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THE SUPPRESSION OF EPSTEIN-BARR VIRUS INFECTION *IN VITRO* OCCURS AFTER INFECTION BUT BEFORE TRANSFORMATION OF THE CELL[1]

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It has been demonstrated that T cells from humans can suppress the transformation of EBV-infected B cells *in vitro*. In this paper, studies are presented on the mechanism of the suppression. The T cells do not act against the virus itself nor the infection process. They appear to suppress the outgrowth of B cells after they are infected but before they are transformed. The T cells are much less effective in suppressing the outgrowth of the B cells once the latter have transformed (24th post-infection). The significance of these observations to EBV infection and other forms of T cell-mediated anti-EBV immunity is discussed.

When Epstein-Barr virus (EBV)[2] infection occurs *in vivo*, a variety of symptoms can arise. Infection during early childhood does not generally lead to the manifestation of clinical symptoms (1); however, infection during adolescence can cause the typical symptoms of the Paul Bunnell monospot positive infectious mononucleosis (IM) (2). In either case, the infected individuals continue to harbor the virus and remain seropositive for the rest of their lives without further expression of clinical symptoms. Under certain conditions EBV infection may also be associated with the development of Burkitt's lymphoma (3) and nasopharyngeal carcinoma (4). The immune responses to EBV infection that limit the lymphoproliferation associated with IM include the generation of killer T cells (5). These T cells presumably destroy the EBV-infected cells, but do not persist beyond the acute phase of the disease. Antiviral neutralizing antibodies are also produced that prevent the spread of free virus (6). The neutralizing antibodies persist during lifetime, as does the virus infection. This is known because healthy seropositive individuals continue to shed virus in the mouth (7) and carry EBV-infected lymphocytes, which will grow out in long-term culture to give rise to EBV-positive lymphoblastoid cell lines without the addition of exogenous virus (8). As well as maintaining neutralizing antibody titers, these individuals must also be immunologically active against the infected lymphocytes to prevent them from proliferating *in vivo*.

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[1] This work was supported by National Institutes of Health Grants R01A115310-01 and 5P01CA21082.
[2] Abbreviations used in this paper: EBV, Epstein-Barr virus; IM, infectious mononucleosis; EBNA, EBV nuclear antigen.

Infection of human lymphocytes *in vitro* results in the eventual outgrowth of immortalized B cell lines (7). It was noted previously that purified adult B lymphocytes transform more effectively than whole lymphocytes (9). Further investigation revealed that adult T lymphocytes were able to suppress and delay the outgrowth of the infected B cells. These observations led to the suggestion that T cells may be capable of a new form of response that allowed them to suppress EBV infection *in vitro* and *in vivo*.

In this paper experiments are presented that suggest that the T cell recognizes and responds to the virus-infected cell within a few hours post-infection. The T cells do not appear to interfere with adsorption and penetration by the virus, nor do they act effectively to suppress the B cells once they express the transformed phenotype.

MATERIALS AND METHODS

The methodology used in these studies has been described in detail elsewhere (9, 10).

Virus. Transforming virus was obtained from the supernatant of the B95-8 marmoset lymphoblastoid line. The cell line (originally obtained from the John L. Smith Memorial for Cancer Research Inc., Maywood, N. J.) was carried in medium RPMI 1640 (Grand Island Biological Co., Grand Island, N. Y.) containing 10% fetal calf serum, 100 μg/ml streptomycin, 100 units/ml penicillin, and 300 μg/ml glutamine. Typical virus preparation contained 10^5 to 10^6 transforming units/ml as assayed by outgrowth of transformed foci.

Cells. Heparinized blood, usually diluted 1:1 with Hanks' balanced salt solution, was layered over Ficoll-Hypaque, and centrifuged at 1000 × G for 30 min. The lymphocytes at the serum Ficoll interface were removed and washed three times with a wash solution (medium containing only 5% fetal calf serum). Adult blood was obtained from donors working in this Institute whose age range was from 20 to 35 years. Fetal cord blood was obtained by courtesy of Boston Lying In Hospital (Boston, Mass).

Cell culture. B and Ig-negative cells were cultured at appropriate concentrations (usually 1×10^6/ml unless otherwise stated) in a Linbro Mk II microtiter plate (Linbro Chemical Co., New Haven, Conn.) at a final volume of 0.2 ml of medium containing 20% fetal calf serum. Infection with virus was usually with a 1:10 or 1:20 final dilution of viral supernatant.

DNA synthesis assay. This method was used to assay the transformation potency of B95-8 virus and is based on the original assay of Robinson and Miller (11). Triplicate cultures of cells (1×10^6 ml, 2×10^5/well) were pulsed for 4 hr with [^{3}H]thymidine (50 μl of 2 μCi/ml, sp. act. 2 Ci/mmole) and harvested onto glass fiber filters.

Radioactivity was assayed by liquid scintillation counting by

using a toluene/Liquifluor scintillation fluid (New England Nuclear, Boston, Mass.). All values for specific [^{3}H]thymidine incorporation were calculated subtracting cpm in mock-infected culture from cpm in virus-infected control. Incorporation into uninfected cultures was always less than 200 cpm.

Outgrowth assay. Outgrowth of transformed lymphocytes was assayed visually as the time of appearance of transformed cells in the culture and assessed on a scale of one to four. A score of one represents only dead cells, judged by phase contrast microscopy; two, living but not necessarily transformed cells and clumps; three, transformed cell clumps and large transformed singled cells in the well (a score of two can sometimes represent a poor three, which would be confirmed within 2 or 3 days); four, the well overrun by transformed and proliferating cells. Each experiment was performed in sextuplicate, and the time of transformation in days was set as either the time when six wells obtain a score of three or alternatively four wells obtain a score of four. The plates were read microscopically every 2 days.

Separation of B and Ig-negative lymphocyte (T) populations. B and Ig-negative lymphocyte populations were prepared by passing whole lymphocyte populations over a rabbit anti-human Fab' immunoabsorbent column as described by Chess and Schlossman (12). Recovery of cells was greater than 90%. The B cell population was 92% pure by the criterion of surface p23, 30 (13) antigens (a marker for B cells and a subpopulation of null cells), and was 95% Ig positive. Both surface markers were assayed by the direct membrane immunofluorescence technique. The Ig-negative population contained 1% surface Ig-positive cells and approximately 2% p23, 30-positive cells due to the p23, 30-positive null cell population.

Assay for short-term B cell proliferation in the presence or absence of T cells. B cells (4 ml × 10^6/ml) cultured alone or with T cells (4 ml usually 3 × 10^6/ml) were mock infected or infected as described before (9). The cell suspension was cultured in a Costar 3524 culture plate (2 ml/well). At the appropriate time the cells from four wells were removed, pooled, and passed over a small immunoabsorbent column (~3 ml packed beads) as described above. The B cells, reisolated in this manner, were then cultured in 3 × 0.2 ml wells in a Linbro MK II microtiter plate, and proliferation was assessed by measuring [^{3}H]thymidine incorporation as described above.

Anti-complement immunofluorescence. This procedure was carried out as originally described (14).

Rabbit anti-EBV serum. The preparation of this serum has been described elsewhere (15). Briefly, purified pelleted virions from 12 liters of B95-8 culture fluid were used to immunize a rabbit over the course of 1 month. The resulting serum was absorbed with B95-8 cells that had been grown in phosphonoacetic acid (an inhibitor of late viral functions) till it was specific for late viral functions as judged by immunofluorescence on EBV producer and nonproducer cell lines. The serum had a specific virus neutralizing titer of 1:1000.

RESULTS

The outgrowth of transformed foci of EBV-infected B cells takes approximately 2 weeks. This process lasts even longer (2 to 3 weeks) in the presence of autologous T cells (9). Uninfected fresh peripheral adult lymphocytes are in an essentially resting state. Therefore, the proliferation of infected cells, which eventually leads to outgrowth of transformed foci, may be followed by measuring DNA synthesis in the B cells. By studying the time course of B cell proliferation in the presence and absence of autologous T cells, it should be possible to discover at what time the T cells suppress the infected B cell.

Time course of B cell outgrowth in the presence and absence of T cells. The proliferation of EBV-infected adult B cells may be assayed by measuring [^{3}H]-thymidine incorporation (10, 11, 16). B cells were infected with EBV in the presence and absence of T cells. At various times post-infection, the B cells were reisolated from the cultures by means of a rabbit anti-human Fab' immunoabsorbent column. The B cells were placed in culture, and their levels of DNA synthesis were assessed by pulsing with [^{3}H]-thymidine. It was necessary to reisolate the B cells, because the T cells, unlike the B cells, have a high background level of DNA synthesis (10) that would otherwise obscure the proliferation of the B cells. The results of such an experiment (Fig. 1) demonstrate that the proliferation of the B cells is reduced in the presence of T cells within 2 to 3 days post-infection. This suggests that the B cells are suppressed before detectable levels of EBV-stimulated DNA synthesis takes place. An increase in DNA synthesis 7 days post-infection and a reduction 14 days post-infection was noticed in several experiments. This was due to the development of highly cytotoxic T cells 7 to 10 days post-infection that would kill [^{51}Cr]-labeled EBV-transformed lymphoblasts *in vitro*. These cytotoxic T cells had no specificity for EBV, since they would lyse both EBV-positive and -negative lymphoblasts (see *Discussion*).

The ability of T cells to suppress the proliferation of EBV-infected B cells within a few days post-infection, as measured by reduced [^{3}H]-thymidine incorporation, is quite a general phenomenon, as demonstrated in Figure 2. This figure shows the combined results of experiments carried out on eight seropositive individuals. The presence of T cells led to a greater

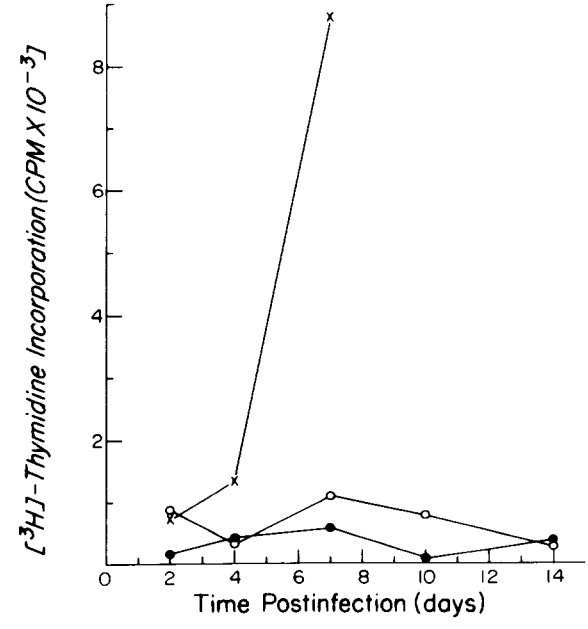

Figure 1. Proliferation of EBV-infected B cells in the presence and absence of T cells. B cells and T cells were separated by immunoabsorbent chromatography. B cells (10^6/ml) were infected in the presence and absence of T cells (3 × 10^6/ml) or were mock infected. At various times postinfection, the cells were taken and the B cells recovered by immunoabsorbent chromatography. The rate of proliferation of the B cells was assayed by pulsing for 4 hr with [^{3}H]-thymidine. ×, B cells infected alone; ●, B cells uninfected; ○, B cells infected in the presence of T cells.

than 50% reduction in the level of proliferation in all cases, with a 90% or greater reduction in four out of eight examples.

At what stage during infection and transformation of the B cell does the T cell act? During the first 2 days post-infection the level of DNA synthesis induced by EBV infection is low. Therefore, it was necessary to use indirect methods to study the events occurring during this time. The B cells were left in contact with T cells for various times post-infection, then they were reisolated and placed back into culture. The level of [^{3}H]thymidine incorporation by these B cells was assayed several times during the following week. Thus, the effectiveness with which the infection process had proceeded by a given time in the presence of T cells could be estimated by removing the T cells at that time and measuring the rates of proliferation of the B cells at later times. When such an experiment was performed (Fig. 3), it was seen that contact between the B cells and T cells for 3 hr post-infection was as effective as contact for 2 days. It is known that adsorption and penetration of the B cell by the virus is complete within 1 to 2 hr (10). It seemed likely, therefore, that the T cell would interfere with this process. In order to test this, three experiments were performed:

a) To test the effect of T cells on viral activity, EBV was incubated at 37°C in the presence or absence of T cells for up to 3 hr. The cells were then removed, and the ability of the virus to induce proliferation in B cells was tested. It was found that preincubation with T cells had no effect on the viral activity (Table I).

b) To determine how late post-infection the T cells may be added to still be effective, T cells were added to B cells at various times post-infection, incubated for 3 days, and then the ability of the B cells to proliferate (after removal of the T cells) was compared with infected B cells that had never been cultured with T cells. The result, shown in Figure 4, indicates that T cells added between 0 and 2 to 3 hr were equally effective in suppressing the B cells. Furthermore, these B cells were not able to proliferate after removal of the T cells. If, however, the T cells were added 24 hr or later post-infection, they were ineffective in suppressing the B cells. This effect could not be accounted for by an increase in the number of EBV-transformed B cells during the 24-hr time periods, because cell division does not start till at least 30 hr post-infection (10, 17). Neither could it be due to elimination of the T cell activity by preincubation because 24-hr reincubated T cells were able to suppress the infection of 24-hr preincubated B cells (data not shown).

EBV-neutralizing anibodies will also block infection (10) if added to the B cells at the same time as the virus. In contrast to the T cells, however, the antibodies become ineffective when added to infected B cells 2 to 3 hr post-infection (Table II), because viral absorption and penetration occur within 1 to 2 hr (10). Since T cells still suppress the infected B cells when added

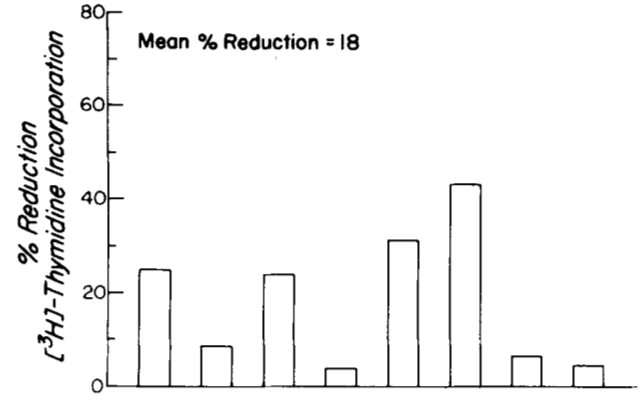

Figure 2. Proliferation of EBV-infected B cells in the presence and absence of T cells. This figure presents summary data from experiments carried out on eight individuals. The experimental procedure is described in the legend to Figure 1. The % reduction of [^{3}H]-thymidine incorporation was calculated as follows:

$$\frac{\text{Incorporation into B cells infected in the presence of T cells} - \text{Incorporation into uninfected B cells}}{\text{Incorporation into B cells infected alone} - \text{Incorporation into uninfected B cells}} \times 100\%$$

The cells were usually assayed 7 days postinfection. The same preparations of immunoabsorbent column material and EBV were used for each individual.

TABLE I

Effect on viral infectivity of preincubating[a] EBV with T cells

	Proliferation of B Cells 7 days Postinfection Assessed by [^{3}H]-Thymidine Incorporation (cpm × 10^3)	
	Virus Dilution	
	1/2	1/10
+T cells	6.8 ± 0.4	3.6 ± 0.6
−T cells	7.7 ± 0.4	3.6 ± 0.5

[a] Virus was incubated for 2 hr at 37°C with or without T cells (3.10^6/ml) before infecting pure B cells.

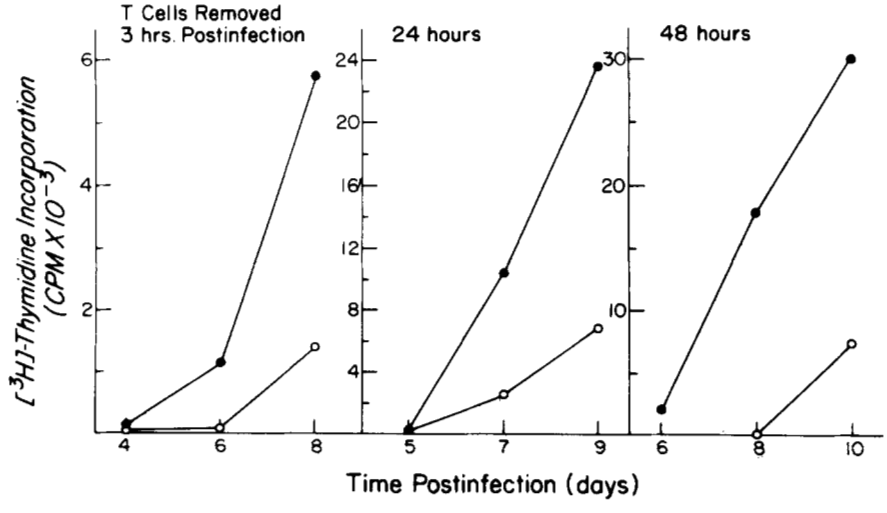

Figure 3. Proliferation of infected B cells after incubation with T cells for various lengths of time. Separated B cells were infected in the presence (○) and absence (●) of T cells. After various times the B cells were reisolated and set up in culture. The proliferation of the B cells was then assayed at various times by [^{3}H]-thymidine incorporation. For details see legend to Figure 1 and *Materials and Methods*. Note different scales.

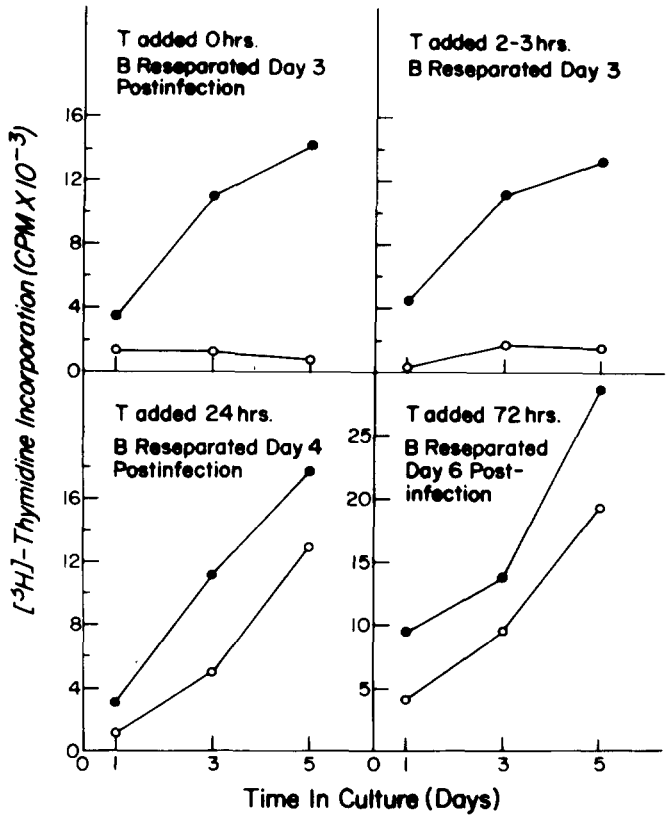

Figure 4. Proliferation of infected B cells after T cells added for a fixed period at different times postinfection. For details see legend to Figure 1.

TABLE II

Proliferation of EBV-infected B cells after addition of autologous T cells or EBV neutralizing serum before and after infection

T Cells or Serum Added	Proliferation of B Cells 7 Days Postinfection Assessed by [^{3}H]-Thymidine Incorporation (cpm × 10^3)	
	+T cells[a]	+Serum[b]
hr postinfection		
−1	0.5	0.2
2 to 3	1.0	8.0
24	13.0	8.0
No additions	14.0	7.5
Uninfected control	0.2	0.1

[a] Twenty microliters of T cells at 30 × 10^6/ml were added to wells containing B cells (0.2 ml 1 × 10^6/ml).

[b] Twenty microliters of a rabbit anti-EBV serum (titer 1:1000) prediluted 1:10 was added to wells containing B cells (0.2 ml 1 × 10^6/ml).

2 to 3 hr post-infection, they must be inhibiting processes that occur after viral absorption and penetration.

c) To test the effect of removal of free virus before addition of T cells: if the free virus was washed out from the infected B cells before the addition of the T cell, the proliferation of the B cells was stilled suppressed (Table III).

These three experiments lead to the conclusion that the T cells were both recognizing and responding to the virus-infected B cells. The T cells did not affect the virus itself nor viral absorption and penetration.

Is the EBV nuclear antigen (EBNA) expressed normally in the presence of T lymphocytes? Previous studies have shown that the first phenotypic expression of EBV-induced transformation (DNA synthesis (11) and EBNA induction (14)) occurs by 24 hr post-infection (10, 18). As the T cells were unable to suppress the infected B cells after 24 hr (Fig. 4), it may be concluded that the T cells were unable to suppress the infected B cells once they were transformed. Thus, an experiment was performed to examine whether induction of EBNA was also suppressed in the presence of autologous T cells.

B cells were infected in the presence or absence of T cells. After 30 hr the B lymphocytes were reisolated by passage over the immunoabsorbent column, smeared onto slides, and stained for EBNA (12). When cultures containing B cells alone were passed over the column, 10 to 20% of the bound cells were EBNA positive compared with less than 1%, when the B cells had been infected in the presence of T cells (Fig. 5). This result is difficult to assess with certainty because the recovery of bound cells from the immunoglobulin column was two to three times greater from cultures containing T cells. Nevertheless, this difference in recovery could not account for the difference in the percentage of EBNA-positive cells. Thus, T cells appear to suppress EBNA expression as well as the stimulation of DNA synthesis and outgrowth of transformed B cells.

Is the early suppression event responsible for the delay in outgrowth of EBV-infected B cells? It has previously been shown that adult T cells can delay the outgrowth of B cells infected with EBV *in vitro* (9). It was also demonstrated that a ratio of one or more T cells for each B cell was required and that fetal T lymphocytes were not able to delay outgrowth. To test whether the early suppression event was responsible for delay in outgrowth of infected B cells, the properties of the two phenomena were compared and the results were summarized in Table IV. An experiment with lymphocytes from the same donor indicated that the same number of T cells would delay the outgrowth of infected B cells as was required for the early suppression event. Similarly, fetal T lymphocytes were less effective than adult lymphocytes at reducing the level of proliferation of the infected B cells (Table V). The results of four experiments gave a mean of only 24% reduction in B cell proliferation by fetal T cells compared with 77% by adult T cells.

Last, in both phenomena, the T cells were insensitive to prior irradiation with levels of x-ray just sufficient to inhibit DNA synthesis (1000 R); however, they became sensitive at doses of 4,500 R or higher (Table VI).

These data provide evidence that the early suppression of B cell proliferation by T cells may be the primary mechanism for the delay in outgrowth observed in long-term culture.

DISCUSSION

In a previous study it was reported that adult T cells could delay the outgrowth of EBV-infected B cells (9). The studies

TABLE III

Suppression of EBV-infected B cells in the presence and absence of free virus[a]

	Virus Present		Virus Absent	
	+T	−T	+T	−T
Proliferation [^{3}H]thymidine incorporation 6 days postinfection	1803	3312	1387	2497
% suppression		46		45

[a] B cells (1 × 10^6/ml) were infected for 1 hr at 37°C with EBV. Free virus was removed from the B cells by washing once with medium (see *Materials and Methods*) and resuspending to the original volume. T cells (3 × 10^6/ml) or medium were then added to the cultures.

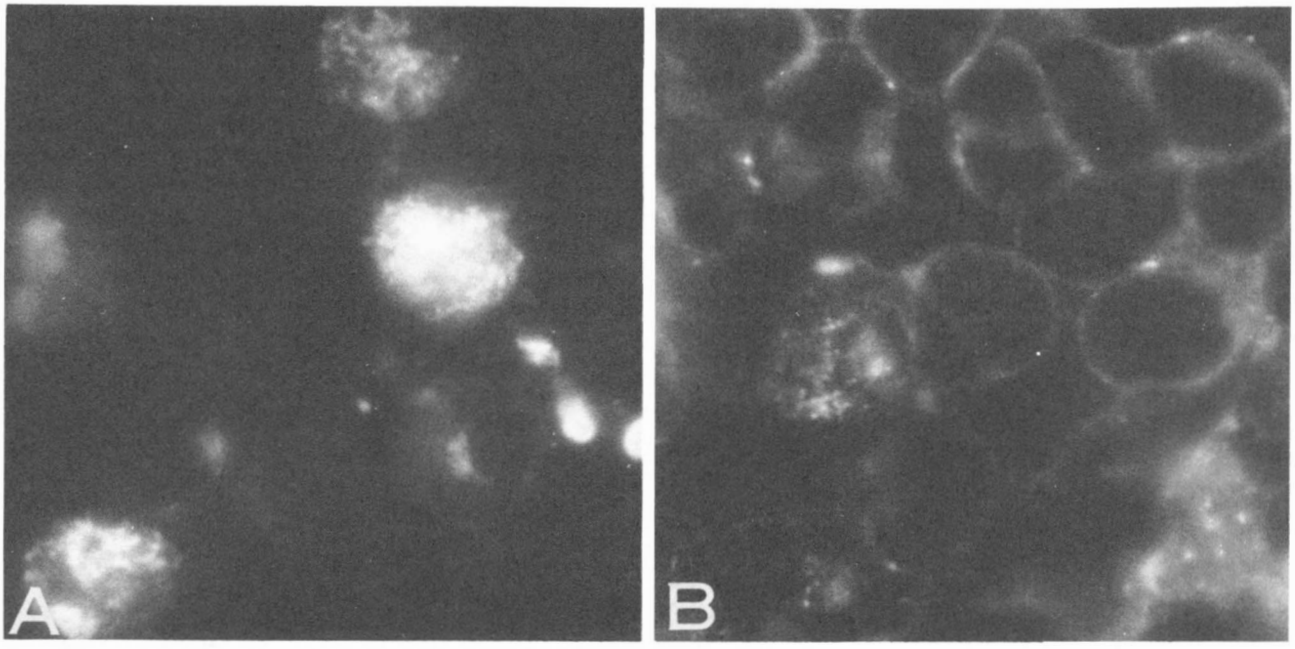

Figure 5. Expression of Epstein-Barr virus nuclear antigen (EBNA) in B cells infected in the presence and absence of T cells. The B cells were obtained 30 hr postinfection as described in the legend to Figure 1. Except they were stained for EBNA instead of assayed for proliferation. A, B cells infected alone; B, B cells infected in the presence of T cells.

TABLE IV

Comparison of the ability of T cells to delay the outgrowth of infected B cells and inhibit early proliferation

	Delay in Outgrowth	Early Suppression Event
Effectiveness of adult lymphocytes		
Cell number required	≥ 1 T : 1 B	≥ 1 T : 1 B
Effectiveness of fetal lymphocytes		
Cell number required	> 4 T : 1 B	> 3 T : 1 B
Sensitivity of x-ray treatment of T cells	Insensitive to 1000 rad Sensitive to 4500 rad	Insensitive to 1000 rad Sensitive to 4500 rad

TABLE V

Comparisons of the effectiveness with which fetal and adult T cells suppress the outgrowth of autologous EBV infected B cells

Expt. No.	Fetal B Alone	Fetal B + Fetal T[a]	% Reduction	Adult B Alone	Adult B + Adult T[a]	% Reduction
1	8400	6200	26	2500	810	67
2	495	160	68	470	15	97
3	4600	4300	6	5400	650	88
4	9500	10100	−6	2600	1200	54
Mean			24			77

[a] Cultures contained B cells at 1×10^6/ml and T cells at 3×10^6/ml. Total volume was 4 ml.

TABLE VI

Sensitivity of the suppression of early proliferation and outgrowth to x-irradiation

	Dose of X-Rays	Delay in outgrowth	EBV-Induced Proliferation 7 Days Postinfection ([^{3}H]-Thymidine Incorporation)
	rads	*days*	
	0	15	1,100
	1,000	7	1,400
	4,500	0	9,500
B cells alone		0	11,000

presented here indicate the T cells suppress the outgrowth of infected B cells within 24 hr post-infection. The suppression did not seem to interfere with the virus, since virus preincubated with T cells was fully infectious. Similarly, viral adsorption to and penetration of the B cell, which takes 1 to 2 hr, were not affected, since the T cells were equally effective when added after this time. The observation that the T cells became much less effective when added 24 hr post-infection is important, because the B cells are stably transformed by this time. This is known, since EBNA induction (18) and the stimulation of early cellular DNA synthesis (10) are first detected by 24 hr although cell division probably does not start till 48 to 72 hr post-infection (10, 19). Thus, the number of infected cells probably remains constant throughout the first 24 to 48 hr. Furthermore, the T cells are fully active against the virus-infected cells in the absence of free virus. It may be concluded, therefore, that the T cells are interfering with transformation in response to viral determinants left on the B cell surface after infection, and that the T cells are unable to suppress infection once the B cells are transformed. The observation that EBNA expression is suppressed implies similarly that the process of outgrowth is arrested early.

It is known from immunofluorescence studies (20, 21) that EBV leaves antigens on the surface of the B cells when it infects and uncoats. These antigens are probably the ones that induce the T cells to suppress the transformation of the B cell. The possibility that a dual recognition of viral and self antigens is required cannot be excluded at this time.

Although the suppression is not always complete, i.e., some B cells escape, it does not seem to be reversible. This is most strongly suggested in Figure 4, where B cells in contact with T cells for 3 days post-infection show no signs of proliferating after removal of the T cells. This observation and the suppression of EBNA induction distinguishes the early suppression of

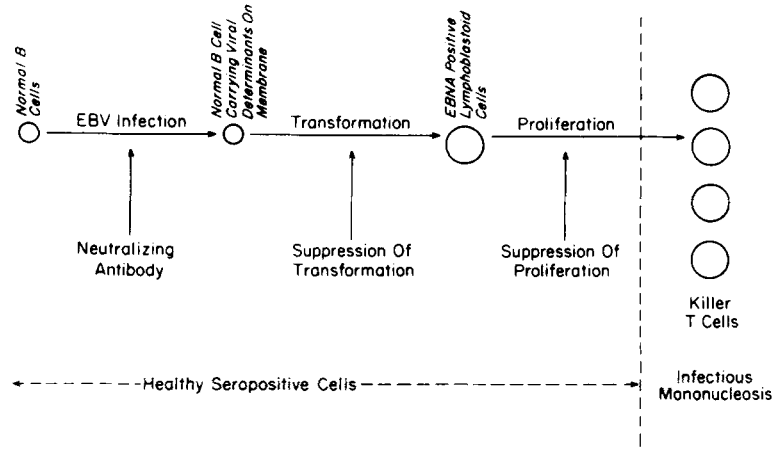

Figure 6. A diagrammatic demonstration of the possible role of humoral and cell-mediated responses in controlling Epstein-Barr virus infection, *in vivo*.

outgrowth described here from that described by Moss *et al.* (22), who demonstrated arrest of transformation in the presence of mononuclear cells from seropositive donors. In their system, a fibroblast feeder layer from an adult seropositive donor was required, the stage of arrest was after EBNA induction, and arrest was reversed upon removal of the feeder layer. More recently these authors have presented data suggesting that there is a T cell-induced regression of outgrowth 7 to 10 days post-infection (23). The studies presented here indicate that this regression is due to the induction of nonspecific killer T cells at that time. This type of killer cell has been demonstrated *in vitro* before (5), but has not been shown to have any significance *in vivo*.

Given the observations described here and previously (5, 6, 9, 22), a picture emerges of a series of cell-mediated responses to EBV infection (Fig. 6). Thus, in the normal healthy seropositive individual there are neutralizing antibodies (6) to block the virus before it infects the cell. If virus can pass this block and infect the B cell, T cells will prevent the B cells from transforming (this paper). Any infected B cell that transforms will be further suppressed by T cells and prevented from proliferating (22). Presumably, when the adult first becomes infected, these three mechanisms are insufficient to prevent the emergence of proliferating EBNA-positive transformed B cells (19), and the more powerful intervention by specific killer T cells is invoked. Once infection is reduced to a subclinical level, the killer cells disappear and the continuing low level of persistent EBV infection is held in check by the first three immune responses.

Thus, these mechanisms can account for the containment of both acute IM-associated and subclinical persistent EBV infection. They do not, however, explain why EBV infection in childhood does not result in IM (1). It is known that the majority of atypical lymphocytes in IM are T cells, and it has been suggested previously by several authors (e.g., 5) that the clinical symptoms of IM are a consequence of this large T cell response. The lack of IM in young children could then be accounted for by a lack of large-scale proliferative T cell responses in children. There is some clinical evidence to support this notion, since children do not make inflammatory responses to some infections that do cause inflammatory responses in adults (F. Rosen, personal communication). Therefore, it may be that antiviral neutralizing antibody and the suppression of infected cells before transformation are adequate to control EBV infection in newborn and adult.

This is completely consistent with the observation that the vast majority of EBV-infected cells *in vivo* during acute IM are small EBNA-negative untransformed B lymphocytes (24). Presumably, these lymphocytes are being continually generated by infection and destroyed by suppression before they transform. If this were true, then the massive T cell responses of IM, including killer T cells, may be less relevant in controlling EBV infection and may arise only while the other responses are bringing the infection under control. The possibility that the killer T cells may be of secondary importance in controlling B cell proliferation after transformation by EBV is reinforced by the observation that African Burkitt's lymphoma biopsies are infiltrated with killer T cells (25), but they, presumably, are unable to prevent the growth of the tumor.

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