Cytotoxic mechanisms in vitro against Epstein-Barr virus infected lymphoblastoid cell lines in rheumatoid arthritis

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SUMMARY Impaired regulation of latent infection with Epstein-Barr virus (EBV) may contribute to the pathogenesis of rheumatoid arthritis (RA) by allowing uncontrolled polyclonal B cell activation. The control of EBV infection in vitro is dependent on several cytotoxic lymphoid cell populations. The present report examines the suppression of early lymphoblastoid outgrowth by natural killer (NK) like cells and the ability to form cytotoxic T lymphocytes (CTLs) specific for EBV in vitro. The latter was measured by a regression assay of EBV induced lymphoblastoid transformation. In this assay the regression of B cell outgrowth at four and six weeks is due to the generation of CTLs specific for EBV. Patients with RA were defective in this ability to generate CTLs. Eight out of nine patients with RA had a geometric mean at the 50% regression end point equal to or greater than 20×10^5 cells/ml. In contrast, the geometric mean for all control donors was less than 4×10^5 cell/ml. NK activity was measured by a conventional ^{51}Cr release assay with K562 targets. Patients with RA did not have significantly different activity from that of controls (RA patients, $n=4$, $45.6 \pm 19.7\%$ ($\bar{x} \pm \text{SD}$) at 50:1, effector:target; normals, $n=5$, $56.6 \pm 5.7\%$). No spontaneous NK activity was detected against allogeneic or autologous EBV infected B cell targets. When peripheral mononuclear cells from patients were incubated for six days with interleukin-2, lysis of EBV infected targets was seen. No difference in this activity was seen between RA and control studies. Overall, these studies show that patients with RA are defective in their ability to generate CTLs specific for EBV in vitro.

Key words: natural cytotoxicity.

During the last several years it has become clear that patients with rheumatoid arthritis (RA) have several antibodies present which react with Epstein-Barr virus (EBV) antigens. Thus it has been proposed that a latent infection with EBV might contribute to the pathogenesis of RA by inducing polyclonal B cell activation.¹ If such a chronic EBV infection were present in RA patients, one might expect that an increased incidence of EBV related lymphomas might result, such as is the case in patients with the X linked lymphoproliferative syndrome² or in re-

cipients of organ transplants who receive therapeutic immunosuppression. Such an increase in neoplasms, however, is not seen in RA patients. The control of early lymphoblastoid cell outgrowth is mediated by mononuclear cells with natural killer (NK) like properties and interferon.³⁻⁴ The later control of EBV induced immunoglobulin secretion and the lysis of EBV infected cells is mediated by suppressor and cytotoxic T lymphocytes respectively.⁵⁻⁷ In order for a chronic EBV infection to occur which would result in persistent polyclonal B cell activation it would be logical to suggest that RA patients lack the ability to generate EBV specific cytotoxic T lymphocytes (CTLs). It has been reported, however, that RA patients do not have an

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impairment in their ability to generate EBV specific CTLs.⁷

The purpose of the present report was to re-examine whether there are defects in several cytotoxic mechanisms which could control EBV infection in RA patients. In contrast with the report mentioned above we have shown that generation of EBV specific CTLs in an outgrowth regression assay is defective in RA.⁸ In addition, we have shown that an interleukin-2 (IL-2) induced cytotoxic killer cell (LAK cell) can be generated in vitro which kills autologous B cells infected with EBV.

Patients and methods

BLOOD DONORS

Control subjects were normal individuals of both sexes between the ages of 22 and 50 years. Patient donors were selected from the rheumatology clinics at the University of New Mexico Hospital and the Albuquerque Veterans' Administration Medical Centre. Patients with RA met the criteria of the American Rheumatism Association for definite or classical RA,⁹ and were rheumatoid factor positive. The majority of patients had chronic active disease and were receiving multiple drug therapy, including non-steroidal anti-inflammatory agents. Several patients also received gold, penicillamine, or methotrexate. Patients with osteoarthritis had conventional clinical and radiographic evidence of disease and were receiving non-steroidal anti-inflammatory agents. Donors were immune to EBV as determined by an indirect immunofluorescence test for IgG antibody to the viral capsid antigen.¹⁰

PREPARATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS

Peripheral blood mononuclear cells (PBMC) were isolated from heparinised venous blood by Ficoll-Hypaque density centrifugation,¹¹ washed twice in phosphate buffered saline pH 7.2, and suspended in RPMI 1640 medium supplemented with 10% heat inactivated, fetal bovine serum (Hyclone Laboratories, Logan, UT), 1% penicillin and streptomycin, and 3 mM glutamine.

VIRUS

The EBV producer cell line, B95-8,¹² was a gift from Dr D Carson, Scripps Clinic and Research Foundation. Seven day culture supernates were prepared by ultracentrifugation and stored in culture medium with 10% dimethyl sulfoxide (Sigma, St Louis, MO) at -70°C. The virus stock, assayed by transformation of human cord blood lymphocytes,¹³ had 10³ transforming units/ml.

REGRESSION OF EBV INDUCED

LYMPHOBLASTOID TRANSFORMATION

This assay measures the concentration of PBMC at the start of culture required to generate 50% regression of EBV induced lymphoblastoid transformation.⁸ PBMC at 1×10⁷ cells/ml were incubated with the virus stock for one hour at 37°C. After washing in medium the cells were resuspended in RPMI 1640 supplemented with 20% heat inactivated, fetal bovine serum, 1% penicillin and streptomycin, and 3 mM glutamine. Serial one to two dilutions of cells, from 2×10⁶ cells/ml to 1.25×10⁵ cells/ml, were added to flat bottomed microtitre Linbro plates (Flow Laboratories, Inc., Hamden, CT) in a volume of 0.2 ml. Six wells were plated per dilution. The plates were placed at 37°C in a 5% CO₂ incubator and replenished with 0.1 ml fresh medium weekly. Cultures were observed by inverted stage light microscopy at four and six weeks for evidence of lymphoblastoid transformation. Viable transformation cultures had two or more large clumps of viable cells per well. Regression cultures contained only dead cells or only one small clump of viable cells per well. The cultures were scored for each initial cell concentration as the number of wells out of six showing transformation at six weeks. Negative control cultures consisted of uninfected PBMC and positive control cultures consisted of EBV infected PBMC incubated with cyclosporin A (gift of J Borel, Sandoz Ltd, Basel, Switzerland), 100 ng/ml, during the first week of culture. Cyclosporin A allows the outgrowth of lymphoblastoid cells at high cell concentration.¹⁴ The initial cell concentration required to achieve a 50% regression end point was calculated by the method of Reed and Muench.¹⁵

CELL LINES

The K562 cell line derived from a human myeloid leukaemia was used as an NK target.¹⁶ Lymphoblastoid cell lines (LCLs) were derived from EVB infected cultures in the regression experiments. The cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum replenished with fresh medium every three or four days. LCLs were shown to contain the EBV genome by the anticomplement immunofluorescence test for Epstein-Barr nuclear antigen.¹⁷

NATURAL CYTOTOXICITY ASSAY

A standard four hour chromium release assay was used as previously described.¹⁸ Cells from log phase cultures of K562 and the LCLs were labelled with chromium-51 sodium chromate, specific activity 200–500 Ci/g chromium (New England Nuclear, Boston, MA) for one and six hours respectively.

Target cells at $1 \times 10^5/\text{ml}$, and unfractionated PBMC at $5 \times 10^6/\text{ml}$ were suspended in RPMI 1640 supplemented with 10% fetal bovine serum and plated in round bottomed microtitre Linbro plates in a final volume of 0.2 ml. The plates were incubated for four hours in a 5% CO_2 incubator at 37°C . 0.1 ml of supernatant was aspirated from the top of the wells, and the radioactivity released was counted in a Packard A 800 C gammacounter. Control counts for spontaneous release were obtained from wells containing only target cells.

Maximal release was determined with labelled target cells lysed with detergent. Specific lysis was calculated from triplicate data by the formula:

$$\text{Percentage specific lysis} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximal release} - \text{spontaneous release}} \times 100$$

Spontaneous release from the K562 cell line was less than 10% of maximal release, and spontaneous release from the LCLs averaged 30% of maximal release.

LYMPHOKINE ACTIVATED KILLER CELLS

1×10^7 PBMC were cultured in RPMI 1640 medium with 10% human AB serum (in preliminary experiments fetal bovine serum was cytotoxic), 1% penicillin and streptomycin, 3mM glutamine, and 10% IL-2 (Electro-Nucleonics, Inc., Silverspring, MD) in 15×60 mm plastic Petri dishes (Lab Tek Division, Miles Laboratories, Inc., IL). The IL-2 was a column purified preparation from lectin stimulated pooled human PBMC containing 640 units/ml, and was free from lectin or gamma interferon.¹⁹ Untreated control cultures that did not contain IL-2 were set up in parallel with RPMI 1640. Cultures were supplemented with 10% IL-2 or RPMI 1640 at 48 and 96 hours. After six days of culture non-adherent cells were recovered, washed twice in RPMI 1640, counted, and resuspended in culture medium with 10% human AB serum and 10% IL-2. At this stage cells from IL-2 supplemented cultures and from untreated control cultures were treated in the same way. In preliminary experiments IL-2 present in the assay did not augment lysis by the untreated control PBMC. Cell recovery averaged 25–50% of the starting cell number, and viability averaged 80% by trypan blue dye exclusion. Cytotoxic effector function was assayed as described for the natural cytotoxicity assay.

STATISTICAL METHODS

The statistical differences between the results were analysed by the following methods: firstly, the difference between geometric differences was deter-

mined by an analysis of variance (non-parametric, one way; Kruskal-Wallis); secondly, the difference in proportions was evaluated by χ^2 or Fisher's exact test, or both.

Results

REGRESSION OF EBV INDUCED LYMPHOBLASTOID TRANSFORMATION

After *in vitro* infection of PBMC with EBV and subsequent culture clumps of proliferating, growth transformed B lymphocytes emerge after 14 days.²⁰ In cultures of PBMC from immune normal donors these EBV infected cells die and result in dead cell clumps after four to six weeks. This phenomenon, regression of B lymphoblastoid outgrowth, is dependent on the cell concentration at the start of culture, with regression observed in cultures plated at 2, 1, or 0.5×10^6 cells/ml.⁸ No regression occurs in cultures of PBMC from non-immune donors. Regression is due to the generation of CTLs in culture that kill the virus infected B lymphocytes.^{20–21} These CTLs are specific for EBV and HL-A restricted.²² Cultures can be scored visually for the presence or absence of clumps of proliferating cells by inverted stage light microscopy, and replicate data can be used to calculate a 50% regression end point. The regression end point is reproducible in a given donor over time.⁸

In the present experiments, after EBV infection of PBMC from normal donors and from patients with RA or osteoarthritis, the cells were plated in serial dilutions from 2.0 to $0.125 \times 10^6/\text{ml}$ in flat bottomed microtitre plates and incubated at 37°C . Cultures were replenished weekly with fresh medium. Lymphoblastoid transformation or regression was scored at four and six weeks. The final score at six weeks was used to calculate a 50% regression end point (Table 1). The geometric mean 50% regression end point for nine normal donors was $1.8 \pm 0.7 \times 10^5$ cells/ml. Normal donor number 10 was non-immune, and his PBMC were unable to generate regression (Table 1). PBMC from nine RA donors, all immune to EBV, had a 50% regression end point ranging from 1.9×10^5 cells/ml to greater than 20×10^5 cells/ml, the upper limit of detection in this assay (Table 1). RA donor number one, the only RA donor with a value in the normal range, had had definite RA for one and a half years but was in clinical remission for six months at the time of the assay. RA donor number two had had definite RA for six months, with active disease at the time of the assay and was receiving no medication other than occasional aspirin in low dose. The other RA donors had longstanding, chronically active disease. The four control patients with osteoarthritis had 50%

Table 1 RA patients have decreased EVB specific CTLs as measured in outgrowth regression assays

Normal donor No	Cells×10 ⁻⁵ /ml	Rheumatoid donor No	Cells×10 ⁻⁵ /ml	Osteoarthritis donor No	Cells×10 ⁻⁵ /ml
1	< 1.25*	1	1.9	1	1.25
2	< 1.25	2	19.1	2	1.5
3	1.6	3	20.0	3	1.6
4	1.6	4	20.0	4	1.9
5	1.7	5	> 20.0		
6	1.8	6	> 20.0		
7	1.8	7	> 20.0		
8	2.5	8	> 20.0		
9	3.6	9	> 20.0		
10	>20.0				

*Cell concentration for 50% regression of EBV induced lymphoblastoid transformation. All donors, except for normal donor No 10, are immune. Data are 50% regression end point calculations by the method of Reed and Muench¹⁵ (see 'Patients and methods'). There is a significant difference between RA patients and normal subjects or patients with osteoarthritis ($p<0.01$).

regression end points in the normal range. In several experiments monocyte depletion by adherence to glass Petri dishes for one hour before infection with EBV and culture had no effect on the poor regression in cultures of RA donors (data not shown).

NATURAL CYTOTOXICITY AGAINST EBV INFECTED LCLs

NK lysis of virus infected or tumour derived cell lines by PBMC is well defined in normal donors and is defective in some patients with chronic disease.²³ Using unfractionated PBMC in a four hour chromium release assay at an effector:target ratio of 50:1, lysis of autologous and allogeneic EBV infected LCLs was compared with lysis of a standard human NK sensitive target, K562. Experiments were performed with five normal donors and four of the RA donors used in the regression assay experiments. The mean percentage specific lysis of K562 was 56.5% with the normal donors and 45.6% with the RA donors (Table 2). There was no lysis of autologous or

Table 2 Natural cytotoxicity against K562, autologous or allogeneic EBV infected LCLs

	K562	Allogeneic LCLs	Autologous LCLs
Normal (n=5)	56.5±5.7*	0	0
RA (n=4)	45.6±19.7	0	0

*Data are mean ± 1 SD, percentage specific lysis, based on a four hour chromium release assay, effector:target ratio=50:1.

allogeneic LCLs by either normal or RA donor PBMC (Table 2). In other experiments lytic activity against EBV infected LCLs could not be generated from normal donor PBMC that had been infected with EBV and cultured for one to seven days, while lysis of K562 remained raised for that time (data not shown).

LYMPHOKINE ACTIVATED KILLING OF EBV INFECTED LCLs

Culture of PBMC for several days in the presence of exogenous IL-2 yields LAK cells which can lyse both

Table 3 Measurement of LAK cytotoxicity in RA patients

Cell source	IL-2 present	Targets*		
		K562	Autologous LCLs	Allogeneic LCLs
Normal, immune	-	22.4†	0	0
	+	90.0	49.8	52.9
Normal, non-immune	-	19.2	0	0
	+	90.1	35.9	31.6
RA, immune	-	22.9	0	0
	+	83.1	35.5	29.8

*Cytotoxicity against K562, autologous and allogeneic EBV infected LCLs is recorded as percentage specific lysis from quadruplicate data for the donor in a four hour chromium-51 release assay at effector:target ratio of 50:1. The data represent one of five experiments with different donors.

†The SD of all experimental values was less than 10%.

Fig. 1 NK and LAK cytotoxicity of patients with rheumatoid arthritis. Results are expressed as percentage specific lysis, mean $\pm$ 1 SD. There was no difference between normal and rheumatoid patients using either K562 or EBV infected lymphoid cells.

conventional NK sensitive and NK resistant targets.²⁴ Unfractionated PBMC from normal and RA donors were cultured for six days with or without exogenous IL-2. Fresh non-toxic human AB serum was used in this culture and in the subsequent chromium release assay. The cultures were supplemented with fresh IL-2 or RPMI 1640 at two and four days. At the end of culture non-adherent cells were recovered, washed, and plated in a four hour chromium release assay in fresh medium with 10% IL-2. Target cells, either K562 or autologous or allogeneic EBV infected LCLs, were added at an effector:target ratio of 50:1. Percentage specific lysis was calculated as in the NK assay. Spontaneous release from the LCL targets averaged 30% of maximal release. Data from one of five experiments are presented in Table 3. IL-2 in culture augmented lysis of K562 cells and expanded the target cell repertoire to NK resistant, EBV infected LCLs. When the immune and non-immune normal donors were compared no antigen specificity or HL-A restriction in LCL target lysis was found (Table 3). The PBMC from RA patients were able to generate cytotoxicity against EBV infected LCLs at a level comparable with that of the normal donors (Table 3). Data from the five experiments are shown in Fig. 1. There was no significant difference between RA patients and normal subjects in the level of cytotoxicity against EBV infected LCL targets by LAK cells.

Discussion

We have examined the ability of RA patients to

control EVB induced lymphoblastoid outgrowth by three distinct cytotoxic mechanisms in vitro: classical virus specific CTLs, NK function based on lysis of K562 in a standard chromium release assay, and LAK cell cytotoxicity. Regression of EBV induced lymphoblastoid transformation due to classical, virus specific CTLs is dependent on cell concentration. Long lived memory T cells are diluted to a critically low level in this assay, so that the 50% regression end point is an approximation of the relative number of precursor CTLs in peripheral blood. The differentiation of CTLs in vitro, however, involves a complex interaction of multiple cell types and lymphokines.²⁵ It is not known which cell type is critically diluted in the regression assay. The poor regression or absence of regression in our RA samples is supported by the work of other laboratories, which showed poor regression by PBMC from RA donors compared with normals and patients with osteoarthritis.²⁶⁻²⁸ Tsoukas *et al*, however, have shown that when EBV specific CTLs are generated in vitro by another method—coculture of peripheral blood lymphocytes with mitomycin C treated, autologous EBV infected LCLs in two stages over 23 days—normal levels of EBV specific cytotoxicity are generated by RA patients in a four hour chromium release assay.⁷ It must be concluded from the later experiments that precursors of EBV specific CTLs present in the peripheral blood of RA patients can differentiate under optimal conditions of antigen presentation in culture. EBV specific CTL precursor frequency analysis by limiting dilution in normals or RA patients has not been reported to our knowledge. The significance of poor outgrowth regression in RA is thus unknown. In this assay perhaps inhibitory cells or soluble factors suppress differentiation of CTLs at high cell concentrations in RA: a condition not present in the culture of normal PBMC.

Natural cytotoxicity was normal in the RA patients, while there was no lysis of autologous or allogeneic EBV infected LCLs by PBMC from normals or RA patients. Other investigators have found normal levels of natural cytotoxicity in RA.^{23, 28} EBV infected LCLs derived from non-malignant B lymphocytes are relatively NK resistant. One group of investigators reported that RA lymphocytes do not lyse EBV infected LCLs in a short term chromium release assay.²⁹ In marked contrast with these observations, there was definite lysis of EBV infected LCLs by normal and RA LAK cells generated in vitro. The LAK cell, defined by Grimm *et al*,^{24, 30} is a unique lymphoid cell with phenotypic markers, kinetics of development, stimulus requirement, and target cell specificity distinct from that of both CTLs and NK cells. The induction of

LAK cells is IL-2 specific: interferons, IL-1, or migration inhibitory factor will not induce their formation.³¹ The in vivo significance of the LAK cell remains unknown. We have shown that LAK cells are also cytolytically competent against virus infected targets that are NK resistant in normals and in RA patients. We have not, however, shown by growth kinetics or cell phenotype analysis that our LAK cell is the same as that described by Grimm *et al.*²⁴ CTL precursors can differentiate in the presence of IL-2 without specific antigenic stimulation.³² It is unlikely that an EBV specific CTL precursor is the major cell type that differentiates in response to IL-2 in the LAK assay because we have shown that non-immune PBMC are as effective as immune PBMC. Cells with phenotypic similarities to the LAK cell that can lyse EBV infected LCLs have been described in the blood of patients with infectious mononucleosis and were generated in vitro by T cell growth factor or by autologous mixed lymphocyte culture.³³ A relevance of the LAK cell phenomenon to the pathology of RA is suggested by the findings of IL-2 activity in rheumatoid synovial fluid,³⁴ and of NK like cells in rheumatoid synovial fluid that can lyse EBV infected LCLs, although at low levels.²⁹

In summary, patients with RA possess defects in their ability to generate EBV specific CTLs in outgrowth regression assays. In addition, despite the presence of normal NK activity against conventional NK sensitive targets, RA patients are able to generate LAK cells in vitro that kill EBV infected autologous B cells. It still remains unclear what is the relation between these in vitro findings and the potential role of persistent EBV in the pathogenesis of RA.

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