HETEROGENEITY OF IMMUNOLOGIC MEMORY T CELLS SPECIFIC FOR H-2 ANTIGENS AND DETECTION OF THEIR RECEPTORS

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The formation of cytotoxic T lymphocytes (CTL) *in vitro* requires cooperation between macrophages [11] and at least two subpopulations of T cells [6]. One of these consists of CTL precursors carrying Lyt 2,3 markers, the other consists of cells carrying Lyt 1 amplifiers. After activation by antigen, presented on living stimulator cells the T amplifiers produce a humoral mediator, interleukin-2 which, in the presence of the corresponding antigen, induces proliferation and differentiation of CTL from their precursors [12, 13]. Conversely, during immunization of lymphocytes *in vitro* by killed cells or soluble alloantigens no CTL are formed, because T amplifiers are not activated [8, 12]. This defect is compensated by the addition of interleukin-2 to a mixed lymphocyte culture (MLC), and this leads to CTL formation.

The secondary immune response (immunologic memory) is characterized by a more intensive and rapid response of the T cells to antigens of the H-2 complex than the primary response [16]. This is due both to an increase in the number of CTL precursors after immunization [17] and to a qualitative change in the properties of the T memory cells (MC): they acquire the ability to react to H-2 antigens presented not only on living cells, but also on killed stimulator cells [2, 8, 16]. At least two categories of T cells also are involved in the secondary immune response. Unlike primary, secondary T amplifiers can be activated by antigen presented not only on living, but also on killed cells, and after γ-ray irradiation they can still cooperate with CTL precursors [14].

The object of this investigation was to study differences in the properties of receptors of two categories — secondary amplifiers and precursors of secondary CTL.

EXPERIMENTAL METHOD

To induce H-2^d anti-H-2^b (d anti-b) MC, B10.D2 (H-2^d) mice were immunized intraperitoneally with 2×10^7 ascites leukemia EL-4 cells from C57BL/6 (H-2^b) mice. After 8-10 weeks, 5×10^6 spleen cells from immune or intact B10.D2 mice were stimulated in MLC by incubating them with equal numbers of stimulating C57BL/6 spleen cells, heated for 1 h to 45°C [16], in 2 ml of RPMI-1640 with additions, in FB-16-24-TC plates (Flow Laboratories, England) at 37°C for 4 days in an atmosphere with 5% CO_2 [2].

Activity of the CTL was tested for 16 h by incubation at 37°C with target cells (TC), namely, peritoneal macrophages labeled with ^{51}Cr and cultured for 48 h in a 96-well FB-96-TC plate (Linbro, USA). The cytotoxic index (CTI) was calculated by the equation

$$CTI = \frac{a - b}{c - b} \times 100,$$

where a, b, and c denote liberation of ^{51}Cr after incubation of TC with CTL, medium, and 2% sodium dodecylsulfate solution, respectively [3].

Adsorption of MC on monolayers of macrophages in large (No. 3024) and small (No. 3012) plastic flasks (Falcon Plastics, USA) and subsequent elution of adherent lymphocytes with pronase were carried out by the method described previously [1, 4].

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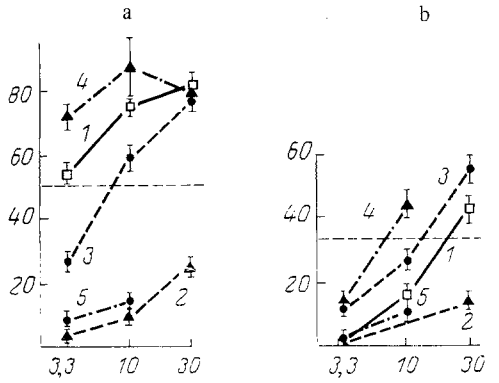

Fig. 1

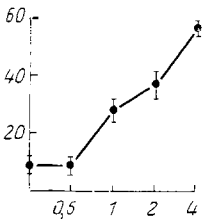

Fig. 2

Fig. 1. Enrichment of eluted B10.D2 lymphocytes with anti-C57BL/6 MC. a, b) Two independent experiments. Abscissa, dose of secondary CTL ($\times 10^4$) in reactions to C57BL/6 TC; ordinate, CTI (in %). 1) MC not fractionated, 2) not adherent to monolayer of C57BL/6 macrophages, 3) the same for B10.D2, 4) eluted from C57BL/6 monolayer and incubated in MLC with heated C57BL/6 stimulators, 5) the same for B10.D2.

Fig. 2. Dependence of cytotoxic effects of lymphocytes on ratio of secondary amplifiers and primary CTL precursors in MLC, stimulated by heated cells. Abscissa, number of immune B10.D2 anti-C57BL/6 spleen cells ($\times 10^6$) not adherent to C57BL/6 macrophages, mixed with 4×10^6 normal B10.D2 spleen cells and 4×10^6 heated C57BL/6 stimulators; ordinate, CTI (in %) of 3×10^5 secondary CTL, lysing C57BL/6 TC.

TABLE 1. Interaction between Secondary Amplifiers and Precursors of Primary CTL in MLC

Variants	Reacting B10.D2 spleen cells in MLC			CTI %*
	immune d anti-b		normal	
	unfractionated	not adherent to C57BL/6 macrophages		
1				66.0±4.5
2				12.0±3.4
3				14.0±2.7
4**				52.0±5.4

Legend. *) Dose of CTL 3×10^5 (M ± m, 10 experiments), **) 4×10^6 cells of each type were mixed in culture.

EXPERIMENTAL RESULTS

To detect receptors for antigens of the H-2 complex on d anti-b MC, immune B10.D2 anti-C57BL/6 lymphocytes were adsorbed on a monolayer of C57BL/6 or B10.D2 macrophages for 2 h at 37°C, and lymphocytes not adhering to the monolayers were incubated in MLC with heated C57BL/6 stimulators. It will be clear from Fig. 1 that the ability of MC to generate secondary CTL was lost after their adsorption on a C57BL/6 cell monolayer, but it was only very slightly changed after control adsorption on a monolayer of syngeneic B10.D2 macrophages.

To enrich the MC, lymphocytes adherent to the C57BL/6 monolayer were eluted, washed free from pronase, and stimulated by C57BL/6 cells in MLC as indicated above. Lymphocytes not ad-

herent to the monolayer accounted for about 87%, and those eluted for 3% of the cells added to the monolayer. It will be clear from Fig. 1 that activity of secondary CTL generated by MC eluted from the C57BL/6 monolayer was trebled compared with activity of CTL generated by unfractionated MC, as reflected in the 50% or 33% lysis (Fig. 1a, b) of TC. This increase in MC activity was specific, for lymphocytes eluted from a monolayer of B10.D2 macrophages did not contain MC.

The adsorption—elution method can thus be used to demonstrate antigen-binding receptors on MC and to remove them or, on the contrary, to enrich the lymphocyte population with them. In the next experiments, using this same method, heterogeneity of MC was discovered.

Immune d anti-b lymphocytes not adherent to a monolayer of C57BL/6 macrophages were divided into two parts, and one of them was mixed with heated C57BL/6 stimulators alone, whereas the other was treated, in addition to these stimulators, with an equal number of lymphocytes from intact B10.D2 mice. It will be clear from Table 1 that the activity of the secondary CTL generated by nonadherent MC (variant 2) was much lower than activity of CTL obtained from unfractionated MC (variant 1). Equally low activity of CTL was found after stimulation of splenic lymphocytes of intact mice by killed allogeneic cells (variant 3). Conversely, when a mixture of two types of reacting B10.D2 lymphocytes was used — normal and immune, not adherent to a monolayer of C57BL/6 macrophages (variant 4) — activity of the secondary CTL obtained from such cultures was restored almost to its original level.

Abolition of the activity of MC after their adsorption on a monolayer of the corresponding target cells is thus connected with selective adhesion of only the precursors of secondary CTL to this monolayer, whereas secondary amplifiers do not adhere to the monolayer under the given conditions.

To study the quantitative relations between precursors of primary CTL and secondary amplifiers required for CTL generation in MLC by killed stimulators, the dose of amplifiers not adherent to the C57BL/6 monolayer was varied. It will be clear from Fig. 2 that activity of the CTL thus formed falls proportionally to the decrease in the fraction of secondary amplifiers. An eightfold reduction in the dose of secondary amplifiers completely abolishes their aid for CTL precursors.

This investigation thus showed that specificity of formation of secondary CTL from MC immune to H-2 antigens is connected with the presence of receptors for H-2 antigens on the surface of MC, by means of which these cells are able to adhere selectively to a monolayer of macrophages of the corresponding H-2 haplotype. Similar results were obtained previously by the use of spleen cells as the adsorbing monolayer [9, 10]. Since an increase in the concentration of secondary CTL was observed only if their precursors were eluted from a monolayer of corresponding allogeneic (but not syngeneic) TC, it must be assumed that this increase was due to quantitative enrichment of the cell population with specific MC. Dependence of the magnitude of the effect of secondary CTL on the number of their direct precursors present in the MC population was definitely proved by the method of limiting dilutions of MC [17] and also by the use of stable CTL lines and their clones [7]. Nevertheless, the possibility still remained that enrichment of MC after adsorption and elution in the present experiments was due to an increase in the concentration, not of precursors of secondary CTL, but of amplifiers, promoting CTL differentiation. However, the present investigation showed that secondary amplifiers cannot adhere to a monolayer of macrophages carrying the corresponding H-2 antigens (Table 1). The adsorption—elution procedure thus enables the two categories of MC mentioned above to be separated physically. This difference between them is evidently associated with lower affinity of the receptors of secondary amplifiers than of receptors of secondary CTL precursors, in agreement with data obtained previously in primary MLC: T cells proliferating on contact with antigen, unlike precursors of primary CTL, are not adsorbed on a TC monolayer [5]. The ability of secondary amplifiers, separated from precursors of secondary CTL either by the method of adsorption on a TC monolayer (Table 1), or by the use of antibodies against their different markers [18], or by the difference in their radiosensitivity [14], to react, unlike primary amplifiers, to killed stimulating cells, is their distinguishing quality.

The ability of MC eluted with TC and separated from the main mass of nonadherent secondary amplifiers to form highly active secondary CTL demonstrates that help of amplifiers is necessary for precursors of secondary CTL to differentiate. The possibility that a small dose of secondary amplifiers adheres to the monolayer and is contained in the population of eluted cells is unlikely, for the behavior of this cell population soon leads to their inactivation

(Fig. 2). The alternative possibility is increased sensitivity of precursors of secondary CTL compared with those of primary CTL to the action of the amplifiers, more especially because secondary precursors of CTL, unlike primary, can differentiate in the absence of proliferation [15]. Whatever the case, the inability of precursors of primary CTL, unlike precursors of secondary CTL, to adhere specifically to the fixed cells of a monolayer carrying the corresponding H-2 antigens [4] indicates peculiarities of structure of the antigen-binding receptor of MC (precursors of secondary CTL), and their essential differences from precursors of primary CTL. The hypothesis that secondary CTL can differentiate from their precursors without the help of amplifiers requires further study.

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COMPARATIVE STUDY OF METHODS OF PERFRINGENS TYPE A ANTITOXIN ASSAY

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To determine the level of perfringens antitoxin in human and animal blood sera the toxin neutralization test (TNT), which exists in two variants, is as a rule chosen. One of these is based on neutralization of the test toxin followed by determination of residual α-toxin activity *in vivo* in albino mice. The use of the second variant, namely, titration of antitoxin *in vitro* is associated with the enzymic nature of the α-toxin (phospholipase C, or PLC). After neutralization of the test toxin, residual activity of PLC is determined by the use of hen egg lecithovitellin as the substrate. The use of the passive hemagglutination test (PHT) with an erythrocytic diagnostic serum obtained by sensitizing erythrocytes with highly purified *Clos-*

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