CYTOTOXIC T LYMPHOCYTE CONTROL DURING ECTROMELIA (MOUSEPOX) VIRUS INFECTION: INTERACTION BETWEEN MHC-RESTRICTED CELLS ANALYZED BY NON-RADIOACTIVE FLUOROMETRY

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Summary. – Cytotoxic T lymphocyte (CTL) activity of draining lymph node (DLN) cells isolated from BALB/c mice infected with ectromelia virus (EV) was examined using a fluorometric cell-mediated cytotoxicity (CMC) assay. Specific lysis of target cells A20 and EMT-6 primed with EV was demonstrated. The classical $CD8^+$ cytolytic pathway dominated (72.7%) as compared to that of $CD4^+$ (27.3%) in the cellular response during acute EV infection. Also an alternative method for determining CMC, employing a bisbenzamide dye for labelling target cells, is described. Coefficient variations of relative fluorescence were below 6%, that makes the method sensitive and reliable.

Key words: ectromelia virus; cytotoxic T lymphocytes; fluorometry

Introduction

The major T lymphocyte subset involved in the cytotoxicity is MHC class I of restricted cells of $CD4^-CD8^+$ phenotype; however, $CD4^+CD8^-$ CTL also occur and may even represent the predominant phenotype in some instances (Schmid and Rouse, 1992; Doherty, 1993; Rourke and Mescher, 1993). This is true for CTL response to HSV-1 in man. In murine systems, however, the great majority of studies emphasize $CD8^+CD4^-$ class I restricted CTL and ignore the class II ($CD4^+CD8^-$) restricted cells. In this study we have used EV as model for studying virus-specific MHC class I and class II restricted CTL.

EV belongs to the *Chordopoxvirinae* subfamily of the Poxviridae family (Murphy *et al.*, 1995; Niemiałtowski *et al.*, 1996a,b). It is a pathogen that naturally infects mice, especially in mouse breeding colonies worldwide. This fact calls for attention to the effect the virus may have on important biomedical research performed on mice susceptible to EV. On the other hand, the biological model that arises from the combination of EV and susceptible mouse strains cannot be overlooked. It provides means for investigation of immune mechanisms involved in viral infections. Apparently, infection with EV triggers immune response that results in generation of antibodies and CTL. Presumably, the cellular response is accountable for recovery from primary infection (Gardner *et al.*, 1974; Rourke and Mescher, 1993), and the lymphocyte-mediated cytotoxicity is thought to be a crucial effector mechanism in immune protection against viruses (Niemiałtowski *et al.*, 1994a, Kägi *et al.*, 1995).

Mice infected with EV may die at the peak of the disease or survive. This usually occurs between day 6 and 14 of

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Abbreviations: CMC = cell-mediated cytotoxicity; CMV = cytomegalovirus; CTL = cytotoxic T lymphocytes; DLN = draining lymph node; DMSO = dimethyl sulphoxide; E/T = effector/target ratio; EV = ectromelia virus; FCS = foetal calf serum; H33342 = Hoechst 33342 stain; HSV-1 = herpes simplex virus type 1; MEM = Eagle's Minimal Essential Medium; PBMC = peripheral blood mononuclear cells; p.i. = post infection; RFU = relative fluorescent unit; VV = vaccinia virus

infection. In this paper, we present a study aimed at determining the cytolytic activity of CTL during the acute phase of mousepox. We concurrently assessed the suitability of a fluorometric method for CMC assay. Cytotoxicity was measured in DLN cells, the second site at which the virus replicates before spreading to other organs. The fluorometric technique, that was developed to measure the CTL activity, involves the use of a bisbenzamide stain, Hoechst 33342 (H33342). This dye has been used for diverse investigations, ranging from subclassification of lymphocyte populations (Loken, 1980), measurement of cell number and proliferation (Blaheta *et al.*, 1991) to DNA quantification (Lutz and Yayanos, 1985). H33342 is specially designed to penetrate the living cells (Blaheta *et al.*, 1991) upon which it binds preferentially to A-T base pairs of DNA. The non-covalent binding of DNA makes the dye to fluoresce intensively. In the method described in this paper, H33342 was used to label the target cells. After appropriate incubation with effector cells, the cytolytic activity was determined by quantifying the fluorescence of the released DNA in supernatants of tested samples. Our investigations show the suitability of a non-radioactive fluorometric method for determination of virus-specific activity of CTL. It is likely that the method can replace the ^{51}Cr-release assay, which for the last 30 years has dominated in viral immunology in analyses of the interaction between activated CTL (both $CD8^+$ and $CD4^+$) and target cells.

Materials and Methods

Mice and virus. Six- to eight-week-old BALB/c mice (H-2d haplotype) were purchased from the Children Memorial Health Institute, Warsaw. EV strain Moscow was kindly provided by Dr. R.M.L. Buller, NIAID, NIH, Bethesda, MD, USA.

Effector T cells. Mice were inoculated into the hind footpads with EV (103.5 TCID50) and observed daily for clinical signs. At day 6 post infection (pi), mice were sacrificed by cervical dislocation and DLN were aseptically removed. Cell suspensions were prepared from the lymph nodes by passing them through a stainless steel sieve into a Petri dish containing medium RPMI-1640 (Gibco). Traces of red blood cells were lysed with Tris-HCl buffer. The cells were then washed at least three times in RPMI-1640 and the viability (at least 94%) was assessed by the trypan blue exclusion method. Finally, the cells were suspended in RPMI-1640 supplemented with L-glutamine, 2-mercaptoethanol and 10% foetal calf serum (FCS, Gibco), and plated at a density of 1 x 107 cells/well in 6-well-microplates (Corning). Meanwhile a dose of EV, appropriate to reach a multiplicity of infection (MOI) of 5 TCID50 per cell (before inactivation), was inactivated under a germicidal lamp for 2 mins and added to the cell suspension for additional stimulation. DLN cells were then cultured at 37°C for 3 days in 5% CO_2. On day 3 the cells were harvested, washed in RPMI-1640 and the recovery rate was determined by the trypan blue exclusion method. The recovery rate was us usually above 70%.

Target cells. Syngeneic A20 (a mouse B cell lymphoma continuous cell line that expresses both MHC class I and class II proteins) and EMT-6 cells (a BALB/c mammary adenocarcinoma cell line that expresses only class I MHC proteins), and allogeneic EL4 cells (a mouse lymphoma cell line [H-2b haplotype]) were used as targets (all these cell lines were obtained from Dr. B.T. Rouse, Department of Microbiology, University of Tennessee, Knoxville, TN, USA) (Niemialtowski and Rouse, 1992). EMT-6 cells were prepared in Eagle's Minimal Essential Medium (MEM) with 10% FCS, A20 cells in RPMI-1640 with 10% FCS, and EL4 cells in MEM with 10% horse serum (Biomed, Lublin, Poland) in 75-cm^3-tissue culture flasks (Corning, USA) at least 48 hrs before the cytotoxicity assay. All sera used were heat-inactivated. Shortly before the assay, cells were harvested, washed three times in appropriate medium, counted, infected with EV at MOI of $5TCID_{50}$/cell and incubated at 37°C for 2 hrs in 5% CO_2. H33342 (5 μg/ml in distilled water) was added to the cell suspension for 2 hrs. Finally, the cells were washed, counted, resuspended in the appropriate medium and kept for 30 mins to allow exflux of the unbound stain.

Cell viability assay. As there were reports on toxic and mutagenic effects of H33342 on various cell types, the suitability of H33342 for cytotoxicity measurements was tested. A fluorometric method was adopted to measure the viability and activity of target cells after incubation with H33342. A calibration curve for each target cell line was constructed. Serial twofold dilutions of target cells ($2 \times 10^4 - 6 \times 10^2$) were stained with 5 μg/ml FDA for 45 – 60 mins at 37°C in 5% CO_2 (Table 1) and the fluorescence was read. Before use, FDA was prepared in Ca^{2+}-free phosphate buffered saline from a stock solution in dimethyl sulphoxide (DMSO).

To determine the number of cells still active after incubation with H33342, diluted target cell suspensions were dispensed in 96-well Costar tissue culture plates (10^4 cells per 100 μl per well) in triplicates. One-hundred μl of H33342 (5 μg/ml) was then added to the cells and the plates were incubated for 2 hrs. Afterwards, the cells were washed, resuspended in appropriate media and incubated at 37°C in 5% CO_2 for the next 2 hrs. The plates were spun at 1000 rpm, 100 μl of medium was removed and 100 μl of 5 μg/ml FDA was added. Cells were incubated for 45 – 60 mins and finally the fluorescence was measured. The resulting relative fluorescence values were used to derive from the calibration curves the number of cells still active after the incubation with H33342.

FDA is hydrolyzed by cytoplasmic esterases in living cells to highly fluorescent fluorescein that allows the assessment of cell activity and viability (Larsson *et al.*, 1992). The excitation and emission spectra of H33342 and FDA do not overlap and therefore the cell viability evaluation in the dual staining procedure employed in this assay is not obstructed by fluorescence interference from H33342.

Cytotoxicity assay. Two-hundred μl aliquots of effector and target cells prepared as described earlier (Niemialtowski and Rouse, 1992; Toka *et al.*, 1996) were dispensed successively into conical bottomed 96-well tissue culture plates at an effector: target (E:T) ratio of 100:1 in triplicates. The plates were centrifuged at 1000 rpm for 5 mins and 0.5% Triton X-100 was added to the wells. Uninfected EMT-6 and A20 cells were used as controls, while EL4 cells were used as an MHC restriction control. The plates were incubated at 37°C in 5% CO_2 for 4 hrs, centrifuged, 100 μl aliquots of the supernatant from each well were transferred to corresponding wells on black fluoroplates (Labsystems) and the fluorescence was read immediately.

Fluorometry. A 96-well scanning fluorometer, Fluoroskan II Neonat version 5 (Labsystems) was employed. H33342 and FDA are excited at 355 nm and 485 nm, respectively, and they emit light at 460 nm and 538 nm, respectively. The latter was sequentially read for each well within 70 secs at integration rate of 0.1. The instrument was operated from an attached Packard Bell PC and the data were transmitted for calibration purposes to Genesis software (Labsystems). The relative fluorescence data for cytotoxicity calculation was transmitted from the PC by Transmit software (Labsystems) to a customed spredsheet for the cytotoxicity correction. The following formula was adopted for the cytotoxicity calculation:

$$\text{corrected cytotoxicity} = (H_{exp} - H_{sp})/(H_{tt} - H_{sp}) \times 100$$

where H_{exp} = H33342 fluorescence of DNA released from tested samples, H_{sp} = spontaneous release and H_{tt} = total release upon action of Triton X-100. The results presented here are averages of 3 independent determinations.

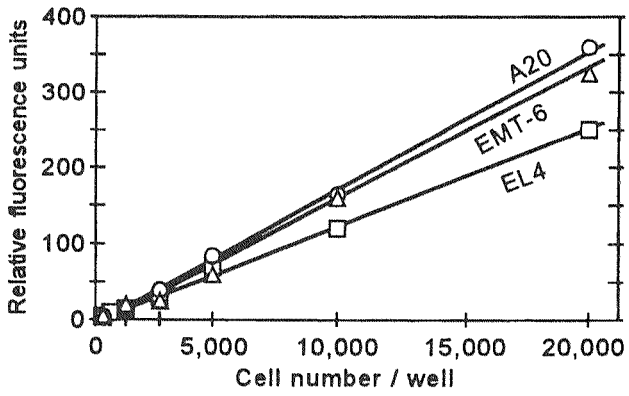

Fig. 1
Calibration curves for determination of target cell number with FDA
Cells were stained with 5 μg/ml FDA as indicated in Table 1.

Results

At the time of sacrificing the mice (6 days p.i.), typical mousepox clinical manifestations were observed. These included swelling of the feet, ruffled hair, hunched posture and, in some mice, encrustation on the ears. *Post mortem* it was found that the livers and spleens were enlarged as compared to those of uninfected control mice.

Optimum concentrations of H33342 and FDA used in the assay were determined in preliminary experiments (data not shown). Initially, the crucial point was to ascertain the preservation of target cell viability and activity upon staining and incubation with H33342. We observed (Fig. 1) a linear relationship between the hydrolyzed FDA fluorescence and the target cell number. This calibration curve was used to determine the number of cells surviving the incubation with H33342. The target cell number determined in this way (Table 1) indicated a minimal cell activity loss after incubation with H33342. We observed that higher concentrations of H33342 gave higher values of spontaneous release (data not shown). This was in agreement with the observations of Durand and Olive (1982) who reported initial rapid disappearance of H33342 from the cells within the first 2 hrs. Therefore we decided to suspend the stained target cells in RPMI-1640 for 30 mins before mixing with effector cells. The concentration of the serum during the cytotoxicity assay was reduced to 2%, because higher concentrations (e.g. 10%, used for cell cultivation) increased the fluorescence background, rendering the assay insensitive. In this way the spontaneous release was reduced to acceptable values below 6%. The inter- and intra-assay coefficients of variation of fluorescence values obtained were below 6% in triplicates of samples.

The optimal parameters were employed in the analysis of cytotoxic activity of cells isolated from DLN of BALB/c mice infected with EV. The cytotoxic activity was detected with both A20 and EMT-6 target cells (Fig. 2). The highest cytotoxicity values (52.92 ± 0.4%) were obtained with A20 cells at E:T ratio of 100:1. The same E:T ratio gave a higher cytotoxic activity with EMT-6 cells (38.48 ± 0.97), but lower when compared to A20 cells. The cytotoxic of EV-primed DLN cells was expressed against infected target cells but not against mock-infected A20 and EMT-6 cells (4.87 ± 0.45 and 4.39 ± 0.44%, respectively) (Fig. 3). As an additional control, DLN cells isolated from uninfected mice did not show significant level of cytotoxicity; the latter was much the same as that of mock-infected A20 and EMT-6 cells (data not shown). This demonstrated the specificity of CTL response during EV infection. Further evidence was provided by a lower level of non-specific cytolysis of allogenic EL4 cells (4.72 ± 0.78%, Fig. 3). This level was comparable to the non-specific lysis observed in mock-infected A20 and EMT-6 cells.

Table 1. Number of active target cells after 4 hrs of incubation with H33342

Cell type	Staining time (mins)	FDA fluorescence (RFU)	Cell number
A20	45	153 ± 12	9250 ± 45
EMT-6	60	151 ± 15	9051 ± 65
EL4	45	124 ± 21	9105 ± 51

Cell number was calculated from calibration curves (Fig. 1). Cell number and PFU values are averages of 3 independent determinations.

Discussion

CMC as one of the mechanisms by which the immune system defends the organism against the non-self has been described extensively (Vignaux and Golstein, 1994; Nie-

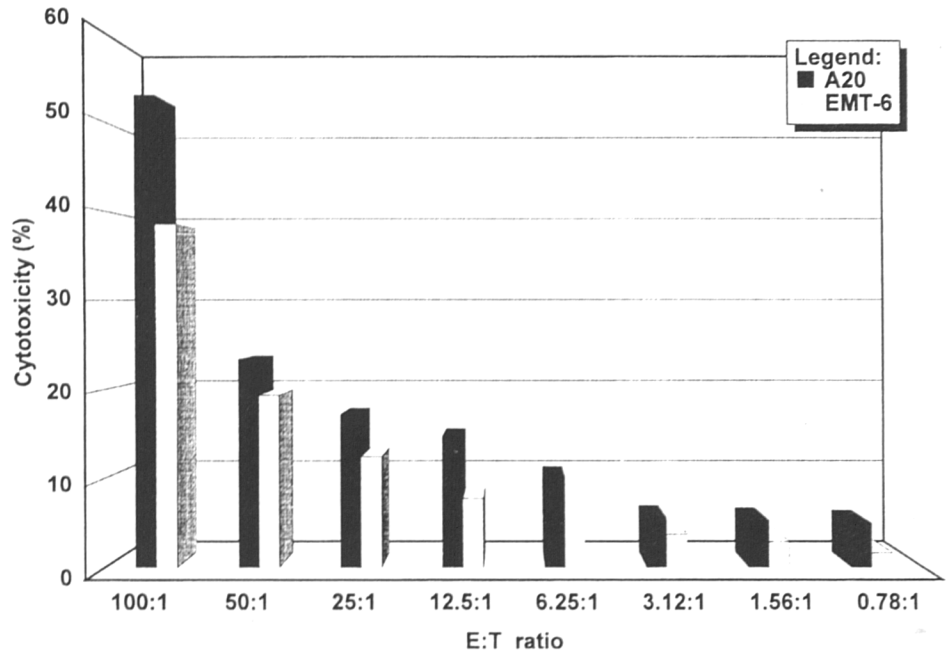

Fig. 2

Cytotoxic activity of T lymphocytes isolated from EV-infected BALB/c mice against EV-primed syngeneic target cells A20 and EMT-6

Target cells were labelled with 5 μg/ml H33342 and incubated with effector DLN cells for 4 hrs. Fluorescence of labelled DNA of lysed target cells was measured.

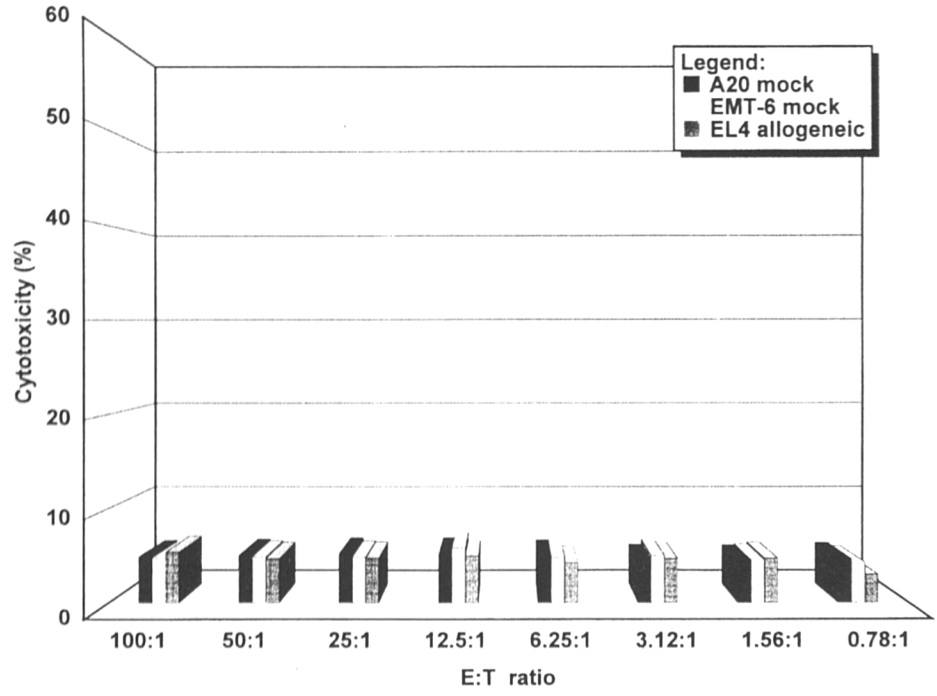

Fig. 3

Non-specific lysis of mock-infected syngeneic target cells A20 and EMT-6 and allogeneic EL4 cells

miałtowski and Rouse, 1994c) and has been found to play a very important role in the recovery from viral infection. The full spectrum of cytotoxic effectors includes CTL, NK and lymphokine activated killer (LAK) cells (Ahmed, 1992; Schmid and Rouse, 1992; Doherty, 1993; Niemiałtowski and Rouse, 1994c; Kägi *et al.*, 1995). CTL, which are able to recognize and destroy virus-infected cells, have been experimentally demonstrated to play a crucial role in this process. However, a number of animal viruses have been found to evade T cell recognition by down-regulating MHC class I expression or by expressing specific proteins that alter or block the MHC class I antigen presentation pathway (York *et al.*, 1994). Vaccinia virus (VV) and EV cause a decrease in expression of MHC molecules. HIV, hepadnaviruses, papillomaviruses, rhabdoviruses, herpesviruses (CMV and HSV-1, 2) and adenoviruses (Maudsley and Pound, 1991) behave similarly. E.g., by expressing immediate early protein ICP47 (ICP47 is both necessary and sufficient to block transport of class I MHC proteins and to inhibit lysis by $CD8^+$ CTL), HSV-1 can evade detection by $CD8^+$ T lymphocytes, perhaps explaining the predominance of $CD4^+$ T cells rather than $CD8^+$ HSV-1 specific CTL *in vivo* (York *et al.*, 1994).

Several aspects of the infections associated with some orthopoxviruses are remarkably similar to those associated with other viruses, e.g. HSV-1 (Kolaitis *et al.*, 1990; Schmid and Rouse, 1992; Yasukawa *et al.*, 1992; Nash and Cambouropoulos, 1993). In our communication, some evidence which implicates anti-EV CTL as a principal controlling mechanism is discussed.

It is known from the early works of Blanden *et al.* (1977) performed with EV that virus-specific immune response involving CTL plays a crucial role in the destruction of infected target cells. It has been shown that VV-specific $CD4^+$ and $CD8^+$ are present *in vivo* and that $CD4^+$ CTL could be cloned from PBMC taken from an immune individual (Littaua *et al.*, 1992). The studies of Demkowicz *et al.* (1992, 1993, 1996) contributed valuable information that VV-specific $CD4^+$ and $CD8^+$ T lymphocyte-dependent immunity can persist in humans for up to 50 years after immunization against smallpox in childhood in the presumed absence of exposure to VV. Precursor frequency analysis under limiting-dilution conditions of the VV-specific memory $CD4^+$ T cells from a donor immunized earlier revealed a frequency of 1 in 65,920 $CD4^+$ CTL. Studies with mice infected with orthopoxviruses shown that $CD8^+$ CTL-mediated effector pathway is more effective than that of $CD4^+$ CTL.

Our report extends previous observations (Blanden *et al.*, 1977; Buller *et al.*, 1987; O'Neill and Brenan, 1987; Binder and Kunding, 1991) on murine CTL showing the presence of both phenotypes after EV infection and compares the cytotoxic activity of both phenotypes. We show that DLN T cells from BALB/c mice infected *via* footpads contained both $CD8^+CD4^-$ and $CD8^-CD4^+$ CTL with the latter phenotype in 2 times lower concentration. This is evident from the difference between the levels of cytotoxicity against the two target cell types used: A20 cells that express both class I and class II MHC proteins (these cells are vulnerable to recognition and lysis by both $CD8^+CD4^-$ and $CD4^+CD8^-$ CTL), and EMT-6 cells that express only class I MHC proteins recognized by $CD4^+CD8^-$ only. Our results confirmed the findings reported earlier by Gardner *et al.* (1974) showing that the cytotoxic activity of DLN and spleen T cells against EV was highest at day 6 p.i. Therefore it can be deduced that T cells in the EV system acquire effector function at a relatively shorter period following antigenic stimulation during acute infection. In a separate paper (Toka *et al.*, 1996) we have demonstrated an enhanced mobilization of Ca^{2+} in CTL isolated from BALB/c mice at day 6 p.i. with EV. Investigations are under way to examine the dynamics of the cell cytotoxic activity against EV during the memory state.

Ever since the cell-mediated cytolysis was first described (Govaertz *et al.*, 1960; Brunner *et al.*, 1970), different types of assays have been developed, including microscopy, inhibition of various metabolic activities (Perlmann and Holm, 1969), and dye release and retention (Kolber *et al.*, 1988). The ^{51}Cr-release has set the gold standard in T cell cytotoxicity assays (Cerottini, 1993). Our assay system provides another alternative to the radioactive system, moreover, it is advantageous when safety and cost are considered. The sensitivity of the assay demonstrated with H33342 is comparable to that of the ^{51}C-release used by Gardner *et al.* (1974) with EV. However, stringent conditions in the H33342 assay concerning the dye concentration, use of low-serum or serum-free media, incubation temperature and time should be followed to keep its sensitivity abreast of that of other systems.

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