD lymphocytes alone, was significantly less for EBV-seropositive donors than for EBV-seronegative donors ($0.12 \pm 0.04$ versus $1.01 \pm 0.21$, $p < 0.001$).

Studies in Patients With IM

Patients with IM exhibited one of two $^3$H-thymidine incorporation patterns. TCE lymphocytes from three
of individuals, subsequently termed "nonsuppressors," failed to inhibit outgrowth of autologous EBV-transformed TCD lymphocytes (Fig. 3). In 10 other individuals, subsequently termed "suppressors," outgrowth inhibition by TCE lymphocytes of a magnitude similar to that observed by TCE lymphocytes from EBV-seropositive individuals was observed (Fig. 4). The opportunity for restudy of lymphocytes from one nonsuppressor arose several weeks after the first determination. During the convalescent stage of IM, inhibition of B-lymphocyte outgrowth was demonstrated by both \(^{3}H\)-thymidine incorporation and visual estimation of EBV-induced B-cell outgrowth. The capability of T lymphocytes to inhibit outgrowth of autologous EBV-infected B lymphocytes appeared before the end of the second week of clinical illness (Fig. 5). White blood cell counts and the absolute number and percentage of peripheral blood atypical lymphocytes were significantly higher in patients prior to the development of the capability for outgrowth inhibition (Fig. 6).

**Capability for Suppression of EBV-Induced B-Lymphocyte Outgrowth by Allogeneic T Lymphocytes**

In an effort to determine whether TCE lymphocytes required HLA identity of effector and target cell populations to suppress outgrowth of EBV-infected B lymphocytes, experiments were carried out in which TCE lymphocytes were added to allogeneic EBV-infected TCD lymphocytes. ELA typing at the A and B loci performed for 6 of the 8 donors utilized for allogeneic coculture experiments identified 19 of 24 possible alleles. No shared antigens were detected for the pairs of donors whose lymphocytes were cocultured. As can be seen in Fig. 7, when TCE lymphocytes were obtained from an EBV-seropositive donor, inhibition of outgrowth of cocultured, allogeneic, EBV-infected B lymphocytes was as effective as when the B lymphocytes were autologous.

**DISCUSSION**

The present study has demonstrated by two quantitative techniques the inhibition of outgrowth of EBV-transformed TCD lymphocytes by autologous or allogeneic T lymphocytes from healthy EBV-seropositive donors, or from convalescent IM patients. T lymphocytes from EBV-seronegative individuals or from patients studied early in the course of IM were unable to inhibit outgrowth of autologous EBV-transformed TCD lymphocytes. The emergence of the capability for outgrowth suppression occurred within 2 wk of the onset of illness, near the time at which clinical symptoms peaked and convalescence began.

Blast transformation to viral antigens has been a useful means for assessment of the cell-mediated immune response in several viral illnesses. Prior attempts at utilization of this technique in IM involving short-term lymphocyte cultures have focused on
T-lymphocyte proliferation resulting both from in vitro antigenic stimulation by the virus and from the autologous MLR. Extension of the in vitro culture period to 3 wk allows for resolution of the autologous MLR, and of purely antigenically mediated lymphocyte proliferation. Residual $^{3}$H-thymidine incorporation after 3 wk in culture is a manifestation of proliferation of EBV-transformed B lymphocytes. Suppression of this late $^{3}$H-thymidine incorporation may be taken as a quantitative measure of suppression of the proliferation of EBV-transformed B lymphocytes by cocultured T lymphocytes.

Recently, Thorley-Lawson has reported another technique based on $^{3}$H-thymidine incorporation for assessment of B-lymphocyte outgrowth inhibition. In this system, cultured lymphocytes are refractionated into slg-positive and negative subpopulations by passage over an immunoabsorbent column. $^{3}$H-thymidine incorporation by the slg-positive fraction of each culture is then determined. Each $^{3}$H-thymidine-based system has advantages and disadvantages. The second cell separation performed in Thorley-Lawson's system allows a shorter in vitro culture period (8–10 versus 14–21 days), but requires 20-fold more separated T and B lymphocytes at the outset for each outgrowth inhibition determination.

Assessment of outgrowth suppression by $^{3}$H-thymidine incorporation is far less subjective than observation of cultures for delay in the appearance of transformed foci. It is more quantitative and requires significantly less time in culture than does inspection
from EBV-seropositive donors. The data represent the mean cocultures of EBV-infected TCD lymphocyte and TCE lymphocytes, continuously cultivatable cell cultures at 6 wk for the presence or absence of suppressor T cells in EBV-induced IM.

Fig. 7. $^3$H-thymidine incorporation patterns of allogeneic cocultures of EBV-infected TCD lymphocyte and TCE lymphocytes from EBV-seropositive donors. The data represent the mean ± 1 SEM of triplicate determinations for 8 cocultures from 8 individual donors. Symbols are as shown in Fig. 1.

of cultures at 6 wk for the presence or absence of continuously cultivatable cell lines. The capability for B-lymphocyte outgrowth inhibition was absent from T lymphocytes from EBV-seronegative donor and emerged during acute IM. The data regarding the capability for outgrowth inhibition by T lymphocytes from EBV-seronegative individuals are in agreement with the data of Rickinson et al., but differ from those of Shope and Kaplan. The latter investigators reported that Tg lymphocytes from one normal adult EBV-seronegative donor were capable of mediating B-lymphocyte outgrowth inhibition of a magnitude similar to Tg lymphocytes from adult EBV-seropositive donors. Our data suggest that rather than being a maturational function, the capability of T lymphocytes to inhibit outgrowth of autologous EBV-infected B lymphocytes is acquired during primary EBV infection. Studies of additional EBV-seronegative individuals may help resolve the apparent differences between the two studies regarding the timing of the acquisition of this function.

In the present study, although more limited in scope than that reported by Rickinson et al., outgrowth inhibition by allogeneic TCE lymphocytes was found to be as effective as that manifested by autologous TCE lymphocytes. Initial studies of cell-mediated immunity to EBV in $^{51}$Cr release cytotoxicity systems did not demonstrate a requirement for HLA identity between effector and target lymphocytes, but recent studies suggest that an HLA preference may exist if natural killer activity is removed from the effector lymphocyte population. Further studies in this system with typing at the C and D loci may help to resolve the apparent differences between our findings and those of Rickinson et al.

The T-lymphocyte subpopulation capable of mediating B-lymphocyte outgrowth suppression in our system was found within the E-rosetting fraction of peripheral blood mononuclear cells. These data are in agreement with the data of Thorley-Lawson and Strominger. Shope and Kaplan, however, have found maximal outgrowth suppression activity among E-rosette-negative, T-cell antigen-positive lymphocytes bearing receptors for the Fc portion of IgG (Tg lymphocytes), and have found very little activity within the E-rosetting fraction. The apparent differences between the studies may reflect differences in the cell separation procedures. Tg lymphocytes form low avidity rosettes with sheep erythrocytes, and thus may be pelleted with the E-rosette-negative fraction. We have recently demonstrated that between 70% and 90% of the atypical lymphocyte population lacks receptors for the Fc portion of either IgG or IgM, and hence these cells fall within the T non-$\mu$, non-$\gamma$ lymphocyte subpopulation. Taken together, these findings and the observation that the degree of outgrowth inhibition correlates inversely with the percentage of circulating atypical lymphocytes are consistent with the observation that the capability for outgrowth inhibition resides within the non-atypical Tg-lymphocyte subpopulation. The further observation of the persistence of the capability for outgrowth inhibition by T lymphocytes from adult EBV-seropositive individuals years after primary EBV infection is additional evidence that outgrowth inhibition is not a direct function of the atypical lymphocyte.

In another series of experiments reported elsewhere, involving the patients studied in the present report, we have demonstrated the transient appearance of suppressor T cells for in vitro pokeweed-mitogen-induced Ig synthesis. Suppression of Ig production was always manifested early in the illness and disappeared with recovery. On the other hand, patients studied soon after the onset of symptoms of IM often lacked the ability to suppress B-lymphocyte outgrowth. Suppressor cells of pokeweed-mitogen-driven Ig production disappeared with recovery from illness, whereas the ability of T cells to suppress B-cell outgrowth remains years after recovery from primary EBV infection. These observations suggest that these two forms of suppression may be mediated by different mechanisms or by different T-lymphocyte subpopulations.

The immune response to EBV during primary infection is complex and involves several interrelated,
simultaneously evolving, humoral and cell-mediated immune components. The incorporation of \(^{3}H\)-thymidine as a measure of T-lymphocyte suppression of EBV-induced B-lymphocyte outgrowth provides a quantitative in vitro model for the investigation of cell-mediated immune functions that may play a role in the control of EBV infections in vivo. An in vitro system may be particularly useful in the study of immunologic complications of IM, since the restricted host range of the virus has prevented the development of suitable animal models for the study of IM. The potential deleterious clinical effects of antiviral agents with immunosuppressive properties have been amply demonstrated in other herpesvirus infections.31,32

Investigations of the effects of antiviral compounds on immunologic functions in in vitro systems, such as the one described in this report, may aid in the development of agents that may be useful in the treatment of EBV infections. In addition, an understanding of the basic mechanisms involved in the recognition and inhibition of proliferation of EBV-infected B lymphocytes may provide insights into the immunobiology of other latent viral infections of man.

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