# INTERACTION OF LYMPHOKINE-ACTIVATED KILLER CELLS WITH SUSCEPTIBLE TARGETS DOES NOT INDUCE SECOND MESSENGER GENERATION AND CYTOLYTIC GRANULE EXOCYTOSIS

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A unified model to explain lysis of target cells by different lymphoid cell populations with cytotoxic activity (e.g., CTL, NK cells, and lymphokine-activated killer [LAK][1] cells) holds that target cell binding triggers effector cell secretion of cytotoxic granules directed against the target cell membrane (1, 2). These granules contain pore-forming proteins, called perforins, which show functional and structural similarities to the C9 component of complement (3, 4), cytotoxic peptides immunologically related to TNF (5), as well as a number of enzymes, such as proteoglycans and serine esterases, whose role in the lytic process has not been completely elucidated (6, 7). In agreement with the observation that $Ca^{2+}$ is required for perforin polymerization on the target cell membrane (8), target cell killing generally depends on the extracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_o$) (9). More recently, an equally important role has been attributed to the intracellular free $Ca^{2+}$ concentration ($[Ca^{2+}]_i$), as we (10) and others (11) have shown that CTL–target cell interaction leads to a rapid rise in $[Ca^{2+}]_i$, mediated in part by $Ca^{2+}$ release from intracellular stores, due to inositoltrisphosphate ($InsP_3$) formation, and in part by $Ca^{2+}$ influx from the medium through a still unknown mechanism (12). With the $[Ca^{2+}]_i$ rise, another second messenger is generated: diacylglycerol, the physiological activator of protein kinase C (13). A rise in $[Ca^{2+}]_i$ and the activation of protein kinase C are considered necessary to reorient the cytoskeletal apparatus of the effector cell and trigger granule secretion (14).

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[1] *Abbreviations used in this paper:* BLT, *N*-α-benzyloxycarbonyl-L-lysin thiobenzyl ester; $[Ca^{2+}]_i$, cytosolic-free $Ca^{2+}$ concentration; $[Ca^{2+}]_o$, extracellular $Ca^{2+}$ concentration; $InsP_1$, $InsP_2$, and $InsP_3$, inositol phosphate, bisphosphate, and trisphosphate, respectively; LAK, lymphokine-activated killer cells; M-MuLV, Moloney-murine leukemia virus.

Although this cytotoxic mechanism is supported by a number of direct and indirect experimental observations, it has been challenged by the recent finding that CTL-induced lysis can occur even when effector cells lack lytic granules, or granule content release is absent (15, 16). Moreover, some CTL clones induce lysis even when extracellular $Ca^{2+}$ has been chelated (17). Therefore, $Ca^{2+}$-independent cytolytic mechanisms have been proposed in addition or as an alternative to the classical $Ca^{2+}$-dependent granule exocytosis model (18).

In vitro stimulation of lymphocytes with supraoptimal concentrations of IL-2 leads to generation of cytolytic lymphocytes, designated as LAK cells, which, unlike CTL, lyse a broad spectrum of syngeneic and allogeneic tumor cells (19, 20). We report here that interaction of LAK cells with susceptible targets does not induce $[Ca^{2+}]_i$ increase, $InsP_3$ formation, and secretion of cytolytic granule-associated enzymes. Furthermore, using an antigen-specific CTL clone, which acquires LAK-like activity when cultured in medium containing high IL-2 doses, second messenger generation and cytolytic granule content secretion were not detected during lysis of unrelated target cells, while killing of specific targets triggered both these processes. Therefore, two lytic pathways seem to coexist in the same effector cell population: a second messenger-dependent pathway involving degranulation, which is activated after TCR interaction with specific targets, and another pathway independent of any known second messenger generation, which does not require granule secretion and is responsible for unrelated target cell lysis.

## Materials and Methods

*Mice.* Inbred C57BL/6 (B6), BALB/c, and DBA/2 8–10-wk-old mice were purchased from the Charles River Breeding Laboratories (Calco Como, Italy).

*Tumor Cell Lines.* MBL-2, a Moloney-murine leukemia virus (M-MuLV)-induced lymphoma, and P815, a chemically induced NK-resistant mastocytoma, were maintained in ascitic form by weekly intraperitoneal passages in syngeneic B6 and DBA/2 recipient mice, respectively. The NK-sensitive YAC-1 line, a subclone of M-MuLV-induced leukemic cells from A/SN mice, was maintained by continuous in vitro culture in complete medium. Complete medium consisted of DMEM (Gibco Laboratories, Glasgow, Scotland) supplemented with L-glutamine ($2 \times 10^{-3}$ M final concentration), Hepes ($10^{-2}$ M final concentration), 2-ME ($3 \times 10^{-5}$ M final concentration), antibiotics, and 10% heat-inactivated FCS (Flow Laboratories, Inc., Irvine, UK).

*Cell Cultures.* (*a*) LAK cells were generated by culturing spleen cells at a final concentration of $3 \times 10^6$ cells/ml in complete medium containing 100 U/ml rIL-2, (Glaxo Institute for Molecular Biology S. A., Geneva, Switzerland) in 250-ml tissue culture flasks (3024; Falcon Labware, Oxnard, CA). Cultures were incubated at 37°C in a water-saturated atmosphere containing 5% $CO_2$ in air. These cells were used either after 5 d (short-term LAK) or after 15–20 d in rIL-2 containing medium (long-term LAK). Flow cytometry analysis of these cell populations showed that ~60% of short-term LAK cells expressed the Thy-1.2 and Lyt-2.2 antigens, while only 10% were asialo-$GM1^+$; the expression of CTL-associated markers was even more striking in long-term LAK cells, where >85% of the cells were Thy-$1.2^+$ and Lyt-$2.2^+$. (*b*) MLC were prepared in 250-ml tissue culture flasks (3024; Falcon Labware) by mixing $4 \times 10^7$ responding spleen cells and $4 \times 10^7$ irradiated (4,500 rad) stimulator spleen cells in 30 ml of complete medium. (*c*) M-MuLV-specific CTL clone CHM-14 was maintained by weekly passages in bulk cultures together with irradiated MBL-2 stimulator and syngeneic feeder spleen cells in the presence of rIL-2 (10 U/ml) (21). Unspecific cytotoxic activity was induced by culturing these cloned cells for >10 d in complete medium supplemented with higher doses (100 U/ml) of rIL-2 or with supernatant from an IL-2-producing EL-4 lymphoma subclone, obtained as previously described (22).

*Cytolytic Assays.* Cytolytic activity was assessed as previously described (23) by incubating

serial dilutions of effector cells with $2 \times 10^3$ $^{51}$Cr-labeled ($Na_2{}^{51}CrO_4$; New England Nuclear, Dreiech, FRG) target cells in triplicate wells of round-bottomed microtiter plates (Sterilin, Teddington, Middlesex, UK) in a final volume of 0.2 ml. In some experiments, the $Ca^{2+}$ ionophore A23187 (50 nM) and PMA (32 nM) or Con A (10 μg/ml) were added after pelleting effector cells with the targets. After 4 h of incubation the plates were centrifuged and 0.1 ml supernatant was removed for counting.

The percentage of specific $^{51}$Cr release was calculated according to the expression: 100 × (experimental release − spontaneous release)/(maximum release − spontaneous release). In no case did spontaneous release exceed 20% of the maximum release.

*Esterase Activity Assay.* *N*-α-benzyloxycarbonyl-L-lysin thiobenzyl (BLT)-esterase secretion was evaluated by incubating $10^5$ effector cells with and without $3 \times 10^5$ targets, or with a combination of A23187 (50 nM) and PMA (32 nM) or with Con A (10 μg/ml) in 100 μl final volume of DMEM supplemented with Hepes ($10^{-2}$ M) and 2% FCS in round-bottomed microtiter plates (Sterilin). In preliminary studies this E/T cell ratio (1:3) was the most appropriate for evaluating BLT-esterase release; in fact, although target cell destruction increases with increasing E/T cell ratios, the opposite is true for target cell-induced enzyme release. In this case, the effector cell population constitutes a "read out" system, and so at a lower E/T cell ratio, more effector cells will be activated by target cell contact. After a 4-h incubation, 20-μl samples of supernatant were collected and BLT-esterase activity was assayed according to a minor modification of the Coleman and Green method (24). Briefly, duplicate samples were mixed with 180 μl of BLT solution ($2 \times 10^{-4}$ M BLT, $2.2 \times 10^{-4}$ M 5,5′-dithiobis-(2-nitrobenzoic acid) in PBS, pH 7.4) in flat-bottomed microtiter plates (3070; Falcon Labware). After 10 min at 37°C, absorbance was measured at 412 nm in an Titertek multiskan scanner (Flow Laboratories, Inc.) against a cell-free blank solution (DMEM, $10^{-2}$ M Hepes, 2% FCS) treated exactly as above. Total cellular enzymatic activity was evaluated by solubilizing effector cells in a 0.1% Triton X-100 solution. The OD of 1.0 was defined as 1 U of esterase activity, and values were calculated for $10^6$ cells. The percentage of enzymatic activity released was calculated according to the expression: 100 × (experimental release − spontaneous release)/(maximum release − spontaneous release).

*$[Ca^{2+}]_i$ Measurement with the Fluorescent $Ca^{2+}$ Indicator fura-2.* The loading procedure and calibration of the fluorescent signal in terms of $[Ca^{2+}]_i$ are described in detail in reference 10, except that the medium was routinely supplemented with 200 μM sulphinpyrazone, a drug capable of inhibiting fura-2 leakage from intact cells (25). Fura-2 leakage was greater in LAK cells than in CTL clones or lymphocytes from MLC. Controls run in parallel without sulphinpyrazone showed that this drug did not modify $[Ca^{2+}]_i$ under basal conditions, nor did it alter the $[Ca^{2+}]_i$ rises induced by Con A, the $Ca^{2+}$ ionophore A23187, or specific target cells. Furthermore, in sulphinpyrazone-treated cells, baseline fluorescence is stable for at least 15 min, making calibration far more accurate.

The standard protocol for measuring $[Ca^{2+}]_i$ rises after E/T cell interaction is described in reference 10. Briefly, target and effector cells (5:1 or 10:1) were cocentrifuged for 10 s at 1,000 *g*, incubated for 1–2 min at 37°C, and then resuspended in the fluorimeter cuvettte for $[Ca^{2+}]_i$ measurement.

*Inositol Phosphate Extraction and Separation.* Inositol phosphates were extracted and separated as described previously (10, 26). Briefly, lymphocytes were incubated for 24 h in RPMI 1640 inositol-free medium, supplemented with 1% FCS and 5 μCi/ml of [$^3$H]myoinositol (Amersham International, Amersham, UK). Cells were then washed and resuspended in fresh medium. The reaction was stopped by adding to the reaction mixture an equivalent volume of 15% ice-cold TCA. The samples were incubated for 1 min on ice, extracted five times with equal volumes of diethylether, neutralized with Tris, and applied either to Dowex columns to separate $InsP_1$, $InsP_2$, and $InsP_3$, or to a Partisil Sax HPLC column to separate the $InsP_3$ isomers. $InsP_3$ was eluted with a step gradient of $NH_4$-formate as described previously (10, 26).

## Results

*Interaction of LAK Cells with Susceptible Targets Does not Induce a Rise in $[Ca^{2+}]_i$.* We (10) and others (11) have recently observed that activation of CTL by their specific

targets leads to a rapid and transient rise in $[Ca^{2+}]_i$. To investigate whether a $[Ca^{2+}]_i$ rise is also needed to activate the killing process by other types of cytolytic lymphocytes, LAK cells were loaded with fura-2 and challenged with susceptible targets.

We found no significant change in $[Ca^{2+}]_i$ after LAK cell challenge with susceptible P815 mastocytoma and YAC-1 lymphoma target cells; in addition, $[Ca^{2+}]_i$ of LAK cells centrifuged in the absence of targets was the same as that of LAK cells cocentrifuged with susceptible targets (Fig. 1). No rise in $[Ca^{2+}]_i$ was observed when LAK cells were challenged with either the lysis-resistant target HL60 (a human leukemic cell line) or with other susceptible targets, such as MBL-2 lymphoma cells or normal thymocytes (data not shown). In numerous similar experiments, $[Ca^{2+}]_i$ in LAK cells alone was 110 ± 10 nM at the moment of resuspension; cocentrifugation with sensitive targets did not changes this value appreciably (115 ± 10 and 120 ± 20 nM with the P815 and YAC-1, respectively; Fig. 1 *inset*), which remained constant for the following 10 min. No rise in $[Ca^{2+}]_i$ was observed when cocentrifugation (10–60 s) and incubation (1–5 min) times were varied, nor when E/T ratios were changed from 1:5 to 1:20 (data not shown). Addition of the mitogenic lectin Con A to LAK cell cultures instead induced a $[Ca^{2+}]_i$ rise, the kinetics and amplitude of which were independent of previous stimulation with the target cells (Fig. 1). On the other hand, interaction of lymphocytes from MLC (H-$2^b$ anti-H-$2^d$) with their specific target P815 induced a rapid $[Ca^{2+}]_i$ increase (Fig. 2). In five different experiments the mean $[Ca^{2+}]_i$ was 140 ± 10 nM when effector cells were centrifuged alone or with unrelated targets, and 400 ± 20 nM when specific target cells were added (Fig. 2 *inset*); in the latter case, $[Ca^{2+}]_i$ remained above baseline levels for >5 min. Moreover, the net effect of Con A addition was smaller when CTL obtained in MLC were pretreated with specific targets compared with controls where no target or unrelated target had been added (Fig. 2). This result was expected, since, when

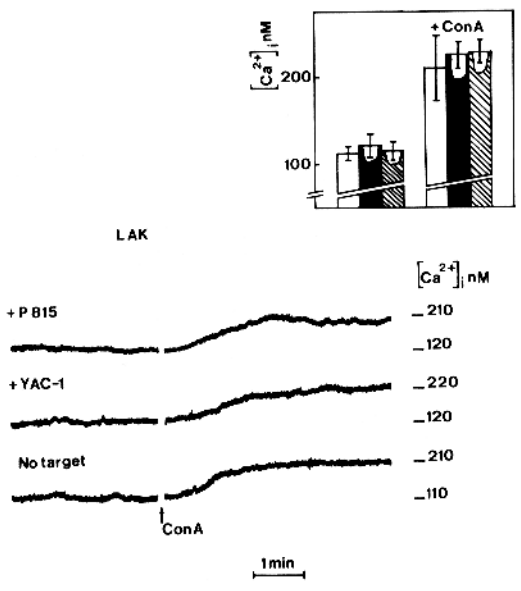


FIGURE 1. Interaction with susceptible targets does not increase $[Ca^{2+}]_i$ in short-term LAK cells. Medium (in millimoles/liter) included NaCl 130, KCl 5, $Na_3PO_4$ 1, $CaCl_2$ 1, $MgSO_4$ 1, glucose 5.5, sulphynpirazone 0.2, and Hepes 20 (final pH 7.4). LAK cells were obtained from 5-d spleen cell cultures, and loaded with fura-2 as described in Materials and Methods. The protocol of cocentrifugation is described in Materials and Methods and in reference 10. Tracing begins at the moment of cell resuspension in the fluorimeter cuvette, i.e., 90 s after the start of centrifugation. In this and in the following figures, calibration of the fluorescent signal in terms of $[Ca^{2+}]_i$ is reported on the right of each tracing. The arrow indicates the moment when Con A (10 μg/ml) was added. The inset shows the mean $[Ca^{2+}]_i$ (± SE) at the moment of resuspension (*bars on the left*) and 3 min after addition of Con A (*bars on the right*). LAK cells centrifugated: (□) without targets ($n$ = 4); (■) with P815 ($n$ = 3), or (▨) YAC-1 cells ($n$ = 6). The E/T ratio in all the experiments was 1:10.

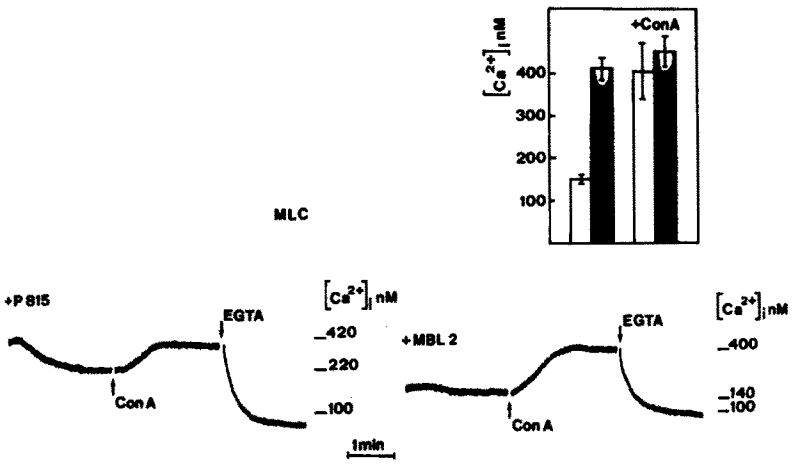


FIGURE 2. Interaction with antigen-specific targets increases $[Ca^{2+}]_i$ in lymphocytes from MLC. Conditions as in Fig. 1, but lymphocytes from MLC (b anti-d) were used against P815 cells. (*Left*) The fura-2-loaded effector cells were cocentrifuged with the sensitive, antigen-specific P815 cells; (*right*) the same effector cells were cocentrifuged with the irrelevant target MBL-2. Where indicated, Con A (10 μg/ml) and EGTA (1 mM) were added. (*Inset*) The bars on the left represent the mean $[Ca^{2+}]_i$ (± SE) at the moment of resuspension in the fluorimeter cuvette, and those on the right represent the maximum increase caused by Con A. CTLs cocentrifuged with (□) MBL-2 ($n$ = 9) or (■) P815 cells ($n$ = 2).

the lectin was added, $[Ca^{2+}]_i$ was already high in CTL interacting with specific targets.

To investigate whether the lack of significant rises in $[Ca^{2+}]_i$ could be attributed to cell heterogeneity, LAK cells from several long-term cultures were used. In five different such cultures, at the time of resuspension, the $[Ca^{2+}]_i$ was 100 ± 20 nM regardless of susceptible target addition (Fig. 3). In these cases as well, Con A raised $[Ca^{2+}]_i$ to the same level, whether targets were present or not.

When antigen-specific CTL clones are maintained in medium supplemented with supernatants from several IL-2-producing cell lines or with high (100 U/ml) rIL-2 doses, they also acquire unrestricted cytotoxic activity. In particular, the H-2-restricted MBL-2-specific CHM-14 clone cultured for >10 d under these conditions becomes able to lyse antigenically unrelated P815 and YAC-1 tumor cells (see below). This clone still exhibited a rise in $[Ca^{2+}]_i$ after challenge with MBL-2, but not when it was assayed against the susceptible P815 and YAC-1 tumor cells (Fig. 4). As far as $[Ca^{2+}]_i$ is concerned, these results are qualitatively the same as those obtained in the parental antigen-specific CHM-14 clone, which lysed only MBL-2 cells (Fig. 4 *inset* and reference 10).

*LAK Cell Interaction with Susceptible Target Cells Does not Stimulate Inositol Phosphate Production.* The $[Ca^{2+}]_i$ rise in antigen-specific CTL clones is associated with a transient formation of inositol phosphates (10, 27). Fig. 5 *a* shows that the interaction of LAK cells from short-term cultures with P815 cells did not increase the $InsP_3$ level above control values (i.e., LAK cells incubated under the same conditions but in the absence of targets). $InsP_1$ and $InsP_2$ phosphate concentrations were also

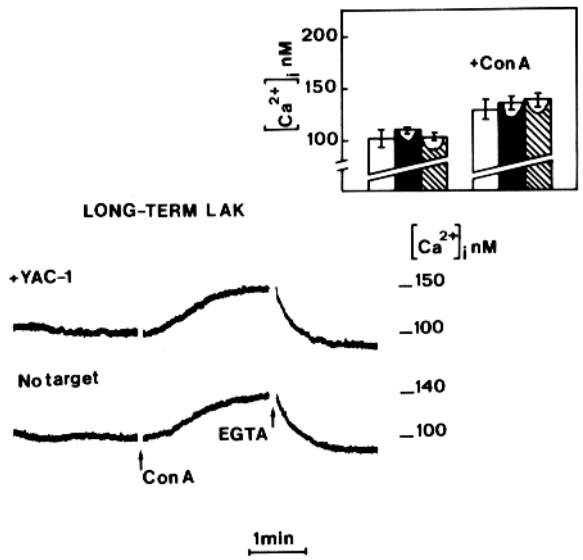


FIGURE 3. Interaction with susceptible targets does not increase $[Ca^{2+}]_i$ in long-term LAK cells. Conditions as in Fig. 1. Where indicated, Con A (10 μg/ml) and EGTA (1 mM) were added. (*Inset*) The mean $[Ca^{2+}]_i$ (± SE) at the moment of resuspension (*bars on the left*) and the maximum rise induced by Con A (*bars on the right*). LAK cells centrifuged: (□) without targets ($n$ = 4); (■) with P815 ($n$ = 3); or (▧) YAC-1 cells ($n$ = 6).

unaffected (Fig. 5 *a*). Similar results were obtained using other susceptible cells, such as YAC-1 and MBL-2 lymphomas and long-term LAK cell cultures as effectors (data not shown). By means of the HPLC technique, at least two different $InsP_3$ isomers can be separated, Ins 1,4,5$P_3$ and Ins 1,3,4$P_3$; only the 1,4,5,$P_3$ isomer is involved in $Ca^{2+}$ mobilization. We were unable to detect any rise in Ins 1,4,5,$P_3$ when LAK cells, from short-term cultures, were incubated with sensitive target cells (Fig. 5 *b*). Similar results were obtained for the Ins 1,3,4 isomer and for both isomers using long-term LAK cultures (data not shown). The most striking dissociation between second messenger generation and killing was observed in the CTL clone CHM-14

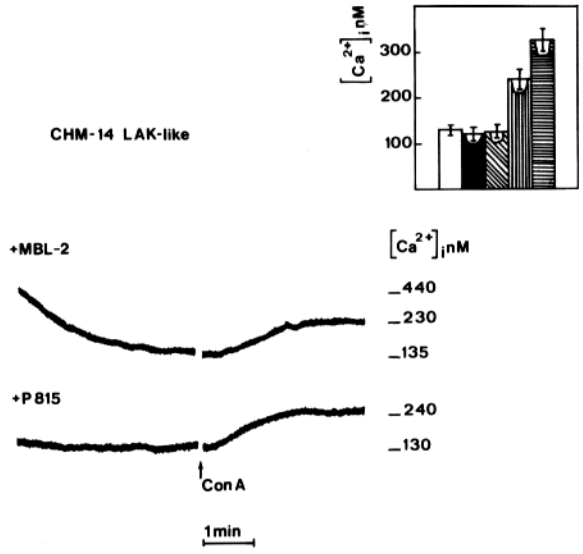


FIGURE 4. Only interaction with antigen-specific targets triggers in CHM-14 clone with LAK-like activity a rise in $[Ca^{2+}]_i$. The clone with LAK-like activity was obtained as described in Materials and Methods. All other conditions as in Fig. 1. The inset reports the mean $[Ca^{2+}]_i$ (± SE) at the moment of resuspension. CHM 14 (LAK-like) centrifuged: (□) without targets ($n$ = 3); with (■) P815 ($n$ = 3); (▧) YAC-1 ($n$ = 3), or (▥) MBL-2 ($n$ = 3). The bar on the extreme right (▤) represents the mean $[Ca^{2+}]_i$ in the antigen-specific CHM 14 clone centrifuged with the relevant target MBL-2.

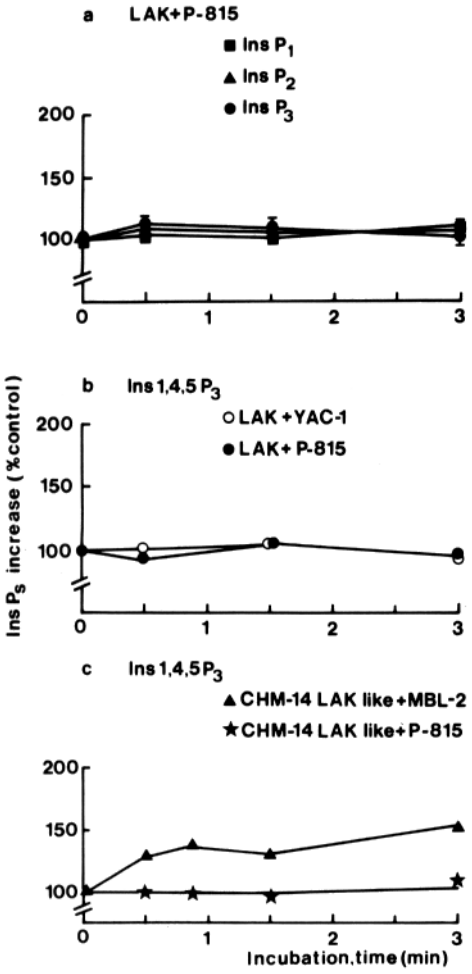


FIGURE 5. InsPs generation in effector cells challenged with susceptible targets. (*a*) short-term LAK cells (labeled with [$^3$H]myoinositol) were cocentrifuged with P815 cells and incubated at 37°C for the indicated time. InsPs were separated by Dowex columns as described in Materials and Methods. Values are expressed as percent of controls treated in the same way but without target cells. Each point is the mean (± SE) of five different experiments. (*b*) Ins 1,4,5$P_3$ was separated from other InsP isomers by HPLC. Each point is the mean (± SE) of duplicate determinations performed in two separate experiments; other conditions as in *a*) (*c*) The CHM-14 clone with LAK-like activity was incubated with two susceptible targets (MBL-2 and YAC-1). Ins1,4,5 $P_3$ was separated by HPLC; other conditions as in *a*.

cultured in the presence of high rIL-2 concentrations; in fact, the level of Ins 1,4,5 $P_3$ did not increase appreciably after interaction with unrelated but lysis-susceptible P815 cells (Fig. 5 *c*). A significant increase, albeit smaller than that observed in the clone with antigen-specific activity, was detected when the same clone was challenged with MBL-2 targets (Fig. 5 *c*).

*LAK Cell Interaction with Susceptible Target Cells Does not Induce BLT-Esterase Release.* BLT-esterase release into the culture supernatant is considered a specific marker of cytotoxic granule content exocytosis (28). In fact, enzyme release may be triggered in CTL by $Ca^{2+}$ ionophores + PMA, lectins, mAb to CD3-TCR complex, and interaction with specific targets (29, 30).

Table I shows that CTL and LAK cells obtained in short-term cultures have very similar enzyme levels, which increased ~10-fold, in long-term cultures. As expected, a high BLT-esterase level was also found in the CHM-14 CTL clone (Table II). The finding that the CTL clone having LAK-like activity contained a higher quantity

TABLE I

*Cytotoxic Activity and BLT-Esterase Release of LAK Cells and Alloreactive CTL*

| Effector cells* | Percent specific $^{51}Cr$ release | | | Percent specific BLT-esterase release | | | BLT-Esterase level |
|---|---|---|---|---|---|---|---|
| | MBL-2 | P815 | YAC-1 | MBL-2 | P815 | YAC-1 | |
| | | | | | | | *U/10⁶ cells*‡ |
| Short-term cultured: | | | | | | | |
| LAK | 24 ± 7 | 57 ± 18 | 59 ± 4 | 0 | 0 | 0 | 3.8 ± 2.6 |
| CTL (b anti-d) | 3 ± 2 | 62 ± 9 | ND | 1 ± 1 | 21 ± 3 | ND | 4.3 ± 1.6 |
| Long-term cultured: | | | | | | | |
| LAK | 26 ± 5 | 36 ± 13 | 46 ± 14 | 0 | 0 | 0 | 52.8 ± 9.9 |
| CTL (b anti-d) | 2 ± 2 | 58 ± 6 | ND | 0 | 22 ± 8 | ND | 48.0 ± 3.2 |

* Effector and target cells were incubated at 37°C for 4 h, at an E/T ratio of 50:1 for $^{51}Cr$ release assay and 1:3 for BLT-esterase release assay. After incubation, specific $^{51}Cr$ release and BLT-esterase activity in the culture supernatants were evaluated as described in Materials and Methods.

‡ Total cellular enzymatic activity was determined by solubilizing effector cells with a 0.1% Triton X-100 solution, and BLT-esterase units for $10^6$ were calculated as reported in Materials and Methods.

of BLT-esterase than the specific clone maintained at low IL-2 doses suggests that IL-2 has a stimulatory effect on enzyme synthesis and/or storage (31).

When BLT-esterase release was evaluated in cytotoxic cell supernatants after interaction with susceptible targets, we observed that CTL-specific lysis was clearly associated with a net enzyme release, while LAK cell-mediated lysis was not (Table I). Accordingly, when the CTL clone having LAK-like activity was tested, BLT-esterase release increased only after specific interaction with MBL-2 lymphoma cells, and lysis of unrelated P815 and YAC-1 cells, occurred in the absence of enzyme release above control level (Table II).

Acquisition of LAK activity is not accompanied by inhibition of stimulated BLT-esterase secretion. In fact, after challenge with pharmacological agents that increase $[Ca^{2+}]_i$ (such as Con A) and/or activate protein kinase C (such as the $Ca^{2+}$ ionophore A23187 + PMA), both LAK cells and the CTL clone having LAK-like activity efficiently release BLT-esterase (Table III).

TABLE II

*Cytotoxic Activity and BLT-Esterase Release of CTL Clone Cells*

| Effector cells* | Percent specific $^{51}Cr$ release | | | Percent specific BLT-esterase release | | | BLT-Esterase level |
|---|---|---|---|---|---|---|---|
| | MBL-2 | P815 | YAC-1 | MBL-2 | P815 | YAC-1 | |
| | | | | | | | *U/10⁶ cells*‡ |
| Specific CTL clone | 43 ± 10 | 2 ± 1 | 10 ± 9 | 17 ± 2 | 1 ± 1 | 1 ± 1 | 82.6 ± 8 |
| LAK-like CTL clone | 49 ± 16 | 36 ± 9 | 57 ± 12 | 7 ± 1 | 1 ± 1 | 0 | 161.9 ± 22 |

* Effector and target cells were incubated at 37°C for 4 h, at an E/T ratio of 10:1 for $^{51}Cr$ release assay and 1:3 for BLT-esterase release assay. Specific $^{51}Cr$ release and BLT-esterase activity in the culture supernatants were evaluated as described in Materials and Methods.

‡ Total cellular enzymatic activity was determined by solubilizing effector cells with a 0.1% Triton X-100 solution, and BLT-esterase units for $10^6$ were calculated as reported in Materials and Methods.

TABLE III

*BLT-Esterase Release After Stimulation with PMA + A23187 or Con A*

| Effector cells* | Percent specific BLT-Esterase release in presence of: PMA + A23187 | Con A |
|---|---|---|
| Short-term cultured: | | |
| LAK | 13 ± 3 | 16 ± 2 |
| CTL (b anti-d) | 12 ± 3 | 11 ± 4 |
| Long-term cultured: | | |
| LAK | 6 ± 1 | 16 ± 2 |
| CTL (b anti-d) | 5 ± 1 | 12 ± 2 |
| CTL clone cells: | | |
| Specific | 12 ± 3 | 27 ± 4 |
| LAK-like | 11 ± 3 | 23 ± 3 |

* $10^5$ effector cells were incubated for 4 h at 37°C with A23187 (50 nM) and PMA (32 nM) or with Con A (10 μg/ml) and BLT esterase activity in the culture supernatants was measured as reported in Materials and Methods.

## Discussion

CTL interaction with specific targets leads to a transient increase in $[Ca^{2+}]_i$, as well as $InsP_3$ generation and activation of protein kinase C (9, 32, 33). mAbs to TCR or the associated CD3 complex mimic the target cell effect to a large extent, and can induce generation of second messengers (34). These signals are likely to be required for cytotoxic granule exocytosis (35).

We observed that LAK cells, whose lytic activity is aspecific and not dependent on CD3-TCR complex triggering, kill susceptible targets in the absence of a rise in $[Ca^{2+}]_i$, $InsP_3$ formation, and BLT-esterase release. These results were obtained using LAK cells from short- and long-term cultures, as well as an antigen-specific CTL clone that acquires LAK-like activity in medium containing high IL-2 doses. In line with our results, Ostergaard and Clark (9) observed a $Ca^{2+}$ influx into cloned CTL loaded with the fluorescent $Ca^{2+}$ indicator quin2 during lysis of antigen-related targets, but not when the same clone, which acquired indiscriminate cytotoxicity after preincubation with PMA, was challenged with unrelated targets. It may be advanced that the heterogeneity of effector cells from 5-d cultures masks the activation signals of a few reacting LAK cells. However, a rise in $[Ca^{2+}]_i$ was readily detected using CTL from MLC that were as heterogeneous as the short-term LAK cells, and showed the same killing efficiency when assayed against the same targets. On the other hand, no $[Ca^{2+}]_i$ increase was observed even in long-term LAK cell cultures that were homogeneous on the basis of morphological features and phenotypic markers. Moreover, the antigen-specific CTL clone with LAK activity exhibited $[Ca^{2+}]_i$ increase and $InsP_3$ formation when challenged with the specific MBL-2 target, but lysed unrelated YAC-1 and P815 cells in the absence of these signals. Therefore, the same effector cell population is able to activate second messenger generation only when challenged with targets bearing the relevant antigen.

The finding that Con A induced the same rise in $[Ca^{2+}]_i$ in LAK cell popula-

tions, whether or not susceptible targets were present, is consistent with these results. Conversely, in antigen-specific CTL the net rise in $[Ca^{2+}]_i$ was blunted by the previous increase caused by target cell triggering.

It has recently been observed that exposure of human or rat NK cells to sensitive targets leads to both a $[Ca^{2+}]_i$ rise and $InsP_3$ generation (36). Therefore, the biochemical events occurring during activation of CTL and NK cells on one hand, and those taking place in LAK cells and in the CTL clone having LAK-like activity on the other, are apparently different.

In agreement with present observations on second messenger generation, CTL-specific but not LAK cell-aspecific lytic activity is invariably associated with an increased serine-esterase release in the supernatant. The excellent correlation between BLT-esterase secretion and $[Ca^{2+}]_i$ increase in both CTL and LAK cells after stimulation with A23187 + PMA or Con A, further confirms the close association of these phenomena.

It has been reported that CTL that have lost antigen specificity after passages in IL-2-containing medium fail to secrete serine-esterase and other enzymes upon interaction with susceptible cells and can kill targets in the absence of extracellular $Ca^{2+}$ (15, 17). However, $[Ca^{2+}]_o$-independent killing cannot explain the lack of $[Ca^{2+}]_i$ increase and granule exocytosis observed in our study. In fact, both effects were elicited by the same target (MBL-2) in CTL but not in LAK cells; moreover, neither specific nor aspecific lysis occurred in the presence of 4 mM EGTA (data not shown).

Although LAK- and NK-mediated cytotoxicity is accompanied by a negligible secretion of BLT-esterase (18), it has generally been considered to depend on granule exocytosis since lysis is blocked, at least in rats, by antiperforin mAbs (37). Moreover, the strict dependency of the activity of these effector cells on $[Ca^{2+}]_o$ is consistent with perforin involvement in the killing process (9).

To reconcile our results with the general belief that LAK cell killing is perforin dependent, it may be suggested that LAK cells use a secretory pathway different from that of classical receptor-mediated exocytosis. As known, two pathways for protein secretion occur in eukaryotic cells: constitutive and regulated. In the former, proteins are secreted in the absence of known second messenger generation; in the latter, proteins are stored in secretory vescicles and released in large amounts and at a high rate only after surface receptor activation and generation of intracellular signals; it has been proposed that both pathways may be operative in cytotoxic cells (38). We suggest that during LAK cell interaction with susceptible targets, a process independent of TCR-CD3, no intracellular second messenger is generated, and granular cytotoxic products are instead continuously discharged, via the constitutive secretion pathway, on the target cell membrane. Regulated secretion coupled to $[Ca^{2+}]_i$ increase can be triggered in IL-2-treated cells by aspecific stimuli (e.g., PMA + ionophore and Con A), or by activation of receptors such as CD16 or CD2 (39). On the other hand, it may be held that CTL that follow the pathway of regulated secretion during interaction with antigen-specific targets, under some conditions, may also use the constitutive route to kill targets.

## Summary

CTL activation by specific targets leads to a rapid rise of inositol phosphates (InsPs)

and of cytoplasmic-free $Ca^{2+}$ concentration ($[Ca^{2+}]_i$). While these events are considered necessary to trigger granule secretion, $Ca^{2+}$-independent cytolytic mechanisms have been recently proposed in addition or as an alternative to the classical $Ca^{2+}$-dependent exocytosis model. We observed that lymphokine-activated killer (LAK) cells, obtained after stimulation with supraoptimal concentrations of IL-2 in short- or long-term cultures, kill susceptible targets in the absence of a $[Ca^{2+}]_i$ rise and $InsP_3$ formation. Moreover, LAK cell-mediated lysis was not associated with an increase in cytotoxic granule exocytosis, as evaluated by BLT-esterase release into the culture supernatant.

Furthermore, using an antigen-specific CTL clone, which acquires LAK-like activity when cultured in medium containing high IL-2 doses, second messenger generation and cytolytic granule content secretion were not detected during lysis of unrelated target cells, while killing of specific targets triggered both these processes.

These findings suggest that two lytic pathways may coexist in the same effector cells: a second messenger-dependent pathway involving degranulation, which is activated after TCR interaction with specific targets, and another pathway, independent of any known second messenger generation, responsible for unrelated target cell lysis.

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