Early transmembrane events in alloimmune cytotoxic T-lymphocyte activation as revealed by stopped-flow fluorometry

(major histocompatibility antigen/membrane fluidity/calcium influx)

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ABSTRACT We have studied early transmembrane events in mouse alloimmune cytotoxic T-lymphocyte (LC7, H-2b) activation by specific target cells (mouse mastocytoma P815, H-2d) and a mitogenic lectin, Con A, by using stopped-flow fluorometry with three different fluorescent probes. After binding to target cells (P815), cytotoxic T lymphocytes (LC7) first increased their membrane fluidity and, then, calcium was released from intracellular stores. After that, there was a calcium influx from the external medium into the T lymphocytes. This calcium influx was blocked by calcium antagonists (verapamil or diltiazem). The same sequence of events was also observed in the activation of T lymphocytes (LC7) by Con A and in the response of specific target cells (P815) after cytotoxic T lymphocyte (LC7) binding. Nonspecific (syngeneic) target cells (mouse lymphoma EL-4, H-2d) did not cause any early transmembrane events in cytotoxic T lymphocytes (LC7, H-2b).

There is a considerable amount of evidence that the activation of T lymphocytes is mediated by an increase in the concentration of cytosolic calcium (1–5). However, the relationships between such an increase in intracellular free calcium and the early transmembrane events involved in lymphocyte activation are still unclear (6, 7). Here, we have traced early events in alloimmune cytotoxic T-lymphocyte (CTL) activation by target tumor cells and a mitogenic lectin, Con A, by using stopped-flow fluorometry with three different fluorescence probes (8).

CTL are believed to play a crucial role in immunity to virus infections, tumors, and allograft rejections (9–12). CTL-mediated cytotoxicity is composed of at least three distinct steps: initial recognition or binding, programming for lysis (a lethal hit), and target cell disintegration. CTL can program target cells for lysis within several minutes of initial contact. A strong calcium dependence of the lethal hit has been suggested, whereas the preceding recognition–adhesion step seems to be calcium independent (6, 7, 13).

In this communication, we show that mouse alloimmune CTL (LC7) first increased their membrane fluidity after binding to specific target tumor cells (P815), then calcium was released from intracellular stores. After that, there was a calcium influx into CTL from the external medium.

MATERIALS AND METHODS

Materials. Quin 2AM was purchased from Dojin Chemicals (Kumamoto, Japan), A23187 from Calbiochem and RPMI 1640 from GIBCO. Chlorotetracycline, verapamil, and diltiazem were obtained from Sigma. Con A was obtained from E-Y Laboratories (San Mateo, CA).

Cells. Interleukin 2-dependent mouse CTL (LC7) is a long-term cultured blast-cell line originating from a secondary mixed-lymphocyte culture between C57BL/6 (H-2b, responder) and DBA/2 (H-2b, stimulator) (14) cells. Cell line LC7 was grown in RPMI 1640 with 10% (vol/vol) fetal calf serum (with Con A supernatant) (14). Specific target cells, mouse mastocytoma P815 (H-2d), were passaged in DBA/2 female mice. Nonspecific (syngeneic) target cells, mouse lymphoma EL-4 (H-2b), were passaged in C57BL/6 mice.

Labeling of Fluorescent Compounds. Fluorescence probes were incorporated into LC7, P815, and EL-4 cells in phosphate-buffered saline (PBS, 150 mM sodium phosphate, pH 7.3). For 10 μM quin 2AM in PBS incubation was for 40 min at 37°C; for 50 μM chlorotetracycline in PBS it was for 40 min at 37°C; and for 10 μM 2-(1-pyrenebutyloxy)steaeric acid or 12-(1-pyrenebutyloxy)steaeric acid in PBS it was for 40 min at 37°C. Quin 2AM was hydrolyzed to quin 2 in the cell by the cell esterases. These processes were monitored by the shift in the emission spectrum from the quin 2AM peak at 430 nm to the quin 2 peak around 490 nm (2, 15). After labeling cells were centrifuged for 1 min at 1000 rpm in a Fisher centrifuge model 59. Then they were washed with PBS and were resuspended in PBS containing 1 mM Ca2+ for stopped-flow measurements. Cell viability (more than 98%) was not changed after stopped-flow measurements as judged by trypan blue dye exclusion.

Methods. Fluorescence spectra were measured with a Hitachi fluorescence spectrophotometer model 650-10S. Stopped-flow fluorescence was measured with a Union Giken stopped-flow spectrophotometer RA-401 in combination with a microcomputer RA-450 system (16). The dead time of this instrument is 0.5 ms. Here, the dead time means the time required for mixing plus the earliest time at which data can be taken. In our present experiment, the sensitivity for stopped-flow measurements was improved by data accumulation with an on-line computer. That is, 64 successive data points for 20 ms were stored as one averaged data point in a computer. The experimental trace (see Figs. 1, 3, and 4) was a result of 6–10 accumulated individual stopped-flow measurements that consisted of a series of averaged data points mentioned above. We used a Hoya Y46 cut filter (which allows the emitted light with wavelength longer than 460 nm to come into the detector) in the fluorescence measurements.

RESULTS

Calcium Influx into CTL. Quin 2 is a fluorescent indicator for intracellular free calcium (2, 15). This tetracarboxylate anion binds Ca2+ with a 1:1 stoichiometry and a concomitant 5-fold increase in fluorescence emission (2, 15). Mouse alloimmune CTL (LC7, H-2b), which mediate cytotoxic reactions against mastocytoma P815 cells (H-2d) (14), were loaded with quin 2, by incubation with an acetoxymethylenelester of quin 2, quin 2AM. In the cytoplasm the ester was

Abbreviation: CTL, cytotoxic T lymphocyte(s).

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hydrolyzed by lymphocyte endogenous esterases to produce the free-acid quin 2 that can bind Ca\(^{2+}\) in the cytoplasm. Fig. 1a shows a stopped-flow fluorometry trace obtained from T lymphocytes (LC7, H-2\(^b\)) loaded with quin 2 and mixed with specific target cells, mouse mastocytoma cells P815 (H-2\(^b\)), at 25°C. After binding of T lymphocyte LC7 to mastocytoma P815, the fluorescence of quin 2-loaded CTL rose in 2-3 s (rate constant = 1.2 s\(^{-1}\)) indicating a rise of intracellular free Ca\(^{2+}\) concentration (0.1 \(\mu M\)) to a new steady-state level (estimated to be 0.5-0.6 \(\mu M\)). The calcium concentration was determined by the procedure of Tsien et al. (15), by using a fluorescence spectrophotometer. It is only an average value, and we assumed here that nearly all of the cells in the population were activated.

When 2 mM EGTA was introduced in the external medium, the fluorescence increase disappeared (Fig. 1b). This fact indicates that the observed fluorescence increase corresponds mostly to the Ca\(^{2+}\) influx from the external medium into T lymphocytes. As a control experiment, we also mixed quin 2-loaded CTL with PBS alone, and no fluorescence increase was observed in the time range (Fig. 1a, Lower).

Distribution of Calcium. Next, we examined the effects of P815 cells on the chlorotetracycline fluorescence of T lymphocytes (LC7). After binding to the specific target cells (P815), the chlorotetracycline fluorescence of T lymphocytes decreased with a rate constant of 1.7 s\(^{-1}\) as seen in Fig. 1c, which was slightly greater than that of quin 2 fluorescence increase. Even when 2 mM EGTA was present in the external medium, a chlorotetracycline fluorescence decrease of T lymphocytes was observed (data not shown). Such a chlorotetracycline fluorescence decrease is considered to correspond to a removal of Ca\(^{2+}\) from internal stores in the cytoplasm (17-19). It is difficult to estimate the absolute amount of calcium ions redistributed between the membrane and cytoplasm, but it should be much smaller than the amount of calcium ions that entered the cytoplasm from the external medium. The observed rate (1.7 s\(^{-1}\)) of the process shows that the removal of membrane bound Ca\(^{2+}\) precedes the transmembrane flux of Ca\(^{2+}\) into lymphocyte cytoplasm (1.2 s\(^{-1}\)).

Membrane Fluidity Change of CTL. We attempted to examine a time-dependent change of membrane fluidity in CTL after binding to the target cells. Membrane fluidity can be measured by the use of a fluorescent pyrene derivative, 2-(1-pyrenebutyloxy)stearic acid, which forms excimer in fluid biological membranes (20, 21). These pyrene-loaded CTL had monomer fluorescence peaks at 382 and 400 nm and a broad excimer fluorescence peak around 480 nm as shown in Fig. 2. After binding to the specific target cells (P815), the excimer fluorescence in alloimmune CTL membranes rose at the rate of 3.6 s\(^{-1}\) as may be seen in Fig. 1d. This indicates a rapid rise of membrane fluidity. When another derivative, 12-(1-pyrenebutyloxy)stearic acid, was used as a probe, the amount of excimer of T-lymphocyte membranes increased at an even higher rate, 4.2 s\(^{-1}\). The difference in the rate of excimer formation of the two pyrene derivatives is probably caused by the microheterogeneity in biological membranes. Another explanation, however, is that there is a difference in the location of the probe in the lymphocyte membranes. That is, the pyrene moiety of 12-(1-pyrenebutyloxy)stearic acid should be located near the end of the hydrocarbon tails of phospholipids, while the pyrene moiety of 2-(1-pyrenebutyloxy)stearic acid should be located near the polar head group of phospholipids. The fluidity of the lipid bilayer is known to increase toward the terminals of the hydrocarbon chains (22-24). Therefore, the mobility of 2-(1-pyrenebutyloxy)stearic acids, located in the less fluid region, may increase slightly later than that of 12-(1-pyrenebutyloxy)stearic acid in the lymphocyte membrane. Anyway, it is clear that these fluorescence changes precede any of the effects ascribed to the calcium changes.

Non-specific Target EL-4 (H-2\(^b\)). To determine the effect of non-specific target cells on the CTL, we did similar experiments with EL-4, non-specific target cells (H-2\(^b\)). When each fluorescent compound [quin 2, chlorotetracycline, or 2-(1-pyrenebutyloxy)stearic acid] labeled CTL (LC7, H-2\(^b\)) was mixed with non-specific target cells (EL-4, H-2\(^b\)), no fluorescence intensity change was observed for any of these three fluorescent compounds in the time range as shown in Fig. 1 (data not shown). Therefore, the non-specific effect of the probe labeled non-specific target cells in the alloimmune CTL activation, described in the present experiments, must be due to the effects of specific initial recognition or binding between CTL (LC7, H-2\(^b\)) and specific target cells (mastocytoma P815, H-2\(^b\)).

Con A Binding to CTL. The early transmembrane events involved in lymphocyte activation were observed also with the mitogenic lectins Con A. The binding of Con A to T-lymphocyte surface receptors is known to induce a series of biochemical events attributable to the activation of T
lymphocytes (4, 25). Fig. 3a shows that a Ca\(^{2+}\) influx of CTL (LC7) took place after binding of Con A (0.5 \(\mu\)M). Rate constants of the fluorescence increase (from 1.6 to 1.2 s\(^{-1}\)) were almost independent of the concentration of Con A (50 nM to 5 \(\mu\)M). After binding of Con A (0.5 \(\mu\)M), redistribution of membrane bound Ca\(^{2+}\) in lymphocytes took place at a rate comparable to that of the Ca\(^{2+}\) influx into lymphocyte cytoplasm (Fig. 3b). The excimer fluorescence of 2-(1-pyrenebutyryloxy)stearic acid-loaded mouse CTL LC7 cells at 25\(^\circ\)C. Excitation wavelength, 340 nm.

![Figure 2](image)

**Fig. 2.** Fluorescence spectrum of 2-(1-pyrenebutyryloxy)stearic acid-loaded mouse CTL LC7 cells at 25\(^\circ\)C. Excitation wavelength, 340 nm.

In the present experiments we showed that specific target cells (P815, H-2\(^b\)) caused the series of the early transmembrane events (membrane fluidity change, redistribution of calcium, and calcium influx into the cytoplasm) in CTL (LC7, H-2\(^b\)). Table 1 shows that the rates of the early transmembrane events in the CTL (LC7) are close to those of the corresponding events in specific target cells (mastocytoma P815). The initial recognition could be initiated by the collision between the CTL and specific target cells. A stable binding between these cells would result from the interaction of the CTL membrane receptors and the target cell antigens (major histocompatibility antigen, H-2\(^b\)). These processes would then initiate the early transmembrane events in both cells, CTL and specific target cells. In contrast, syngeneic (nonspecific) target cells (EL-4, H-2\(^b\)) did not cause the early transmembrane events in CTL (LC7, H-2\(^b\)) at all.

**DISCUSSION**

In the present experiments we showed that specific target cells (P815, H-2\(^b\)) caused the series of the early transmembrane events (membrane fluidity change, redistribution of calcium, and calcium influx into the cytoplasm) in CTL (LC7, H-2\(^b\)). Table 1 shows that the rates of the early transmembrane events in the CTL (LC7) are close to those of the corresponding events in specific target cells (mastocytoma P815). The initial recognition could be initiated by the collision between the CTL and specific target cells. A stable binding between these cells would result from the interaction of the CTL membrane receptors and the target cell antigens (major histocompatibility antigen, H-2\(^b\)). These processes would then initiate the early transmembrane events in both cells, CTL and specific target cells. In contrast, syngeneic (nonspecific) target cells (EL-4, H-2\(^b\)) did not cause the early transmembrane events in CTL (LC7, H-2\(^b\)) at all.

![Figure 3](image)

**Fig. 3.** Stopped-flow fluorometry traces indicating the effects of Con A on CTL (LC7). Fluorescent-labeled T-lymphocyte cells (final, 1 \(\times\) 10\(^{6}\) cells/ml) were mixed with Con A (final, 5 \(\times\) 10\(^{-7}\) M) at 25\(^\circ\)C. Experimental conditions were the same as those in Fig. 1. (a) The effect of Con A on the fluorescence of quin 2-loaded T lymphocytes (LC7). (b) The effect of Con A on the fluorescence of chlortetracycline-loaded T lymphocytes (LC7). (c) The effect of Con A on the fluorescence of 2-(1-pyrenebutyryloxy)stearic acid-loaded T lymphocytes (LC7).
We have found a similar pattern in other systems, for example, mouse myeloma cells X5563 (H-2K$^b$) and mouse lymphoma RDM4 (H-2K$^a$) after binding of anti-H-2K$^a$ monoclonal antibodies (11-4.1) (8) which suggests that the early transmembrane events described here may generally occur with a variety of ligand–receptor interactions. Perhaps the increase in membrane fluidity accompanying these events may be of general significance for cell activation.

It is now evident that in several systems an increase in cytosolic calcium following receptor triggering is preceded by the breakdown of phosphatidylinositol bisphosphate into 1,2-diacylglycerol and inositol triphosphate (26–29). The latter is known to cause a release of Ca$^{2+}$ from intracellular stores. The increase in membrane fluidity that precedes the breakdown of phosphatidylinositol bisphosphate may be required for the activation of phospholipase C. Perhaps the calcium ions needed for cell activation at the initial recognition or binding step of CTL–target cell interaction arise in a similar fashion.

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