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Inhibition of the *in vitro* outgrowth of Epstein–Barr virus-transformed lymphocytes by thymus-dependent lymphocytes from infectious mononucleosis patients

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SUMMARY

Known numbers of thymus-dependent (T) lymphocytes, obtained by positive selection from the blood of acute infectious mononucleosis (IM) patients and from control donors, were added to target cultures of foetal mononuclear cells within 0–7 days of exposure of the target cells to one of a range of doses of Epstein–Barr (EB) virus. The subsequent outgrowth of virus-transformed foetal cells was markedly inhibited by the presence in the cultures of IM-derived T cells, whilst similar numbers of T cells prepared either from cord blood or from adult donors seronegative for EB virus had little or no inhibitory effect. Target foetal cells treated with papain to remove any viral envelope material remaining on the cell surface after infection, were just as sensitive as untreated cells to the addition of IM-derived T cells. It is concluded that the inhibition cannot be mediated through recognition either of viral envelope structures on the surface of infected cells or of the antigenically related virus-determined membrane antigen, MA, but must depend upon recognition of the lymphocyte-detected membrane antigen, LYDMA. The regularity with which IM-derived T cells block the outgrowth of virus-transformed foetal cells suggests that LYDMA consistently appears on the surface of infected foetal cells before the establishment of transformed foci, but is unlikely to be directly associated with the cells' existing histocompatibility antigens.

INTRODUCTION

The Epstein–Barr (EB) virus has for some years been recognized as the cause of heterophile antibody-positive infectious mononucleosis (IM) (Henle, Henle & Diehl, 1968), a self-limiting lymphoproliferative disease characterized by the appearance of large numbers of atypical lymphocytes in the blood. Much of our current view of the pathogenesis of this disease stems from two recent observations; the first is that EB virus preferentially infects bone marrow-derived (B) lymphocytes, at least *in vitro* (Jondal & Klein, 1973; Pattengale, Smith & Gerber, 1973; Greaves, Brown & Rickinson, 1975), and the second is that the vast majority of the atypical circulating cells in IM blood are not B-cells but thymus-dependent (T) lymphocytes (Sheldon *et al.*, 1973; Virolainen *et al.*, 1973; Yata *et al.*, 1973; Enberg, Eberle & Williams, 1974). The inference, that most of the circulating atypical cells are in fact reactive against the viral infection rather than participating in it, is now beginning to receive experimental support.

Denman & Pelton (1974) first showed that lymphocytes from IM blood had some cytotoxic activity *in vitro* against an EB virus-producing lymphoid cell line. This approach has been developed further by other workers and these more recent studies make it clear that the cytotoxic action is mediated only against those cell lines which carry the EB virus genome (Svedmyr & Jondal, 1975; Royston *et al.*, 1975). Thus the immunological specificity of IM-derived cells appears in direct contrast to the rather non-

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specific cytotoxicity generated when normal lymphocytes are stimulated *in vitro* by autologous EB virus-transformed cell lines (Svedmyr *et al.*, 1974).

All the previous work with IM-derived cells has employed a chromium release assay for the demonstration of cytotoxicity. Evidence of a different kind but likewise demonstrating a cytotoxic effect has recently emerged in this laboratory during experiments to determine the frequency of establishment of EB virus genome-containing cell lines in co-cultures between foetal cells and various leucocyte subpopulations from acute IM blood (Crawford *et al.*, submitted for publication), when it was noticed that such lines emerged more readily if IM-derived T cells were first excluded from the co-cultures.

The present report describes subsequent experiments in which purified T cells from acute IM patients or from healthy control donors have been added in known numbers to target cultures of foetal lymphocytes after *in vitro* infection of the target cells by EB virus. The effects of the two types of T cells upon the outgrowth of virus-transformed foetal cells in these cultures were then compared in order to determine any specific cytotoxic action which IM-derived T cells might possess.

MATERIALS AND METHODS

Tissue culture medium. Cells were cultured in RPMI 1640 medium (Flow Laboratories, Glasgow) supplemented with 15% foetal calf serum and 100 iu per ml penicillin and streptomycin.

Preparation of mononuclear cells. 30–50 ml blood samples were taken from the foetal cord of full term deliveries using a heparinized syringe (20 u heparin per ml). Similar samples were also taken by venepuncture from young adult donors, either heterophile antibody-positive IM patients within three weeks of the clinical onset of the disease or laboratory volunteers in normal health and of known serological status with respect to EB virus. Mononuclear cells were prepared by Ficoll–Isopaque centrifugation of whole blood, essentially as described by Böyum (1968) and resuspended in culture medium at a known cell concentration.

Preparation of purified T-lymphocytes. E-rosettes between sheep red blood cells (SRBC) and the T-cell fraction of the mononuclear cell population were prepared by standard methods (Wybran, Chantler & Fudenberg, 1973) and then separated from the remaining non-rosetted cells by a second Ficoll–Isopaque centrifugation. The rosetted cells were collected after lysing the SRBC by exposure for 10 min to 0·81% NH_4Cl solution at 4°C, washed, and resuspended in culture medium at a known cell concentration. The cell population thus obtained was more than 95% viable as assessed by exclusion of neutral red stain and contained less than 5% of non-T cells as recognized by expression of surface-bound immunoglobulin or of phagocytic activity, estimated using standard methods (Brown & Greaves, 1974).

Preparation of EB virus. Supernatant fluid from 7-day-old cultures of the EB virus-producing B95-8 cell line (Miller & Lipman, 1973) was used to prepare a concentrated stock of EB virus by the method of Adams (1973). The same virus preparation was employed throughout.

Preparation of EB virus-infected target cells. Mononuclear cells freshly prepared from the cord blood of a single foetal donor were resuspended at a concentration of 10^7 cells/ml in culture medium containing the EB virus preparation at 10^2, 10^3, 10^4 and 10^5-fold dilutions. After regular shaking over a 60-min period at 37°C, the cells were centrifuged at 150 *g* for 5 min, washed twice and resuspended in fresh culture medium at a known cell concentration.

Papain treatment of EB virus-infected target cells. In some experiments the infected foetal cells were exposed for a further 60 min at room temperature either to a 4 mg/ml solution of papain (Crude enzyme, type II, Sigma Chemical Co., London) in 0·15 M NaCl, 0·01 M Tris HCl buffer containing 0·01 M cysteine, or to the buffer alone, then again washed twice and resuspended in culture medium at a known cell concentration. Treatment with papain is known to remove any viral envelope material remaining on the cell surface after infection with EB virus (Dölken & Klein, 1976).

Preparation and maintenance of experimental cultures. 0·1-ml volumes of suspensions of foetal mononuclear cells, already exposed to known doses of EB virus, were seeded into the wells of microtest plates (Nunclon, A.S. Nunc, Denmark) at a concentration of $2{\cdot}5 \times 10^5$ cells per well. Each plate also accommodated parallel control cultures of uninfected cells from the same foetal donor. All cultures were then incubated at 37°C in a humidified atmosphere of 5% CO_2 in air.

0·1-ml volumes of purified T-cells from IM patients or from control donors were then added to these cultures either on the same day or up to 7 days after the infection of the target foetal cells. The T cells were usually added at three different concentrations, namely $2{\cdot}5 \times 10^5$, 5×10^5 and 10^6 cells per well. The cultures were re-fed by changing half the medium every week and were regularly observed over the next 6 weeks for the appearance of transformed foci.

Measurement of the efficiency of outgrowth of transformed cells. Transformation was recognized by the appearance of progressively growing foci of lymphoblastoid cells, as described elsewhere (Rickinson *et al.*, 1974). In many experiments the transformed nature of particular cultures was confirmed by their continued growth after sub-culture. Knowing the incidence of successful outgrowth in cultures of a particular type over a range of virus dilutions, the efficiency of outgrowth in that type of culture was defined as the negative $\log_{10}$ of that virus dilution which was sufficient to induce transformed foci in 50% of replicate cultures, the dilution being determined by the method of Reed & Muench (1938).

Experimental procedure. In a first set of experiments, T cells from acute IM patients and from either foetal donors or

adult donors seronegative with respect to EB virus, were added to target cultures of foetal cells which had been infected with EB virus 3–7 days earlier.

In a further set of experiments T cells from similar donors were added to target cultures of foetal cells within 1–2 hr of infection with EB virus.

A final set of experiments examined in more detail the effect of T cells added to target cultures of foetal cells on the day of infection; in these experiments some of the infected foetal cells had been treated with papain in buffer and others with the buffer alone. Here the activity of IM-derived T cells was compared with that of T cells from healthy adult donors either seronegative or seropositive for antibodies to EB virus.

RESULTS

Table 1 shows the detailed results from Expt 1 of the first series in which foetal mononuclear cells, exposed to one of four ten-fold dilutions of EB virus and cultured for 4 days, were then mixed with various concentrations of T cells either from IM Patient 1 or from a control adult seronegative donor, SN1. Whilst the presence of control T cells had some slight inhibitory effect upon the outgrowth of transformed foetal cells, and then only at the higher cell numbers, the inhibition was much more marked in cultures receiving IM-derived T cells (Table 1).

TABLE 1. Incidence of outgrowth of transformed cells in cultures of foetal lymphocytes exposed to known dilutions of an EB virus preparation. On day 4 post-infection, half the cultures received known numbers of T cells from IM patient 1 (IM1) and half received T cells from a control seronegative donor (SN1)

Dilution of virus used	Incidence of outgrowth of transformed cells in cultures of $2{\cdot}5\times10^5$ foetal lymphocytes with:						
	No T cells added	IM1 T-cells added:			SN1 T-cells added:		
		10^6	5×10^5	$2{\cdot}5\times10^5$	10^6	5×10^5	$2{\cdot}5\times10^5$
10^{-2}	10/10	2/6	6/6	6/6	6/6	6/6	6/6
10^{-3}	10/10	0/6	1/6	2/6	6/6	6/6	6/6
10^{-4}	6/10	0/6	0/6	0/6	0/6	0/6	3/6
10^{-5}	0/10	0/6	0/6	0/6	0/6	0/6	0/6

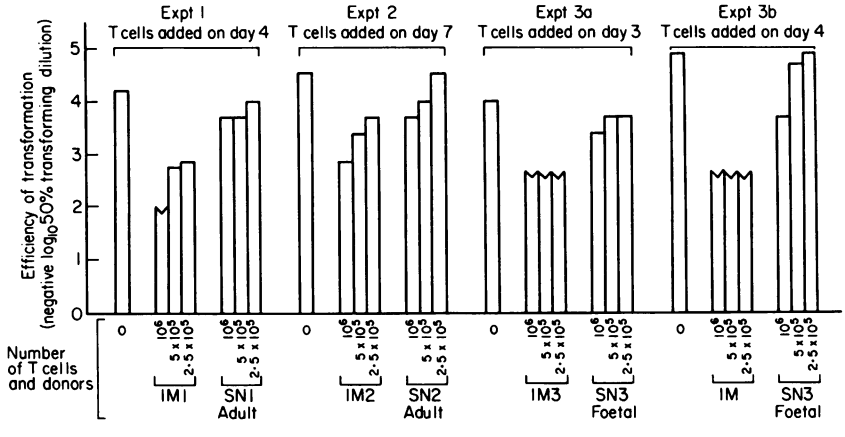

FIG. 1. Efficiency of outgrowth of EB virus-infected foetal lymphocytes co-cultivated 3–7 days post-infection with T cells from IM or control seronegative donors. Where the inhibitory effect of T cells was so marked that even at the highest EB virus dose less than half the cultures developed transformed foci, the efficiency of outgrowth of transformed cells is shown as its maximum value by a bar with an indented head.

The degree of inhibition is better appreciated when the results in Table 1 are converted to express the efficiency of outgrowth of transformed cells for each of the seven types of culture involved in this experiment. Values for the efficiencies of outgrowth from this and from three further experiments in the same series are shown graphically in Fig. 1. Where the inhibitory effect of T cells was so marked that even at the highest EB virus dose less than half the cultures developed transformed foci, the efficiency of outgrowth is represented as its maximum value and this is indicated on the figure by a bar with an indented head (e.g. Expt 1, cultures receiving 10^6 IM1 T-cells). The results in Fig. 1 clearly show that IM-derived T cells were able to block the outgrowth of transformants much more efficiently than could control T cells either from foetal cord blood (Expts 3a and b) or from an adult seronegative donor (Expts 1 and 2). Furthermore the effect appeared to be independent of the identity of the foetal target cells. Thus T-cells from IM Patient 3 were much more inhibitory than were control T-cells when tested against target cells from two different foetal donors (Expts 3a and b).

In the second series of experiments, IM-derived and seronegative donor-derived T cells were added to cultures of foetal target cells within hours of their infection by EB virus. Six such experiments were conducted, each involving a different IM donor, and in every case IM-derived T cells were again able to

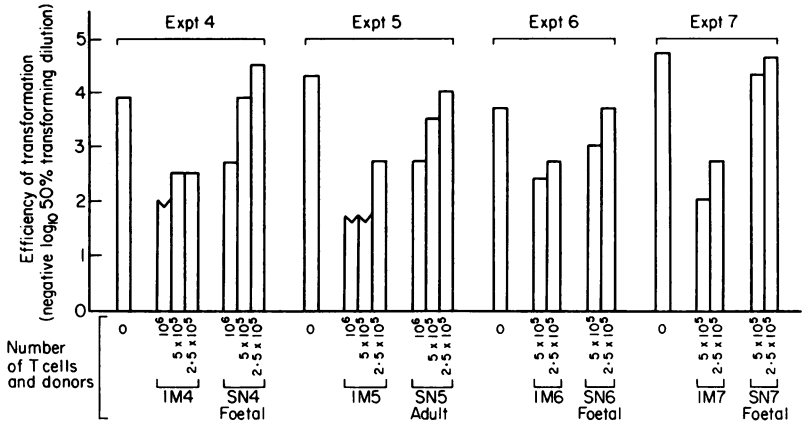

FIG. 2. Efficiency of outgrowth of EB virus-infected foetal lymphocytes co-cultivated immediately post-infection with T-cells from IM or control seronegative donors. Where the inhibitory effect of T cells was so marked that even at the highest EB virus dose less than half the cultures developed transformed foci, the efficiency of outgrowth of transformed cells is shown as its maximum value by a bar with an indented head.

block the subsequent outgrowth of transformants much more efficiently than could control T-cells. Representative results from four of the experiments are presented in Fig. 2 and show the unusually low efficiency of outgrowth in cultures containing IM-derived T cells. Again the source of control T cells (cord blood in Expts 4, 6 and 7; seronegative adult blood in Expt 5) did not appear to affect the results.

The final series of experiments was similar to the above but included target cells from which any viral envelope material remaining on the cell surface after infection had been removed by mild treatment with papain. In addition, adult seropositive donors in normal health provided a third source of T cells to be added to infected target cultures. Fig. 3 shows the results from four complete experiments of this type. It is again clear that IM-derived T cells were consistently more inhibitory to the outgrowth of transformants than were T cells from adult seronegative donors, although in Expt 11 the inhibition was less marked than usual. In two cases T cells from healthy seropositive donors had no effect upon outgrowth (Expts 8 and 10), whereas in Expt 11 such cells appeared just as inhibitory as were T cells from an IM donor. Throughout this series of experiments the results obtained from virus-infected papain-treated target cells, shown in Fig. 3b, matched very closely those obtained from parallel cultures of target cells infected and then exposed only to the buffer (Fig. 3a).

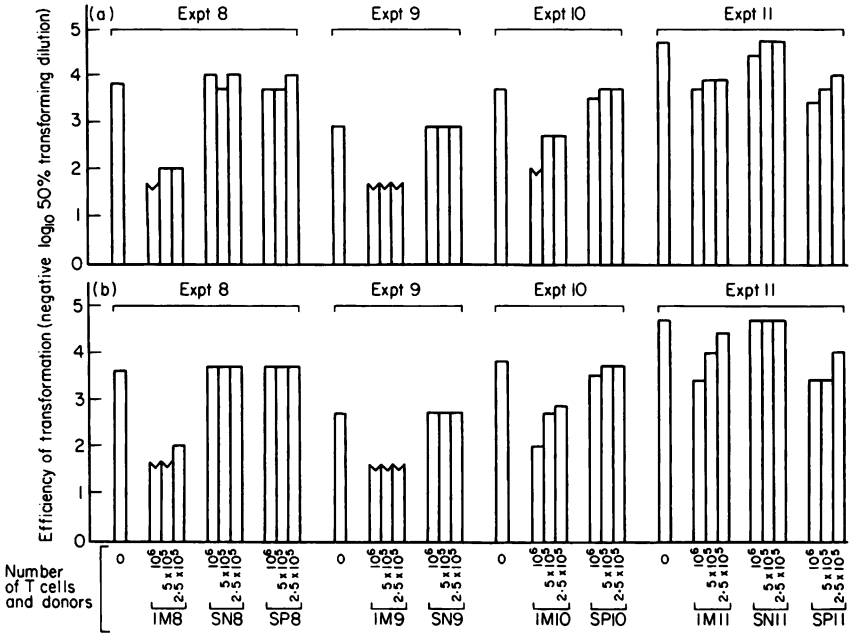

FIG. 3. Efficiency of outgrowth of EB virus-infected foetal lymphocytes co-cultivated with T cells from IM donors or from control adult donors seronegative or seropositive for EB virus. (a) Foetal cells infected and exposed only to buffer before addition of T cells; (b) foetal cells infected and exposed to papain in buffer before addition of T cells. Where the inhibitory effect of T cells was so marked that even at the highest EB virus dose less than half the cultures developed transformed foci, the efficiency of outgrowth of transformed cells is shown as its maximum value by a bar with an indented head.

DISCUSSION

If cells of thymic origin in the blood of acute IM patients are mediating an immune response against EB-virus infection, these same cells should also have the capacity to inhibit the activity of the virus in cell culture systems. In the absence of a naturally permissive system supporting EB virus replication, the *in vitro* transformation of infected foetal cord blood mononuclear cells into permanent lymphoblastoid cell lines represents the most easily quantitated biological assay for this virus (Moss & Pope, 1972). A number of factors other than the initial virus dose have been found to influence the yield of transformed foci in such cultures, notably the numbers of B-lymphocytes (Schneider & Zur Hausen, 1975) and of adherent monocytic cells (Pope, Scott & Moss, 1974) initially present, but once these factors are recognized and controlled the system provides a suitable model with which to assess the *in vitro* activity of T cells from IM patients and from control donors. Throughout the present experiments, EB virus-infected foetal target cell cultures containing T cells from IM patients or control donors remained similar in appearance during the first few weeks, with gradually diminishing clumps of lymphoblastoid cells. Thereafter expanding foci of transformed cells appeared in particular cultures and confirmation of their transformed state was provided by the continued growth of these cells after sub-culture.

In the first set of experiments, T cells were added to target foetal mononuclear cell cultures between 3 and 7 days of exposure of the target cells to one of a range of doses of EB virus. At this stage recognizably transformed foci had not yet developed in any of the cultures of target cells alone, and even in those cultures given the highest virus dose initially, such foci did not appear for a further 1–2 weeks. In each of this set of experiments the outgrowth of transformed cells was not seriously affected by the addition to the cultures of T cells either from foetal cord blood or from an adult seronegative donor, except on occasions when some slight inhibitory effect was seen at the highest T-cell doses (Fig. 1, Expts 2 and 3b). This may have been due simply to the effect of undue cell crowding upon the outgrowth

of transformants, or may perhaps reflect some low grade *in vitro* stimulation of hitherto uncommitted lymphocytes into cells cytotoxic for EB virus-infected target cells co-resident in the culture (Svedmyr *et al.*, 1974). In contrast, the addition of $2{\cdot}5 \times 10^5$ to 10^6 IM-derived T cells markedly reduced the efficiency of outgrowth, so much so that on occasions even those target cultures given the highest dose of virus did not go on to yield transformed foci.

Although these results suggested that IM-derived T cells were able to recognize antigenic changes on the surface of EB virus-infected cells, it was not clear what these changes were or at what time they appeared following infection. The second set of experiments shows that inhibition of outgrowth still occurs if IM-derived T cells are added immediately after infection of target cells, at a time when the infectious cycle could not have developed to the point of expression of virus-induced surface changes. However it is known that EB viral envelope material does remain, sometimes apparently for quite long periods, on the surface of infected cells (Dölken & Klein, 1976) and this might have served as the target antigen in these experiments. That this is not the case is emphasized by the results of the final group of experiments, where papain treatment of infected foetal cells did not alter their efficiency of outgrowth when cultured alone, nor did it in any way protect these cells from the inhibitory action of IM-derived T-cells. Virus-binding studies, carried out in this laboratory using an immunofluorescence assay (Greaves *et al.*, 1975), have confirmed that papain treatment does indeed remove residual envelope material from the surface of infected foetal target cells.

These results suggest that the inhibitory action of IM-derived T cells does not depend upon any recognition of the viral envelope complex on the cell surface, nor presumably upon any recognition of the serologically-defined EB virus-determined membrane antigen, MA (Klein *et al.*, 1966) which is expressed on a variable proportion of cells in at least some transformed lines and which shows antigenic identity with a part of the envelope structure (Ernberg *et al.*, 1974). This is in accord with the results of chromium release assays in which mononuclear cell populations prepared from IM blood and depleted of complement receptor-bearing lymphocytes were cytotoxic against virtually all cell lines carrying the EB viral genome, whether or not the cells expressed MA, but not against EB virus genome-negative cell lines (Svedmyr & Jondal, 1975). Cytotoxic activity therefore appeared to be directed against a surface change common to all EB virus genome-containing cell lines, that is the lymphocyte-detected membrane antigen, LYDMA (Jondal, 1976).

The inhibitory action of IM-derived T-cells described in the present report probably reflects this same cytotoxic activity, mounted here against LYDMA-bearing cells which must appear in cultures of EB virus-infected foetal lymphocytes some time after infection but before the development of obviously transformed foci. Immune IM T-cells, added to foetal target cultures on the day of infection, must retain their cytotoxic capacity until such time as LYDMA-bearing cells appear in the culture, and this perhaps reflects an *in vitro* memory similar to that already described for human T cells in another context (Svedmyr, 1975). The range of EB virus genome-containing cell lines recognised by IM mononuclear cells (Svedmyr & Jondal, 1975) suggests that LYDMA is unlikely to be directly associated with the cells' existing histocompatibility antigens, and this conclusion is supported by the present observation that IM T-cells regularly block the outgrowth of transformed foci in cultures from randomly selected foetal donors.

The present experiments also show that T cells from healthy donors with a history of EB virus infection are usually no more inhibitory to the outgrowth of transformed foci than are T cells from seronegative donors. The significance of the result in Expt 11, where IM-derived and seropositive donor T cells showed similar inhibitory effects, is not understood. However, in three additional experiments of a similar type, one of which actually involved the same seropositive donor as was used in Expt 11, no difference could be detected between the effects of seropositive and seronegative T cells (unpublished observations). This does not deny the existence in seropositive individuals of 'memory' cells with the same immunological commitment as the T cells of IM patients, but merely emphasizes the difficulty of detecting the activity of cells which may be expected to be present in very small numbers in the total T-cell pool.

Finally, it is important to note that the consistent presence of LYDMA on EB virus-transformed cells

in no way makes this antigen exclusive to cells in the transformed state, nor does the generation of a cell-mediated response to LYDMA during IM necessarily imply the existence of EB virus-transformed cells in such individuals. Indeed LYDMA is likely to be expressed *in vivo* during IM both on those cells supporting a productive viral infection in the oropharynx (Gerber *et al.*, 1972) and on a proportion of the virus-carrying B lymphocytes present in blood and in lymphoid tissues, the nature of whose infection is the subject of some debate (Rickinson *et al.*, 1974; Rickinson, Epstein & Crawford, 1975; Dalens, Zech & Klein, 1975; Klein *et al.*, 1976). Why EB virus induces the unusually vigorous T-cell response seen during IM is not known, but it is interesting that a similar response is seen in patients with the mononucleosis-syndrome induced by cytomegalovirus, another human herpesvirus carried in circulating leucocytes (Fiala *et al.*, 1975). In both cases T cells must play a similar key role in limiting the primary viral infection.

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