Clin. exp. Immunol. (1985) **60**, 70–77.

T lymphocytes in infectious mononucleosis. II. Response *in vitro* to interleukin-2 and establishment of T cell lines

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(Accepted for publication 31 October 1984)

SUMMARY

The addition of 20% interleukin-2 (IL-2) significantly reduced the percentage of T lymphocytes dying *in vitro* after being isolated from the peripheral blood of acute infectious mononucleosis (IM) patients. Moreover, the immediate addition of 20% IL-2 to freshly isolated blood allowed IM T cell lines to be readily established from the peripheral blood of acute IM patients. Characterization of seven of these IM T cell lines showed them to be $T3^+$, $T11^+$, $T4^-$, $T9^-$ and generally $T10^-$. Over half of the lines characterized were $T8^+$. It will now be possible to re-evaluate IM T cell effector functions as previous assays of IM T cell functions may have been influenced by the presence of rapid and extensive T cell death *in vitro*.

Keywords T lymphocytes infectious mononucleosis interleukin-2 T cell lines

INTRODUCTION

Infectious mononucleosis (IM), induced by Epstein–Barr (EB) virus, is characterized by the presence of atypical mononuclear cells (Sheldon *et al.*, 1973), the detection of EB virus associated nuclear antigen (EBNA) positive B lymphocytes (Klein *et al.*, 1976) and a marked T cell lymphocytosis (Papamichael, Sheldon & Holborow, 1974). Following infection, EB virus persists for life as an apparently non-productive infection of the host B lymphocytes (Nilsson *et al.*, 1971). Such EB virus infected B cells can be readily established from cultured mononuclear cells of acute IM patients (Pope, 1967).

T lymphocytes isolated from acute IM patients have been ascribed a cytotoxic (Svedmyr & Jondal, 1975; Lipinski *et al.*, 1979) or suppressor function (Haynes *et al.*, 1979; Reinherz *et al.*, 1980a) against EBNA positive B lymphocytes and are thought to be of major importance in the self-limiting aspect of the disease. However, in a previous report (Moss *et al.*, 1985) we have shown that a large proportion of acute IM T cells rapidly die, by apoptosis, when cultured *in vitro*. In view of the death of IM T cells *in vitro* we suggested that the effector functions of IM T cells may need to be re-evaluated, since IM T cell effector functions have generally been defined using *in vitro* assays conducted over periods ranging from 15 h to 7 days, by which time many (if not all) of the T cells have died.

Before a re-evaluation of the effector function of IM T cells could be carried out, it was necessary to minimize the degree of cell death occurring in IM T cell cultures. In the present study we describe the effect of interleukin-2 (IL-2) on the survival of T cells isolated from acute IM patients and report on the establishment of T cell lines from such patients.

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MATERIALS AND METHODS

Culture media. Cells were cultured in RPMI 1640 (Commonwealth Serum Laboratories, Victoria) supplemented with 10% heat-inactivated human serum (HS), penicillin (100 iu/ml) and streptomycin (100 μg/ml). Where indicated, the basic medium was modified by the addition of 20% crude IL-2 (supernatant from MLA 144 T cell cultures, kindly provided by Dr A. Hapel, John Curtin School of Medical Research, Canberra).

Lymphocyte donors. Mononuclear cells were isolated from the peripheral blood of acute IM patients. The clinical diagnosis of IM was confirmed by the presence of atypical mononuclear cells (>15% of the total lymphocyte population), the demonstration of a positive differential Paul–Bunnel test, the presence of a marked T cell lymphocytosis (isolation of $>2{\cdot}5\times10^6$ lymphocytes/ml of blood) and the detection of IgM antibody to EB virus (titre >1/20).

Lymphocyte separation. Unfractionated peripheral blood mononuclear (UM) cells were isolated from heparinized blood by isopycnic centrifugation on Ficoll/Paque (Pharmacia) as described previously (Böyum, 1968). UM cells were mixed with 2-aminoethylisothiouronium bromide (AET) treated sheep erythrocytes and the E rosetting (T) cell population isolated essentially as previously described (Kaplan & Clark, 1974). In some cases, the heparinized blood was added immediately on collection to an equal volume of culture medium containing 40% IL-2, and all solutions used in the subsequent lymphocyte separation contained 20% IL-2.

Characterization of T cell markers. T cell markers were assayed by means of T monoclonal antibodies (MoAb) (Ortho-mune, Ortho Diagnositic Systems, Raritan, New Jersey, USA) and fluorescein labelled goat anti-mouse IgG antibody (Kirkegaard and Perry Laboratories, Gaithersburgh, Maryland, USA) in conjunction with a Becton-Dickinson FACS IV cell sorter, according to manufacturer's instructions (Ortho-mune). The assay was carried out on the cultured UM cell population immediately on isolation from an acute IM patient (time = 0) and then carried out again on T cells separated from the UM cultured population at various times (1, 2, 5, 8 days). The percentage of T MoAb positive cells in the UM population (time = 0) was adjusted according to the number of E rosetting cells (T cells) in this UM population. The assays were carried out on some of the established IM T cell lines and on a number of previously characterized cell lines with known T markers (Misko *et al.*, 1984).

Markers of cell death. Certain histological changes associated with cell death, classically designated as nuclear pyknosis and karyorrhexis, occur in both necrosis and apoptosis (Wyllie, Kerr & Currie, 1980; Searle, Kerr & Bishop, 1982). Necrotic cells also show karyolysis and swelling of the cytoplasm which eventually loses its basophilia, and cell boundaries become indistinct (Trump & Arstila, 1975; Wyllie *et al.*, 1980; Searle *et al.*, 1982). Apoptotic cells appear to be condensed, typically with intensely eosinophilic cytoplasm which frequently shows surface protrusion. These protuberances separate to form roughly spherical bodies sometimes containing basophilic nuclear fragments (Wyllie *et al.*, 1980; Searle *et al.*, 1982).

One commonly employed marker of cell death, dye exclusion, is a poor indicator of cell death by apoptosis. Apoptotic cells exclude dye until they undergo secondary disintegration (Sheridan, Bishop & Simmons, 1981).

Apoptosis Smears were air dried, fixed with methanol and stained with haematoxylin & eosin. The percentage of cell death by apoptosis was estimated from counts of 500 cells in each smear using an oil immersion objective. Where it was impossible to delineate apoptotic bodies in a compact cluster, the cluster was scored as one: the counts were thus only approximate.

Dye excluding cells A twenty microlitre suspension was diluted with 20 μl 0·5% aqueous trypan blue and the stained and non-stained cells were counted in a haemocytometer.

Studies on the response to IL-2. UM cells and T cells from acute IM patients were isolated either in the presence or absence of 20% IL-2 and subsequently maintained in culture media supplemented with 20% IL-2 or in unsupplemented media, respectively. T cell markers were assayed as above. At various times after seeding, the percentage of dye excluding cells and of apoptotic cells were determined in each culture and T cell markers were assayed as above.

Establishment of IM T cell lines. IM T cells were isolated in the presence of 20% IL-2 and seeded into Linbro (2 ml) wells at $2{\cdot}5\times10^5$ cells/ml in culture medium supplemented with 20% IL-2.

cultures were incubated at 36°C in a humidified atmosphere of 5% CO_2 in air. Culture medium was replaced approximately every 7 days until proliferation was evident at which time the cultures were split 1:2 with new culture medium supplemented with 20% IL-2 and with 10^4 irradiated (8,000 rad) autologous LCL/ml (Moss & Pope, 1972). Subsequent splits were made approximately every 7 days with culture media supplemented with 20% IL-2 and irradiated autologous LCL.

Studies on established IM T cell lines. Some IM T cell lines, that had been proliferating for at least 4 weeks, were assayed as above. In some cases, growth kinetics were also studied over a further period of 3 weeks. In other studies, proliferating IM T cells from an established T cell line were washed in unsupplemented medium and seeded into Linbro wells (2 ml) at $2{\cdot}5 \times 10^5$ cells/ml either in culture medium supplemented with 20% IL-2 or in unsupplemented medium. At various times after seeding the percentage of dye excluding cells and of apoptotic cells were determined in each culture.

RESULTS

Response of IM T cells to IL-2 The percentage of cell death by apoptosis in individual cultures of IM T cells isolated from four acute IM patients and incubated either in medium supplemented with 20% IL-2 or in unsupplemented medium is shown in Fig. 1. Twenty-four hours after isolation the mean percentage apoptosis was 65% in cultures incubated in the absence of IL-2, while it was 37% in cultures containing 20% IL-2.

The number of dye excluding cells/ml in cultures of T cells, from one acute IM patient, isolated and incubated either in medium supplemented with 20% IL-2 or in unsupplemented medium is shown in Fig. 2. Eight days after isolation there had been a greater than 40-fold decrease in the number of dye excluding cells/ml in cultures of T cells isolated and incubated in the absence of IL-2, while the number of dye excluding cells/ml in cultures containing IL-2 approached that in the freshly isolated T cell cultures. The number of dye excluding cells in the IL-2 free cultures is likely to

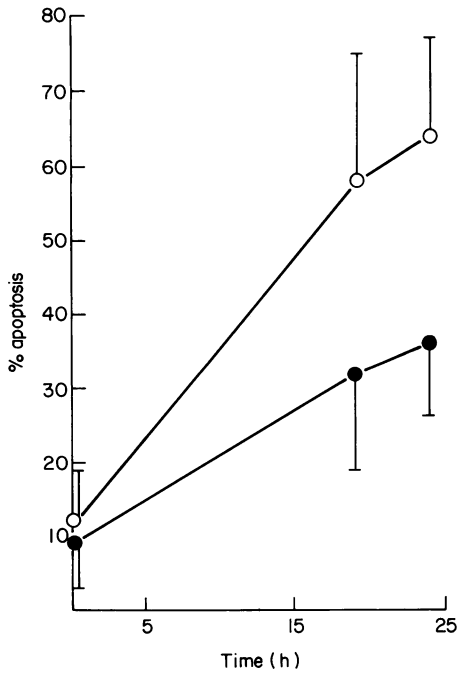

Fig. 1. The percentage of cell death by apoptosis in cultures of T cells, from acute IM patients, isolated and incubated either in medium supplemented with 20% IL-2 (●) or in unsupplemented medium (○). Results are expressed as mean ±s.d. of cultures from four patients.

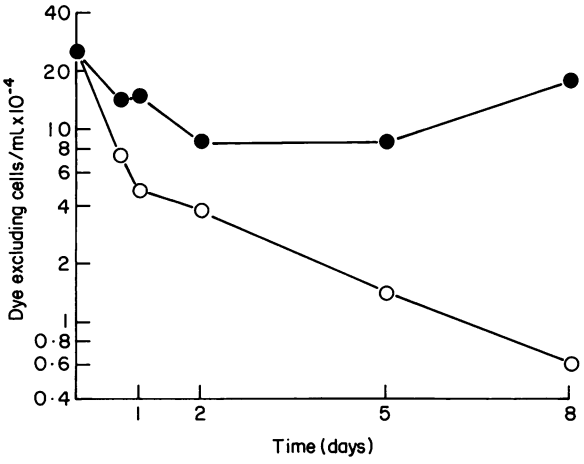

Fig. 2. The number of dye excluding cells/ml in cultures of T cells, from an acute IM patient, isolated and incubated either in medium supplemented with 20% IL-2 (●) or in unsupplemented medium (○).

be an overestimation of the number of viable cells, as apoptotic cells continue to exclude dye until they undergo secondary disintegration. For example, in the IL-2 free cultures 24 h after isolation 32% of the cells did not exclude dye but approximately 70% of the cells were undergoing apoptosis. However, it was not possible to accurately determine the number of apoptotic cells in cultures incubated longer than 24 h as the extensive secondary degeneration of the apoptotic cells made them difficult to recognise.

Sequential changes in the profile of T cell populations isolated from an acute IM patient is shown Fig. 3. Where the T cells were isolated and maintained in the presence of 20% IL-2, there was an initial decrease in the percentage of T cells that were positive to all the MoAb following their separation from the UM cell population. Subsequently there was a further decrease, over 8 days, in the percentage that were $T3^+$ and $T8^+$. However, there was a steady increase in the percentage that were $T11^+$. Where the T cells were isolated and maintained in the absence of IL-2 there was a greater initial decrease in the percentage of T cells that were $T3^+$, $T8^+$ and $T11^+$, but there was no subsequent decrease in the percentage that were $T3^+$ or $T8^+$. There was a subsequent increase in the percentage that were $T11^+$ but this ceased within 5 days.

The significance of T MoAb profiles of IL-2 free cultures was unclear as the percentage of cell death in such cultures was high. The percentage of dye excluding cells in the IL-2 free cultures, characterized in Fig. 3, was 45% after 2 days, 25% after 5 days and 15% after 8 days of culture. This was likely to be an overestimation of the number of viable cells as 70% of the cells were undergoing apoptosis within 24 h in such cultures. In contrast, the percentage of dye excluding cells in cultures of the same T cell population containing IL-2 was 85% after 8 days of culture. The IM T cells characterized in Fig. 3 are from the same cultures described in Fig. 2 and gave rise, in the presence of 20% IL-2 to the established IM T cell line described in Table 1 as patient 7 which was $T3^+$, $T8^+$ and $T11^+$.

Studies on established IM T cell lines

From 32 acute IM patients, 20 T cell lines have been established which have grown, to date, for up to 5 months. When IL-2 was present during the isolation of the T cells, seven lines were established from eight patients. If IL-2 was not added until after T cell isolation, 13 lines were established from 24 patients, but when IL-2 was not added at any stage no T cell lines were established. It was not possible to establish T cell lines from controls even with the addition of IL-2.

The characterization by MoAb of some of the established IM T cell lines is given in Table 1. The percentage of T cells that was $T3^+$ and $T11^+$ was high, while the percentage that was $T4^+$ or $T9^+$ was very low, in all the cell lines. The percentage T cells that was $T8^+$ or $T10^+$ varied considerably among the lines.

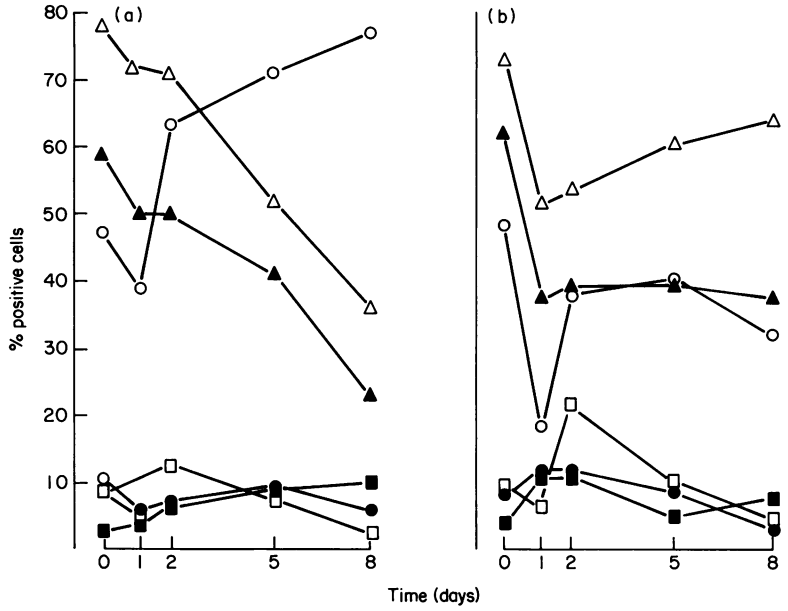

Fig. 3. Sequential changes in the percentage of cells reacting with T MoAb in T cell cultures, from an acute IM patient, isolated and incubated either in medium supplemented (a) with 20% IL 2 or (b) in unsupplemented medium. MoAb. T3 (△), T4 (□), T8 (▲), T9 (■), T10 (●) and T11 (○).

Table 1. Cell surface characterization of established IM T cell lines

	Time in culture (weeks)		% cell reacting with MoAb						
Patient	Total*	At assay	T3	T4	T8	T9	T10	T11	Control
1	24	7	54·0	5·2	51·2	—†	—	—	1·0
2	22	7	80·8	5·4	25·1	—	—	—	2·1
3	18	5	84·9	1·1	6·8	2·8	1·8	95·3	1·2
4	17	4	81·7	6·9	10·1	1·3	1·6	94·1	0·8
5	15	7	87·9	4·6	88·6	8·7	26·2	90·9	1·9
6	10	10	85·7	1·3	8·0	6·9	3·8	86·5	0·8
7	7	7	71·0	4·6	60·2	11·7	6·8	82·0	2·4

* At time of preparation of table.
† Not tested.

Fig. 4 shows the growth kinetics of two of the established IM T cell lines. The lines generally grew slowly under these culture conditions and often showed 'cyclic' patterns of growth and cell death. With the two lines shown there was an eight-fold increase within 3 weeks.

Following the removal of IL-2 from cultures of an established T cell line and subsequent incubation in unsupplemented medium, the percentage of cell death in these cultures, determined by counting apoptotic or dye excluding cells, increased rapidly with time, whereas it remained low in cultures containing 20% IL-2 (Fig. 5). By 2 days, a large number of apoptotic cells in IL-2 deprived cultures were undergoing secondary disintegration and it was not possible to make further counts.

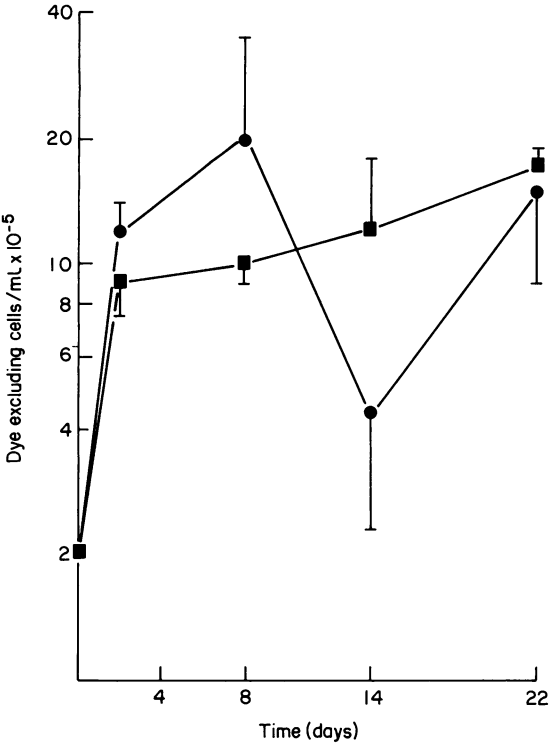

Fig. 4. Growth kinetics of two established IM T cell lines. Results are expressed as mean ± s.d. of triplicate wells.

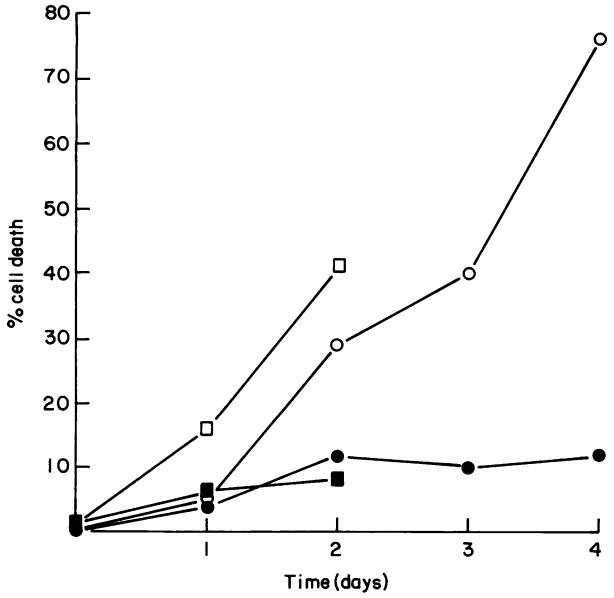

Fig. 5. The percentage of cell death, determined by counting apoptotic (■, □) or dye excluding (●, ○) cells, in cultures of an established IM T cell line following the removal of medium containing IL-2 and subsequent incubation either in unsupplemented medium (○, □) or in medium supplemented with 20% IL-2 (●, ■).

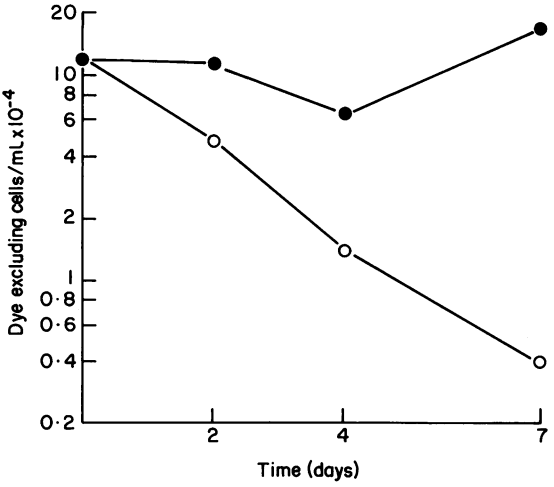

Fig. 6. The number of dye excluding cells/ml in cultures of an established IM T cell line following the removal of medium containing IL-2 and subsequent incubation either in unsupplemented medium (○) or in medium supplemented with 20% IL-2 (●).

However, it again appeared that the number of dye excluding cells was an overestimation of the number of viable cells. Greater than 75% of the cells in IL-2 deprived cultures did not exclude dye within 4 days of the removal of IL-2. Moreover the number of dye excluding cells in IL-2 deprived cultures had decreased 30-fold within 7 days of the removal of IL-2, whereas the number of dye excluding cells in IL-2 supplemented cultures had increased by 50% within this time (Fig. 6).

DISCUSSION

The rapid death *in vitro* of T lymphocytes isolated from the peripheral blood of acute IM patients was significantly reduced by the addition of IL-2. For IL-2 to achieve its maximal effect it had to be added to IM peripheral blood immediately on collection, to all solutions used in the isolation of mononuclear cells and in the separation of T cells and to subsequent culture medium. For example, the percentage of cell death in cultures of IM T cells isolated and maintained in the presence of 20% IL-2 was approximately 37%, 24 h after isolation, whereas it was approximately 65% in cultures of IM T cells from the same patient isolated and maintained for the same period in the absence of IL-2.

Moreover, the immediate addition of IL-2 allowed IM T cell lines to be readily established from the peripheral blood of acute IM patients. When 20% IL-2 was immediately added to acute IM peripheral blood and maintained during subsequent T cell isolation and culture, long-term T cell lines were established from greater than 85% of the patients. If IL-2 was not added until after T cell isolation, IM T cell lines were established from approximately 50% of acute IM patients, but when IL-2 was absent at all stages no IM T cell lines were established. It was not possible to establish T cell lines from controls even with the addition of IL-2. Where the T MoAb profile of a freshly isolated IM T cell population was compared with the IM T cell line established from this population, the percentage of cells reacting with various T MoAb was similar except for a consistant increase in the percentage $T11^+$.

As expected, all of the established IM T cell lines that we have characterized with T MoAb were $T3^+$ (all of seven) and, where tested, $T11^+$ (all of five). All the lines appeared to be $T4^-$ (all of seven), suggesting that they do not contain significant numbers of inducer/helper T cells. Over half of the lines (four of seven) were $T8^+$, indicating that they contained suppressor/cytotoxic T cells. Of these lines only one was $T10^+$ suggesting that it was derived from early haematopoietic stem cells (Reinherz *et al.*, 1980; Terhorst *et al.*, 1981).

IL-2 support of the growth of T cells isolated from acute IM patients was not unexpected as IL-2

has been shown to support the growth of activated T lymphocytes (Smith, 1980). The observation that the lines that were characterized were all $T9^-$ and generally $T10^-$ indicated that these lines were derived from a mature T cell population (Reinherz *et al.*, 1980; Terhorst *et al.*, 1981).

That the withdrawl of IL-2 from these IM T cell lines enhanced cell death (by apoptosis) was also not unexpected. The normal involution and pathological atrophy of adult tissue, including lymphoid tissue, has been shown to involve the enhancement of apoptosis (see Wyllie *et al.*, 1980; Searle *et al.*, 1982); for example, in the normal involution of endocrine-dependent tissues following changes in blood levels of trophic hormones.

Now that we can increase the survival in culture of T cells from acute IM patients and have established T cell lines from such patients it will be possible to re-evaluate IM T cell effector functions as previous assays of IM T cell functions may have been influenced by the presence of rapid and extensive T cell death.

We wish to thank Dr J. Irwin and Sr M. Briggs, Student Health Service, University of Queensland, for collecting blood from patients. This work was supported by grants from the National Health and Medical Research Council of Australia and the Queensland Cancer Fund.

REFERENCES

BÖYUM, A. (1968) Isolation of mononuclear cells and granulocytes from human blood. *Scand. J. clin lab. Invest.* **21 (suppl. 97),** 77.

DE WAELE, M., THIELEMANS, C. & VAN CAMP, B.K.G. (1981) Characterization of immunoregulatory T cells in EBV-induced infectious mononucleosis by monoclonal antibodies. *N. Engl. J. Med.* **304,** 460.

HAYNES, B.F., SCHOOLEY, R.T., PAYLING-WRIGHT, C.R., GROUSE, J.E., DOLIN, R. & FAUCI, A.S. (1979) Emergence of suppressor cells of immunoglobulin synthesis during acute Epstein–Barr virus-induced infectious mononucleosis. *J. Immunol.* **123,** 2095.

KAPLAN, M.E. & CLARK, C. (1974) An improved rosetting assay for detection of human T lymphocytes. *J. Immunol. Meth.* **5,** 131.

KLEIN, G., SVEDMYR, E., JONDAL, M. & PERESON, P. (1976) EBV-determined nuclear antigen (EBNA)-positive cells in the peripheral blood of infectious mononucleosis patients. *Int. J. Cancer*, **17,** 21.

LIPINSKI, M., FRIEDMAN, W.H., TURSZ, T., VINCENT, C., PIOUS, D. & FELLOWS, M. (1979) Absence of allogeneic restriction in human T cell-mediated cytotoxicity to Epstein–Barr virus-infected target cells. Demonstration of an HLA-linked control at the effector level. *J. exp. Med.* 150, 1310.

MISKO, I.S., POPE, J.H., HUTTER, R., SOZYNSKI, T.D. & KANE, R.G. (1984) HLA-DR-antigen-associated restriction of EBV-specific cytotoxic T-cell colonies. *Int. J. Cancer*, **33,** 239.

MOSS, D.J., BISHOP, C.J., BURROWS, S.R. & RYAN, J.M. (1985) T lymphocytes in infectious mononucleosis. I. T cell death *in vitro*. *Clin. exp. Immunol.* **59,** 61.

MOSS, D.J. & POPE, J.H. (1972) Assay of the infectivity of Epstein–Barr virus by transformation of human leukocytes *in vitro*. *J. gen. Virol.* **17,** 233.

NILSSON, K., KLEIN, G., HENLE, W. & HENLE, G. (1971) The establishment of lymphoblastoid cell lines from adult and foetal human lymphoid tissue and its dependence on EBV. *Int. J. Cancer*, **8,** 443.

PAPAMICHAIL, M., SHELDON, P.J. & HOLBOROW, E.J. (1974) T- and B-cell subpopulations in infectious mononucleosis. *Clin. exp. Immunol.* **18,** 1.

POPE, J.H. (1967) Establishment of cell lines from peripheral leucocytes in infectious mononucleosis. *Nature*, **216,** 810.

REINHERZ, E.L., KUNG, P.L., GOLDSTEIN, G., LEVEY, R.H. & SCHLOSSMAN, S.F. (1980a) Discrete stages of human intrathymic differentiation. Analysis of normal thymocytes and leukemic lymphoblast of T-cell lineage. *Proc. Natl. Acad. Sci. USA*. **77,** 1588.

REINHERZ, E.L., O'BRIEN, C., ROSENTHAL, P. & SCHLOSSMAN, S.F. (1980b) The cellular basis for viral-induced immunodeficiency: analysis by monoclonal antibodies. *J. Immunol.* **125,** 1269.

SEARLE, J., KERR, J.F.R. & BISHOP, C.J. (1982) Apoptosis. *Pathol. Ann.* **17,** 229.

SHERIDAN, J.W., BISHOP, C.J. & SIMMONS, R.J. (1981) Biophysical and morphological correlates of kinetic change and death of a starved human melanoma line. *J. Cell Sci.* **49,** 119.

SMITH, K.A. (1980) T cell growth factor. *Immunol. Rev.* **51,** 338.

SVEDMYR, E. & JONDAL, M. (1975) Cytotoxic effector cells specific for B cell lines transformed by Epstein–Barr virus are present in patients with infectious mononucleosis. *Proc. Natl. Acad. Sci. USA*. **72,** 1622.

TERHORST, C., VAN AGTHOVEN, A., LECLAIR, K., SNOW, P., REINHERZ, E. & SCHLOSSMAN, S. (1981) Biochemical studies of the human thymocyte cell-surface antigens T6, T9 and T10. *Cell*, **23,** 771.

TRUMP, B.F. & ARSTILA, A.U. (1975) Cellular reaction to injury. In *Principles of Pathobiology, 2nd edn.* (ed by M. F. Lavia & R. B. Hill) pp. 9–96. Oxford University Press, New York.

WYLLIE, A.H., KERR, J.F.R. & CURRIE, A.R. (1980) Cell death: the significance of apoptosis. *Int. Rev. Cytol.* **68,** 251.