

THE MEASUREMENT OF CYTOTOXIC T LYMPHOCYTE ACTIVITY DURING VIRUS INFECTION

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ABSTRACT. Quantification of the activity and definition of the specificity of virus-specific MHC-restricted human cytotoxic T cells in many diseases is an important part of current research. We compared two methods of measuring target cell death caused by cytolytic cells. The first method measures ^{51}Cr release from the cytoplasm of dead cells, and the second measures DNA fragmentation (apoptosis) and cell death using ^3H -TdR labeling of unfragmented DNA in living cells (JAM assay). We show here that the JAM assay may be used with human CTL and target cells infected with viruses. The method is convenient and simple and yields results comparable to those obtained in the ^{51}Cr release assay. Simplifying CTL activity measurements is a major goal, if such assays are to be performed routinely to assess vaccination protocols designed to stimulate CTL activity in diseases such as HIV.1.

INTRODUCTION

Zinkernagel and Doherty (1979) first described antigen-specific major histocompatibility complex (MHC)-restricted cytotoxic T lymphocytes (CTL), which could be generated during virus infection along with the antibody response. CTL are a population of T lymphocytes expressing the CD3 and CD8 markers, and a clonally restricted recognition molecule called the T cell receptor (TCR). It was originally thought that CTL recognized intact virus glycoproteins, such as haemagglutinin from influenza or envelope from HIV.1, on the surface of virally infected cells. However, a series of experiments by Townsend *et al.* (1986) showed that CTL recognize internal virus proteins, such as influenza matrix protein or HIV.1 gag protein, as well as peptide fragments of viral antigens presented by class 1 molecules of the MHC (Townsend & Bodmer 1989).

When the tripartite complex forms between the TCR class 1 molecule and antigenic peptide, a series of events is triggered that lead first to enzymatic cleavage of the DNA in the target cell into small fragments (apoptosis) (Duke, Chervenak and Cohen 1983), followed by membrane disintegration and leakage of cytoplasm. These events lead to the death of the virally infected target cell while it assembles and before it releases new virus particles.

Sanderson (1964) and Brunner *et al.* (1968) described the usual method for measuring cell death, using ^{51}Cr release to measure membrane disintegration and leakage of cytoplasm. Recently, Matzinger (1991) described a method (the JAM test) for measuring DNA fragmentation (apoptosis) in cells and cell death. This method measures ^3H -TdR-labeled unfragmented DNA retained by that proportion of labeled cells that have not been killed. This method is simple, fast, inexpensive and yields results comparable with the ^{51}Cr release assay. Both cytolysis measured by ^{51}Cr release and apoptosis measured by ^3H -TdR-labeled DNA retention may be quantified accurately using the Wallac BetaplateTM counter.

However, Matzinger (1991) used alloreactive murine CTL and PHA blast cells or P815 tumor cells as target cells. In experiments where virus-specific human CTL are tested for activity, one must use human Epstein Barr Virus (EBV) transformed B cells that are infected with virus or pulsed with peptide as target cells. Sometimes the B cell lines used as target cells are unavoidably contaminated with mycoplasma. Thus, we conducted a short series of experiments using influenza virus-specific MHC class 1 restricted human CTL with virally infected B cell target cells in both

the conventional ^{51}Cr release assay and the JAM assay. In this way, we were able to ascertain that the JAM assay could be used to measure the activity of virus-specific human CTL.

METHODS

Cytotoxic T Lymphocytes (CTL)

Peripheral blood lymphocytes were separated from whole blood and stimulated with A/X31 influenza virus. The CTL were maintained in culture by repeated stimulations with the matrix peptide, M57-68, as described by McMichael *et al.* (1988). These influenza matrix protein-specific CTL were restricted through HLA A2.

Target Cells

EBV transformed lymphoblastoid cells in the log phase of growth were pelleted by centrifugation. They were also infected with: 1) recombinant vaccinia virus (rVV) expressing the influenza matrix protein (5 plaque-forming units/cell), and 2) 100 μl A/X31 influenza virus (1000 HAU). Alternatively they were pulsed with matrix peptide 57-68 for 1 h or untreated. The cells were then washed and labeled in two ways: 1) 1×10^6 infected or non-infected cells were pelleted by centrifugation and labeled with ^{51}Cr (300 mCi) (Amersham UK) for 1 h. They were then washed, resuspended in medium and left for 3 h, so that viral antigens might be expressed on the surface of the cells. The cells were then rewashed and resuspended to $1 \times 10^5 \text{ ml}^{-1}$ in assay medium; 2) 1×10^6 infected or non-infected cells were made up to 2 ml in assay medium. $^3\text{H-TdR}$ (Amersham, UK) was added to a final concentration of 5 mCi ml^{-1} ($1.85 \times 10^5 \text{ Bq ml}^{-1}$). The cells were incubated for 4 h at 37°C to allow incorporation of the label and the expression of the viral antigens. The cells were then washed and resuspended to $1 \times 10^5 \text{ ml}^{-1}$ in assay medium.

Cytolytic Assay

CTL and target cells were incubated together in the wells of round-bottomed microtitration plates at 37°C for 4 h as McMichael *et al.* (1988) described previously.

^{51}Cr released into the medium was measured by spotting 20 μl supernatants onto a spot-on filtermat laminated with plastic on one side. The filtermat was dried and bagged with 10 ml scintillant; the Auger electron component of ^{51}Cr decay was measured on the Wallac BetaplateTM counter. Percent target lysis was calculated from the formula $(E-M/D-M) \times 100$, where E = experimental ^{51}Cr release, M = release in presence of culture medium with no CTL present, and D = release from target cells by 5% Triton[®] $\times 100$.

To harvest the JAM assay, the cells and their medium were aspirated onto glass-fiber printed filter mats using a Wallac 1295-001 cell harvester. Fragmented DNA from dead cells was washed through the filters, in which intact DNA was trapped. The filters were dried and bagged with 10 ml scintillant; activity reflecting the number of living target cells remaining in each well was measured in the Wallac BetaplateTM counter. Percent apoptosis was calculated from $(S-E/S) \times 100$, where S = retained DNA in the absence of killers, and E = experimentally retained DNA in the presence of killers.

RESULTS

Influenza matrix protein-specific, HLA A2-restricted CTL were tested with A2 matched peptide-pulsed B cell target cells labeled with ^{51}Cr or $^3\text{H-TdR}$. Initial experiments indicated that B cells

labeled well with $^3\text{H-TdR}$ in 4 h, and that a 4-h assay time gave comparable percent lysis of ^{51}Cr or $^3\text{H-TdR}$ -labeled target cells (data not shown).

The same CTL were then tested with HLA A2-matched BCL target cells that were labeled in both ways and 1) infected with rVV expressing the matrix protein of influenza virus, 2) infected with influenza A virus, 3) pre-incubated with peptide 57-68 from the matrix protein, or 4) untreated. Figure 1 shows that the percent lysis or apoptosis was comparable. When an identical experiment was carried out using mycoplasma-infected or non-infected target cells, we saw that the specific lysis or apoptosis was comparable when the cells were mycoplasma-infected. We also found that comparable results could be obtained in the JAM assay if the experimental microtitration plates were frozen after the 4-h assay time, and then thawed before harvesting (data not shown).

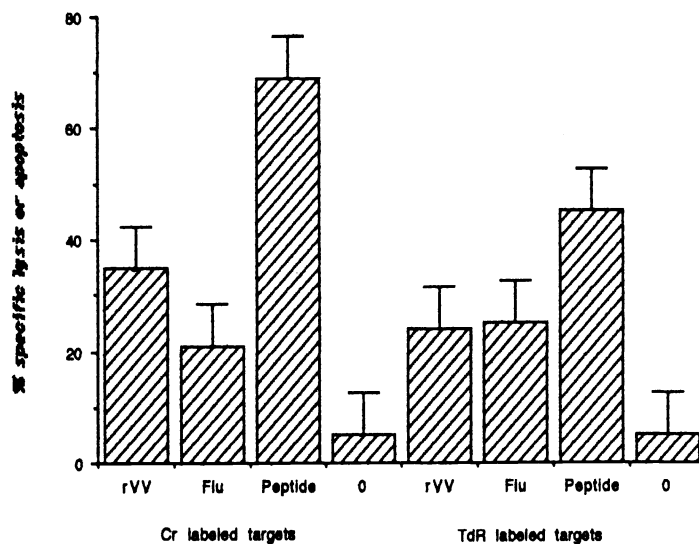


Fig. 1. CTL were prepared as in the methods section and were used at an effector-to-target-ratio of 4:1 with HLA A2-matched EBV-transformed B cells as targets. Target cells were labeled with ^{51}Cr (LHS) or $^3\text{H-TdR}$ (RHS) and: 1) infected with rVV expressing the matrix protein of influenza virus (rVV); 2) infected with influenza virus (flu); 3) pulsed with peptide 57-68 from matrix protein (peptide); 4) uninfected (0). The assay time was 4 h.

CONCLUSION

We have shown that the JAM assay may be used to quantify the lytic activity of human virus-specific CTL with virally infected B cells as targets that may or may not be infected with mycoplasma. The JAM assay is simple, inexpensive, safe and yields results comparable with the ^{51}Cr release assay. However, when infected target cells are used, the JAM assay is no faster than the ^{51}Cr release assay, except that harvesting with a cell harvester is faster than spotting supernatants individually onto filtermats.

Quantitation of the activity and definition of the specificity of CTL in virus diseases, such as influenza, HIV.1, HIV.2, HTLV.1, EBV and other diseases, such as malaria, are very important in current medical research. We believe that CTL form an important part of the cellular immune mechanism, and may exert overall control of viral replication. In some acute viral infections, CTL may be able to clear virus and terminate infection. It is becoming apparent that vaccination against

some of the above viral diseases may be more effective if strong CTL responses are raised. Also, in individuals infected with certain viruses, such as HIV.1, post-infection vaccination may enhance CTL responses. Thus, simple measurement of CTL activity is important, because such assays may be performed routinely with many patients. The Wallac Betaplate™ counter facilitates measurements with both the ⁵¹Cr release assay and the JAM assay.

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