Colorimetric *In Vitro* Evaluation of T-Cell Cytotoxicity

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We propose a new colorimetric method for evaluation of specific T lymphocyte cytotoxicity based on the capacity of viable cells to incorporate alamar blue dye, an indicator of cell metabolic activity. This method is effective for highly specific strains of cytotoxic T lymphocytes and for primary T killers formed *in vivo* and constituting a small part of analyzed cell population. This test is a simple nonradioactive method for evaluating specific cellular cytotoxicity exhibiting sufficient sensitivity for evaluation of this parameter in the presence of effector cells.

Key Words: *cytotoxic T lymphocytes; target cells; alamar blue; optical density*

Several methods for evaluation of cell cytotoxicity are known and used, *e.g.* the method of radioactive label based on recording of ^{51}Cr release from destroyed target cells, colorimetric methods, *etc*. Colorimetric methods are based on measurement of dye release from target cells [3] or evaluation of the number of viable cells. Methylene blue, crystal violet, thiazolyl blue (MTT), and other dyes are used for these purposes [1,4]. These dyes are used mainly for evaluation of the toxicity of chemicals. The main drawback of colorimetric methods in the evaluation of the cytotoxic effect of effector cells is that the dye is incorporated by not only the target cells, but also effectors, which decreases reliability of the analysis. We propose a method for evaluating T lymphocyte cytotoxicity using intravital staining with alamar blue dye [6]. The method is not radioactive and is more sensitive than staining with the above-mentioned dyes.

MATERIALS AND METHODS

The study was carried out on allospecific cytotoxic T lymphocytes (CTL). CTL strains were derived by two-staged priming. The mice were injected with splenocytes (20×10^6) from allogenic donor in the paw pads. Four days after immunization the cells from regional lymph nodes of immune mice were cultured *in vitro* with stimulators of the donor haplotype in a 1:2 ratio. Splenocytes treated with mitomycin C (25 μg/ml, 30 min at 37°C) served as stimulators.

The cells were cultured (37°C, 5% CO_2) in RPMI-1640 medium with additives in the presence of 10% fetal calf serum and 10 U/ml interleukin-2 (IL-2). The cells were subcultured once a week with fresh portions of stimulators and IL-2. The following CTL strains were used: C57BL/10-anti-B10.D2 (haplotypes H-2^b-anti-H-2^d) and BALB/c-anti-C57BL/10 (H-2^d-anti-H-2^b) after 4-5 immunizations *in vitro*. Flow cytofluorometry showed that 90-95% resultant killers carried a T cell receptor, coreceptor CD8, and FasL on their surface.

The 4+3 protocol was used for preparing primary CTL [4]. The mice were immunized in the paw pads as described above. Four days after immunization the cells from regional lymph nodes of these mice were cultured *in vitro* for 3 days, after which the cells were tested for cytotoxicity.

Cytotoxic activity of T killers was evaluated by the capacity of target cells to incorporate intravitally alamar blue dye (Nalgene), an indicator of their metabolic activity [6]. This dye can bind to a wide spectrum of cell elements, which determines higher sensitivity of this method [3,4]. After metabolic oxidation the dye turns from blue to bright pink. The dye was added to the culture 18-24 h before the end of the experiment in a dose of 15 μl/well. The results were evaluated on a Multiscan device (Labsystems) by the difference of optical density (OD) at 540 and 620 nm.

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Transformed cell strains of different origin (P-815 mastocytoma (H-2^d), EL-4 thymoma (H-2^b), RDM-2 (H-2^k), and mouse fibroblasts transformed by SV-40 virus: C57BL/6SV (H-2^b), B10D2SV (H-2^d), and C57BRSV (H-2^k) served as the target cells.

When measuring cytotoxicity, the target cells were transferred to a 96-well plate (20×10^3/well) simultaneously with CTL for suspension targets (P-815) or 2 h after addition of adhesive targets (all fibroblasts).

CTL were removed using supernatant from H-35-17-2 hybridoma containing antibodies to CD8 coreceptor and rabbit complement pretested for the absence of spontaneous toxicity.

Activity of CTL was evaluated by the percentage of suppression of metabolic activity in the system and calculated by the formula:

$$\frac{\text{OD control-OD experiment}}{\text{OD control}}\times100\%.$$

The results were statistically processed using Student's t test. The differences were considered significant at $p<0.05$.

RESULTS

Alamar blue is a dye previously used for evaluation of the toxicity of chemical compounds and microorganisms [4]. In order to develop a method for testing cytotoxic activity of T lymphocytes by the intensity of alamar blue incorporation it was necessary to determine the optimal effector/target ratio. To this end we analyzed the intensity of alamar blue incorporation by different target cells. OD of analyzed cells was directly proportional to their quantity (Fig. 1, *a*).

At the next stage we studied the possibility of using alamar blue for assessing specific cytotoxicity of T lymphocytes (Fig. 1, *b*). Transformed mouse fibroblasts of different haplotypes were used as the targets. The effect of two CTL strains was studied: C57BL/10-anti-B10.D2 (H-2^b-anti-H-2-^{d}) and BALB/c-anti-C57BL/10 (H-2^d-anti-H-2^b). In order to prevent dye incorporation by T killers, they were removed using antibodies to CD8 and complement marker 18 h after interaction with the targets. The effect of T killers was strictly specific and they did not cross-react

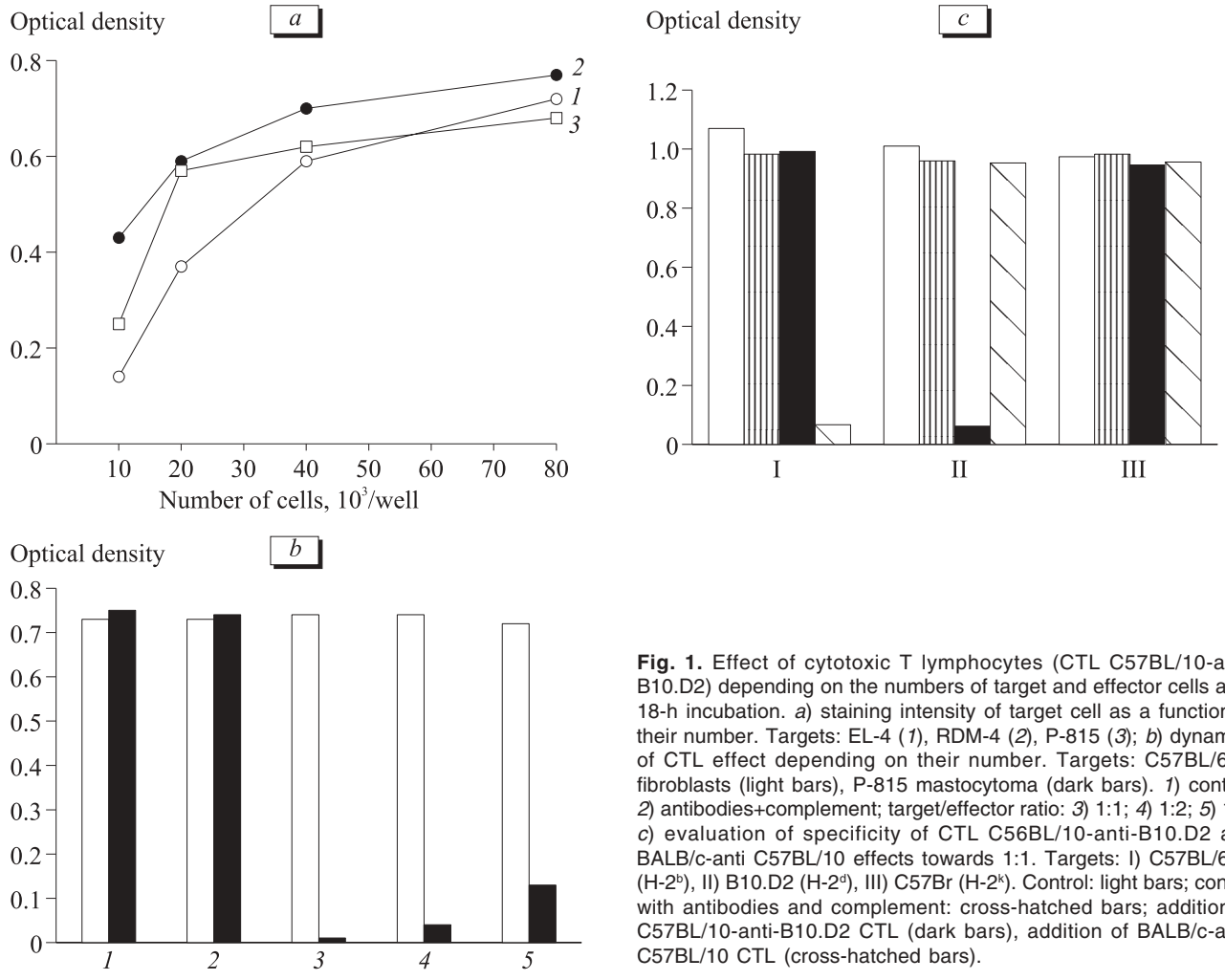

Fig. 1. Effect of cytotoxic T lymphocytes (CTL C57BL/10-anti-B10.D2) depending on the numbers of target and effector cells after 18-h incubation. *a*) staining intensity of target cell as a function of their number. Targets: EL-4 (*1*), RDM-4 (*2*), P-815 (*3*); *b*) dynamics of CTL effect depending on their number. Targets: C57BL/6SV fibroblasts (light bars), P-815 mastocytoma (dark bars). *1*) control; *2*) antibodies+complement; target/effector ratio: *3*) 1:1; *4*) 1:2; *5*) 1:4; *c*) evaluation of specificity of CTL C56BL/10-anti-B10.D2 and BALB/c-anti C57BL/10 effects towards 1:1. Targets: I) C57BL/6SV (H-2^b), II) B10.D2 (H-2^d), III) C57Br (H-2^k). Control: light bars; control with antibodies and complement: cross-hatched bars; addition of C57BL/10-anti-B10.D2 CTL (dark bars), addition of BALB/c-anti-C57BL/10 CTL (cross-hatched bars).

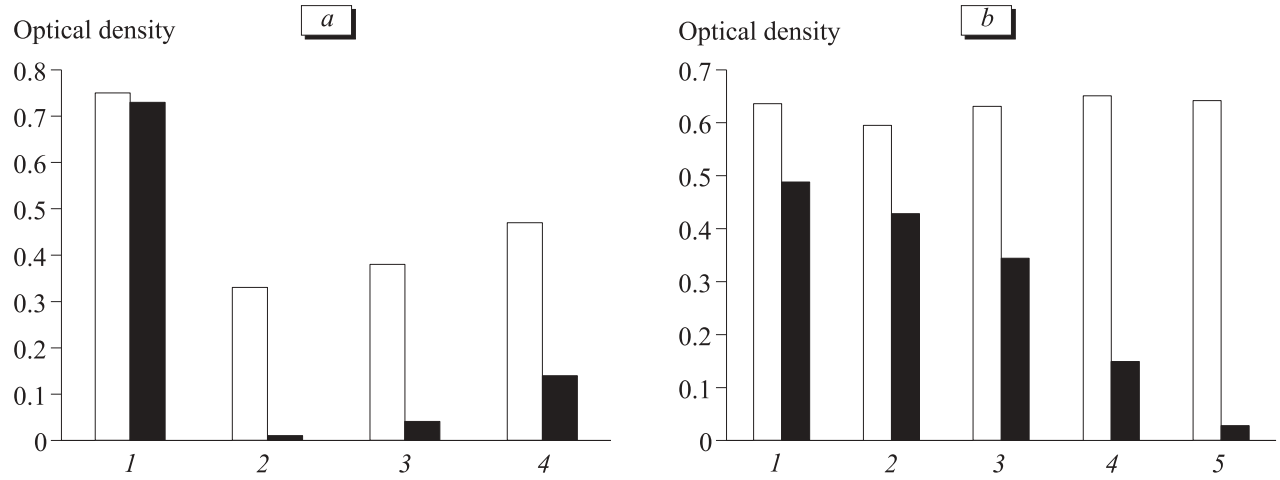

Fig. 2. Testing of CTL (C57BL/10-anti-B10.D2) effect in their presence after 18-h incubation of targets with CTL. *a*) intensity of P-815 target staining in the presence of viable CTL (light bars) and without CTL (dark bars). *1*) control (for targets without CTL: cells treated with antibodies and complement); target/effector ratio: *2*) 1:4; *3*) 1:2; *4*) 1:1. *b*) evaluation of the effect of viable CTL on mouse fibroblasts C57BL/6SV (light bars) and B10.D2 (dark bars). *1*) control; target/effector ratio: *2*) 1:0.25; *3*) 1:0.5; *4*) 1:1; *5*) 1:2.

with syngeneic and foreign targets of the H-2^k haplotype (Fig. 1, *b*).

In the next experimental series we evaluated the relationship between the cytotoxic effect and the amount of CTL used in experiment and removed at the stage of testing (Fig. 1, *c*). All selected ratios gave significantly different results, but we should like to emphasize that the increase of the number of effectors even with lost viability slightly increased OD values in general (Fig. 1, *c*).

In order to simplify the method, we compared OD values in the presence and absence of viable CTL by the moment of testing. Though live CTL incorporate the dye together with the targets, their presence did not abolish significant differences between the control and experimental groups (Fig. 2, *a*).

CTL elimination 18 h after their interaction with allogenic targets (P-815) appreciably increased the sensitivity of the method. Analysis of cytotoxicity in the presence of viable CTL is possible with fibroblasts used as targets (Fig. 2, *b*). The maximum effect was observed at the 1:2 target/CTL ratio.

Allospecific T cell strains derived *in vitro* possess a much higher cytolytic activity compared to primary killers. It was therefore interesting to evaluate the possibility of testing by this method cytotoxic activity of CTL from mouse lymph nodes obtained 1 week after immunization (Fig. 3, *a*). With fibroblasts serving as the targets in the presence of viable primary CTL, this method revealed a pronounced cytotoxic effect (Fig. 3, *a*).

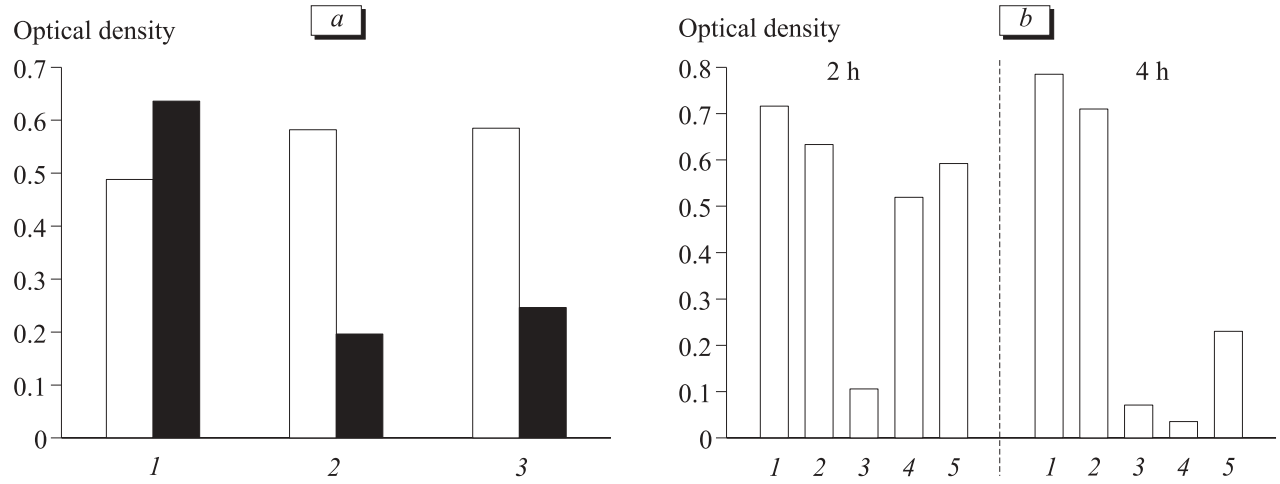

Fig. 3. Evaluation of effects of different CTL. *a*) effect of primary CTL (BALB/c-anti-C57BL/10) on fibroblasts of M10D2SV (light bars) and C57BL/6SV mice (dark bars) after 18-h incubation of targets with CTL. *1*) control; target/effector ratio *2*) 1:3; *3*) 1:10. *b*) effect of CTL (C57BL/10-anti-B10.D2) depending on the time of incubation with target cells (P-815 mastocytoma). *1* — control; *2* — complement; target/effector ratio: *3*) 1:1; *4*) 1:0.5; *5*) 1:0.25.

The targets were exposed to CTL for 18 h in all experiments. When determining the minimum duration of the contact needed for realization and recording of the cytotoxic effect, we incubated target cells with CTL in different ratios for 2 and 4 h (Fig. 3, *b*). After incubation the effector cells were removed using antibodies and complement, and alamar blue was added for the next 18 h. Incubation for 4 h was sufficient for testing a pronounced cytotoxic effect even if the number of killers is 4-fold lower than the number of target cells. Hence, analysis of CTL activity by this method can be completely realized within 18-24 h.

Cytotoxic T lymphocytes are the main subpopulation of T cells protecting the organism in viral infections, tumor growth, and graft rejection. These cells are actively studied, which necessitates analysis of CTL efficiency towards different antigens under experimental and clinical conditions. We proposed a new colorimetric method for evaluation of T-cellular cytotoxicity, based on incorporation of alamar blue dye. This method was used in experiments with allospecific cytotoxic T lymphocytes and with primary T killers constituting a small part of the analyzed cell population. The advantage of this test is simple procedure for evaluation of the intensity of specific cell cytotoxicity, while its sensitivity allows evaluating the cytotoxicity of different CTL.

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