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The effect of leucyl-leucine methyl ester on proliferation and Ig secretion of EBV-transformed human B lymphocytes

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SUMMARY

The selective cytotoxicity of the lysosomotropic methyl esters of leucine or its lysosomal condensation product leucyl-leucine has been used to investigate the effect of cytolytic cells on the clonal outgrowth, cellular proliferation and antibody secretion of Epstein–Barr virus (EBV)-transformed human B cells. Large granular lymphocytes (LGL), monocytes, and a subset of T cells ($CD8/CD11^+$) were permanently eliminated by the ester treatment. These lysosome-rich cells severely inhibit the clonal outgrowth of EBV-infected B cells, as determined by Poisson distribution calculations. Furthermore, leucyl-leucine methyl ester-treated and EBV-infected lymphocytes showed a significant increase in proliferative capability as well as immunoglobulin (Ig) production (three to 11 times) compared to non-treated but similarly infected lymphocytes. Since the effect of leucyl-leucine methyl ester treatment was also detectable in low-density (100 B cells/well) cultures, the suppression was unlikely to be exerted by EBV-specific T-cell clones, but pointed rather to the natural killer (NK) cells as effectors.

INTRODUCTION

Human peripheral blood mononuclear cells (PBMC) have the ability to execute a variety of cell-mediated cytolytic and suppressive functions, known to be mediated partly by large granular lymphocytes (LGL), cytotoxic T lymphocytes, monocytes (Kleinerman & Herberman, 1984), or mixed lymphocyte culture (MLC)-activated NK-like cells (Seeley *et al.*, 1979). These cells also regulate the activation and differentiation of B lymphocytes and NK cells have been suggested to have multiple suppressive activities on antibody secretion by acting via T-helper cells (Arai *et al.*, 1983), on accessory cells (Abruzzo & Rowley, 1983), or directly on B cells (Nabel, Allard & Cantor, 1982; Mason *et al.*, 1988).

The lysosomotropic methyl ester of L-leucine (Leu-OMe) or its lysosomal condensation product L-leucyl-L-leucine methyl ester (LeuLeu-OMe) have a selective toxic effect on lysosome-rich cytolytic cells (Thiele & Lipsky, 1985b). LeuLeu-OMe has been shown functionally to remove monocytes (Thiele, Kurosaka & Lipsky, 1983), cytotoxic T-lymphocyte precursors and effectors (Thiele & Lipsky, 1986), NK cells (Thiele & Lipsky, 1985a), and we have recently demonstrated the regulatory effect exerted by Leu-OMe-sensitive cells on *in vitro* immunization of peripheral blood lymphocytes for the production of human monoclonal antibodies (Borrebaeck, Danielsson & Möller, 1987, 1988; Borrebaeck, 1988a). The antigen-specific activation of B cells was completely abrogated by the cytolytic cell compartment of human peripheral blood lymphocytes (Borrebaeck *et al.*, 1988).

LGL have also been suggested to be involved in the down-regulation of growth (Shope & Kaplan, 1979) and antibody synthesis (Kuwano *et al.*, 1986) of Epstein–Barr virus (EBV)-infected human B cells, independently of the EBV-immune status of the donor. In contrast, suppression of the number of Ig-secreting cells exerted by cytotoxic T cells depends on the serological status of the donor (Tosato, Magrath & Blaese, 1982). To gain further insight into the regulation of EBV-infected human B cells and the establishment of immortal cell lines, an important technology in the production of human monoclonal antibodies, we investigated the effect of LeuLeu-OMe-sensitive cytolytic cells on the clonal outgrowth, cellular proliferation and antibody secretion of EBV-transformed human B cells.

Abbreviations: CD, cluster of differentiation; EBV, Epstein–Barr virus; LeuLeu-OMe, L-leucyl-L-leucine methyl ester; Leu-OMe, L-leucine methyl ester; LGL, large granular lymphocytes; MLC, mixed lymphocyte culture; NK, natural killer; NS, natural suppressor; PBMC, peripheral blood mononuclear cells; PWM, pokeweed mitogen; SRBC, sheep red blood cells.

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MATERIALS AND METHODS

Culture medium

L-glutamine (4 mM), 1% (v/v) 100 times non-essential amino acids, and gentamycin (50 μg/ml) were used to supplement RPMI-1640 (Flow Laboratories Inc., Rickmansworth, Herts).

Fetal calf serum (Gibco Ltd, Paisley, Renfrewshire, U.K.) or human ABO serum (University Hospital Blood Bank, Lund) was also added to the supplemented medium. Human serum was heat inactivated at 56° for 30 min prior to use.

Cell separation

Buffy coats from EBV-seropositive blood donors were obtained from the University Hospital Blood Bank (Lund). Peripheral blood mononuclear cells (PBMC) were prepared by density centrifugation on Ficoll–Paque (Pharmacia Fine Chemicals, Uppsala, Sweden). The lymphocytes were in some experiments further separated into T- and B-cell-enriched fractions by rosetting using AET (Sigma Chemical Co., St Louis, MO)-treated sheep red blood cells (SRBC) (Callard & Smith, 1981). SRBC-rosetted (containing 90–95% $CD3^+$ T cells and 1–2% $CD22^+$ B cells) and non-rosetted cells are referred to below as T and B cells respectively, although, in some experiments, as described in the text, B cells have been enumerated using specific cell-surface markers and flow cytometry.

Treatment with Leu-OMe and LeuLeu-OMe

Mononuclear cells were treated with L-leucine methyl ester hydrochloride (Leu-OMe) (Sigma Chemical Co.) or with L-leucyl-L-leucine methyl ester hydrobromide (LeuLeu-OMe) (Bachem Feinchemikalien AG, Budendorf, Switzerland) using a modification of a previously described procedure (Borrebaeck *et al.*, 1988). Briefly, PBMC isolated by density centrifugation were incubated with freshly prepared 2·5 mM Leu-OMe or 0·25 mM LeuLeu-OMe in serum-free RPMI-1640, for 40 and 15 min, respectively, at room temperature. The cells were then washed three times with culture medium containing 2% human serum and cultured in supplemented RPMI-1640, containing 10% human serum. LeuLeu-OMe is the monocytic, lysosomal condensation product of Leu-OMe and responsible for the observed cytotoxicity (Thiele & Lipsky, 1985a, 1986). The direct use of LeuLeu-OMe instead of Leu-OMe obviates the dependence on the lysosomal condensation reaction.

In vitro *stimulation*

A 6-day *in vitro* stimulation was performed according to a recently described *in vitro* immunization protocol (Borrebaeck *et al.*, 1987, 1988), although no specific antigen was added. During this culture period the supplemented medium also contained 50 μM 2-mercaptoethanol, 5 U IL-2/ml and 25% (v/v) of a supernatant from irradiated (2000 rads) human peripheral T cells stimulated with PWM (10 μg/ml, generously supplied by Dr J. Börjesson, Department of Clinical Chemistry, Helsingborg Hospital, Sweden) (Danielsson, Möller & Borrebaeck, 1987).

Infection of lymphocytes with EBV

Supernatant from the EBV-producing marmoset cell line B95-8 was kindly provided by Dr A. Rosén (Karolinska Institute, Stockholm, Sweden). It was filtered through a 0·45 μm sterile filter, stored at 4° and used without any further treatment. Prior to infection with EBV, PBMC were washed twice with serum-free medium. Cells were then infected for 2 hr at 37° with occasional stirring, using 1 ml of EBV-containing supernatant per 10^7 unseparated PBMC or per 10^6 B cells. B cells were defined by monoclonal antibodies against CD21 (CR2) and CD22 (Leu 14) (Becton-Dickinson, Mountain View, CA). Infected cells were washed twice with supplemented medium, containing 10% fetal calf serum. Finally, cells were seeded in 96-well microtitre plates together with feeder cells [10^4 irradiated (3000 rads) PBMC/well] in supplemented RPMI-1640, containing 10% fetal calf serum.

Proliferation assay

PBMC or LeuLeu-OMe-treated PBMC were infected with EBV and seeded at 100, 500 and 2500 B cells ($CD22^+$) per microtitre well, together with 10^4 irradiated (3000 rads) PBMC as feeder cells. This irradiation abolished all NK activity, as determined by cytolytic effect on K562 cells. Proliferation was determined by incorporation of [methyl-^{3}H]thymidine (Amersham International Ltd, Amersham, Bucks, U.K.; 5 Ci/mmol). The pulse period was 6 hr, using 1 μCi/well. Half of the culture medium (100 μl/well) was exchanged on Days 16 and 30. The immunoglobulin content was measured on Day 16. When the effect of autologous PBMC and T cells was tested on proliferation and immunoglobulin production, the medium was changed on Day 19 and the immunoglobulin content was determined.

Analytical procedures

Flow cytometry analysis was performed on a FACStar Plus cytofluorograph (Becton-Dickinson). Cell surface markers were defined by monoclonal antibodies against CD4 (Leu 3), CD8 (Leu 2), Leu 7, CD16 (Leu 11), CD11 (Leu 15), CD21 (CR2), CD22 (Leu 14) and CD45R (Leu 18), obtained from Becton-Dickinson, or by monoclonal antibodies against CD14 (Mo2) and CD20 (B1) obtained from Coulter Electronics Ltd (Luton, Beds, U.K.).

The immunoglobulin (IgM and IgG) content was determined by a standard sandwich ELISA. Briefly, 50 ng/well of affinity-purified, isotype-specific antibodies (Zymed Laboratories Inc., San Fransisco, CA), diluted in 50 mM sodium carbonate buffer, pH 9·5, were coated in 96-well microtitre plates. Samples, Ig-standard (Serum calibrator, Dakopatts A/S, Glostrup, Denmark), and horseradish peroxidase conjugated to isotype-specific antibodies (40 ng/well) (Zymed Laboratories Inc.) were all diluted in 10 mM sodium phosphate buffer, pH 8·0, containing 0·5 M sodium chloride and 0·1% Tween 20. Ortophenylenediamine (67 μg/well) was used as enzyme chromogen. All washing steps were performed using 0·15 M sodium chloride, containing 0·05% Tween 20.

RESULTS

Effect of Leu-OMe or LeuLeu-OMe treatment on PBMC

To investigate the effect of the methyl esters of leucine or leucyl-leucine on different subpopulations of human PBMC, a number of different cell-surface markers was determined before and after ester treatment, using a cytofluorimeter. Human peripheral lymphocytes were treated with Leu-OMe or LeuLeu-OMe and the cell-surface markers were measured after an over-night culture in supplemented medium, containing 10% human serum. Lysosome-rich cells disappeared quantitatively after the treatment, with apparent equal efficiency using the methyl ester of leucine or the dipeptide (Table 1). It is also evident that the relative number of $CD4^+$ helper T cells increased somewhat after the treatment, due to the removal of lysosome-rich cells, and that the B-cell number was similar to untreated PBMC. To

Table 1. The effect of Leu-OMe and LeuLeu-OMe on human peripheral lymphocytes, measured on Day 0. The values are given as the percentage of total number of cells counted

Donor no.	1		2			3			4		
Treatment	—	L	—	L	LL	—	L	LL	—	L	LL
Marker											
Leu 7	16·3	0·5	2·0	0·5	0·3	2·1	0·1	0·3	4·7	0·1	0·3
CD16	4·0	0·1	0·4	0·1	0·1	5·3	0·1	0·3	7·5	0·4	0·3
CD14	20	0·2	11·0	0·4	0·2	ND	ND	ND	ND	ND	ND
CD8	11	11	28	28	25	33	32	29	34	29	24
CD4	46	53	35	52	53	62	66	73	46	62	64
CD20	8·3	13	18	16	17	8·5	5·9	6·3	17	14	14
CD45R	48	43	68	64	70	ND	ND	ND	ND	ND	ND
CD4/CD45R	10	13	18	27	30	49	51	54	29	39	40
CD8/CD11	6·0	0·0	6·0	ND	0·0	ND	ND	ND	ND	ND	ND

L, L-leucine methyl ester; LL, L-leucine-leucine methyl ester; ND, not determined.

Leu 7 reacts predominantly with natural killer cells and a subset of T cells; CD16 defines the Fc receptor on natural killer cells and neutrophilic granulocytes; CD14 defines monocytes; CD20 defines B cells; CD45R defines a 220,000 MW antigen found on NK cells, B cells and T cells; CD4/CD45R defines suppressor-inducer T-helper cells.

Table 2. The effect of LeuLeu-OMe on human peripheral lymphocytes, measured on Day 0 and after *in vitro* stimulation on Day 6. The values are given as percentage of total number of cells counted

Donor no.	1		2		3		4	
Day	0	6	0	6	0	6	0	6
Marker								
Leu 7	0·5	0·3	0·3	0·5	0·3	0·5	0·2	0·3
CD16	0·1	0·2	0·1	0·2	0·3	0·2	0·5	0·2
CD8/CD11	0·3	1·1	0·7	1·1	ND	ND	ND	ND
CD14	ND	ND	ND	ND	0·2	0·1	0·1	0·1
CD20	13·4	14·5	16·9	ND	6·3	11·6	14·1	18·0
CD21	16·0	18·0	17·0	18·0	ND	ND	ND	ND
CD22	12·5	14·5	14·4	17·2	ND	ND	ND	ND

CD21 defines the EBV receptor.

investigate if the removal of large granular lymphocytes (NK/NS cells), monocytes, and a subset of T cells (CD8/CD11$^+$) was transient, the treated cells were cultured for 6 days in the *in vitro* immunization formate (Borrebaeck *et al.*, 1988), although no specific antigen was added. The cytolytic cells did not re-occur in culture after 6 days (Table 2). It seemed to be a permanent elimination since no re-occurrence was detected even after prolonged culture periods (>30 days) (L. Danielsson, R. Carlsson and C. A. K. Borrebaeck, unpublished results). Its worthwhile noting the increase in CD21- and in CD20-positive cells after an *in vitro* stimulation of Leu-OMe- or LeuLeu-OMe-treated human PBMC (Table 2). The increase in CD20-positive cells is in sharp contrast to *in vitro* stimulations of untreated cells where the number of CD20 positive cells normally decreased by one order of magnitude (L. Danielsson, R. Carlsson and C. A. K. Borrebaeck, unpublished results).

Table 3. Calculation of B-cell transformation frequency mediated by EBV-infection of peripheral blood lymphocytes with or without LeuLeu-OMe pretreatment, as determined by Poisson distribution calculations 21 days after infection ($n = 120$)

Donor no.	Cell pretreatment	B cells/well	Average no. of clones*/well
1	None	100	0·34
	None	2500	0·76
	LeuLeu-OMe	100	0·86
	LeuLeu-OMe	2500	>3†
2	None	2500	1·6
	LeuLeu-OMe	100	1·5
	LeuLeu-OMe	500	>3†
	LeuLeu-OMe	2500	>3†

* Wells containing clones of approximately 50 cells or more were scored as positive.

† Experiments giving >95% growth positive wells.

Effect of Leu-Leu-OMe treatment on the clonal outgrowth of EBV-infected lymphocytes

The clonal outgrowth of EBV-infected cells was scored microscopically and evaluated by Poisson distribution calculations. It was clear that LeuLeu-OMe-treated and EBV-infected lymphocytes showed a several times higher cloning efficiency compared to cells not pre-treated with LeuLeu-OMe (Table 3). Subjective analysis of the clonal size in wells plated with treated and EBV-infected B cells showed a dramatic difference in size. The clones were both larger and more vigorously growing compared to wells plated with cells not pre-treated with LeuLeu-OMe. Furthermore, it was also evident that the cloning efficiency of

Table 4. Production of immunoglobulin isotypes by unseparated EBV-infected lymphocytes, with or without LeuLeu-OMe pretreatment. Cells were seeded at 2500 B cells/well together with 10^4 irradiated allogeneic PBMC. The frequency of B cells in each of the tested cell preparations was 10–14%. Supernatants were collected after 16 days of culture

Donor no.	Cell pretreatment	EBV infection	IgM (μg/ml)	IgG (μg/ml)
1	None	–	<0·01	0·02
	LeuLeu-OMe	–	<0·01	0·05
	none	+	0·76	0·64
	LeuLeu-OMe	+	7·6	7·0
2	None	–	0·08	0·01
	LeuLeu-OMe	–	<0·01	<0·01
	None	+	2·9	0·26
	LeuLeu-OMe	+	7·9	1·3

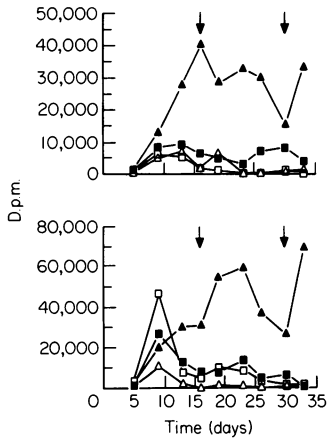

Figure 1. Proliferation of uninfected (open symbols) or EBV-infected (closed symbols) PBMC as measured by [^{3}H]thymidine incorporation. The peripheral lymphocytes were either treated (△) or not treated (□) with LeuLeu-OMe. Arrows indicate a change in culture medium. Cells were originally seeded at 2500 B cells ($CD22^+$)/well together with 10^4 irradiated PBMC as feeder cells. Each value is the mean of six samples. The two diagrams represent cells from different blood donors.

untreated EBV-infected B cells did not show a direct relationship to the number of plated B cells; at the highest cell density the cloning efficiency was relatively low.

Effect of LeuLeu-OMe treatment on proliferation and Ig secretion of EBV-infected lymphocytes

Proliferation of lymphocytes and production of Ig after treatment with LeuLeu-OMe and subsequent EBV-infection were studied. Cells were seeded at 2500 EBV-infected B-cells ($CD22^+$) per well, together with irradiated PBMC as feeder cells. Wells seeded with LeuLeu-OMe-treated and EBV-infected lymphocytes showed a significant increase in proliferative

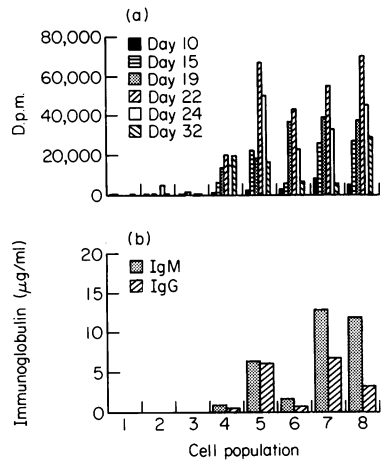

Figure 2. Proliferation (a), measured as the incorporation of [^{3}H]thymidine in DNA. Cells were originally seeded at 2500 B cells ($CD22^+$)/well together with 10^4 irradiated PBMC as feeder cells. Uninfected autologous T cells (10^4) were added in some experiments. Each value represents the mean of six samples. Concentration of immunoglobulin (IgG and IgM) (b) from cultures of uninfected or EBV-infected cells after 19 days of culture. Each value represents the mean of 42 pooled cultures. Cell populations: (1) uninfected PBMC; (2) uninfected LeuLeu-OMe treated PBMC; (3) uninfected B cells (T-cell-depleted PBMC); (4) EBV-infected PBMC; (5) EBV-infected LeuLeu-OMe-treated PBMC; (6) EBV-infected B cells (T-cell-depleted PBMC); (7) cell population 5, with an additional 10^4 uninfected T cells; (8) cell population 5, with an additional 10^4 uninfected and LeuLeu-OMe-treated T cells.

capability as well as in immunoglobulin production (3–11 times) compared to non-treated by similarly infected lymphocytes (Table 4, Fig. 1). Non-infected cells did not produce Ig to any significant extent.

Effect of T cells on the proliferation and Ig production of EBV-infected B lymphocytes

PBMC, LeuLeu-OMe-treated PBMC, and T-cell-depleted PBMC (T cells were removed by SRBC rosetting) were all infected with EBV and proliferation was recorded on Days 10, 15, 19, 22, 24 and 32, whereas Ig secretion was recorded on Day 19. It was evident that LeuLeu-OMe-treated PBMC exhibited the strongest proliferation, followed by T-cell-depleted PBMC (Fig. 2a). In comparison, untreated and EBV-infected PBMC showed a suppressed proliferation; approximately 30% of the proliferation of LeuLeu-OMe-treated PBMC. The addition of 10^4 autologous T cells/well to LeuLeu-OMe-treated PBMC suppressed the proliferation somewhat; the number of PBMC corresponded to 2500 B ($CD22^+$) cells/well. However, if the added T cells were pretreated with LeuLeu-OMe no suppression was observed and the proliferation values were similar as compared to LeuLeu-OMe-treated PBMC (Fig. 2a). Immunoglobulin (IgM and IgG) production was measured on Day 19 and here the difference between the cell populations was even more pronounced. LeuLeu-OMe-treated and EBV-infected PBMC produced 10–15 times more of both IgM and IgG compared to untreated and virus-infected PBMC (Fig. 2b). T-cell-depleted (SRBC rosetted) and EBV-infected PBMC produced only slightly more than untreated and virus-infected PBMC. However, when 10^4 T cells/well were added to the

LeuLeu-OMe-treated and EBV-infected PBMC (corresponding to 2500 B cells/well) a significant increase in IgM production was observed. This was most probably due to T-helper cell-derived growth factors, since a similar effect was observed when the number of feeder cells was increased 10-fold (data not shown). A corresponding increase in IgG production was not observed (Fig. 2b).

DISCUSSION

We have characterized the regulatory effects of lysosome-rich cell populations on the clonal outgrowth, proliferation and Ig-secretion of EBV-transformed human B cells. Quantitative and permanent elimination of cytolytic cell populations was performed easily using the lysosomotropic methyl ester of leucine or of the dipeptide leucyl-leucine. This approach was recently used for the successful design of a general *in vitro* immunization system for human peripheral blood lymphocytes (reviewed by Borrebaeck, 1988b).

The lysosome-rich cell population that is depleted from human PBMC by the ester treatment contains predominantly natural killer cells (Leu 7^+, $CD16^+$), subsets of T cells (Leu 7^+, $CD8/CD11^+$) and monocytes ($CD14^+$), all of which have been implicated in the regulation of B-cell activation. B-($CD20^+$) and T-helper ($CD4^+$) cells are apparently unaffected by the ester treatment, which supports the findings by Thiele *et al.* (1983). Furthermore, the subset of $CD8^+$ T cells, i.e. $CD8/CD11^+$ cells, that we have shown to be completely eliminated by the ester treatment, might be included in the functionally identified cell population that recently was shown to contain both precursors and effects of T lymphocytes cytotoxic against allogeneic stimulator lymphoblasts (Thiele & Lipsky 1986).

The effect of various cell populations on EBV-induced transformation of autologous B cells has been studied by several investigators. Shope & Kaplan (1979) demonstrated that outgrowth of EBV-infected lymphocytes was inhibited by E rosette-positive cells with IgG-Fc receptors and suggested this to be operationally similar to a NK effect. Recently, NK cells were also shown to partly inhibit EBV-induced Ig synthesis in short-term cultures (Kuwano *et al.*, 1986). Furthermore, EBV-specific T-cell clones from immune blood donors have been suggested by Rickinson, Moss & Pope (1979) to inhibit clonal outgrowth of infected B cells. These findings were more recently also supported by Masucci *et al.* (1983) and Bejarano *et al.* (1988), although it was pointed out by Tosato *et al.* (1982) that the frequency of EBV-specific T cells in seropositive donors is low and 2×10^6 T cells were used to suppress the appearance of reverse plaque-forming cells 12 to 14 days after infection with EBV *in vitro*. However, we have shown (Table 3) that the effect of LeuLeu-OMe treatment on the clonal outgrowth of EBV-infected B cells is clearly detectable when as few as 100 B cells (corresponding to a total of approximately 1000 PBMC) are cultured. The suppressive effect is therefore unlikely to be exerted by EBV-specific T cells clones since only a few hundred T cells are present in the cultures, but points rather to the NK cells as effectors. It was also evident that the cloning efficiency of untreated EBV-infected PBMC decreased by one order of magnitude when the number of plated B cells increased from 100 to 2500 cells/well. This demonstrated the strong inhibitory effect exerted by cytolytic cells since a corresponding decrease was not observed when all lysosome-rich cells had been eliminated before EBV-infection. Several-fold increases in both proliferation and Ig secretion were also recorded when LeuLeu-OMe-treated and EBV-infected PBMC were compared to untreated but infected lymphocytes. Since EBV infection, subsequently followed by somatic cell hybridization, is the preferred way to immortalize for the production of human monoclonal antibodies (Kozbor, Lagarde & Roder, 1982; Ohlin *et al.*, 1988), it is worth noting that LeuLeu-OMe treatment in one experiment (Table 4) induced more than a 10-fold increase in immunoglobulin secretion. Furthermore, since the EBV-receptor (CD21) on human B cells was still expressed after 6 days in culture (Table 2), EBV can be efficiently used to immortalize B cells after primary *in vitro* immunization, which was recently shown using a HIV peptide (Ohlin *et al.*, 1988). Human hybridomas produced from LeuLeu-OMe-treated and *in vitro* immunized PBMC routinely exhibit antibody productivity in the range 20–100 $\mu g/10^6$ cells/24 hr (M. Ohlin and C. A. K. Borrebaeck, unpublished results).

The effect of T-cell depletion of PBMC before EBV-infection was also compared to the LeuLeu-OMe treatment of PBMC before infection (Fig. 2) and the amount of Ig secreted from EBV-transformed B cells was measured on Day 19. A several-fold increase in antibody production was recorded when the PMBC had been pretreated with LeuLeu-OMe. This further points to the fact that the inhibitory effect which is removed by the leucine ester predominantly depends on natural killer cells, or an as yet undefined T-cell population, rather than on EBV-specific T-cell clones.

In summary, we have demonstrated the strong inhibitory effect of LeuLeu-OMe-sensitive lymphocytes on the clonal outgrowth, proliferation and Ig secretion of EBV-infected PBMC, an inhibition that was most probably mediated by NK cells rather than EBV-specific T-cell clones.

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