

Flow Cytometric Measurement of $[Ca^{2+}]_i$ and pH_i in Conjugated Natural Killer Cells and K562 Target Cells During the Cytotoxic Process^{1,2}

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We describe a flow cytometric assay that enables one to follow conjugate formation between cytotoxic cells and their target cells during the cytotoxic process. In addition, the internal calcium concentration ($[Ca^{2+}]_i$) and internal pH (pH_i) of the conjugated cells can be monitored and directly compared to the nonconjugated cells. This is achieved by labeling one cell type with the Ca^{2+} -specific dye Fluo-3, while the other cell type is labeled with the pH-sensitive dye SNARF-1. As these fluorochromes have different emission spectra, events positive for both fluorochromes are identified as conjugates. The results show that the conjugates can be clearly distinguished from single cytotoxic cells [natural killer (NK) cells] and

target cells [K562 cells, (TC)]. Upon binding, $[Ca^{2+}]_i$ is increased in the NK cells as well as in the TC. In conjugated NK cells this increase of $[Ca^{2+}]_i$ is temperature dependent and is followed by a decrease to a normal $[Ca^{2+}]_i$ value later on. The $[Ca^{2+}]_i$ in NK cells increases in 2 steps, which may be related to the binding – and lethal hit phase. Upon conjugate formation, NK cells show a slight increase in pH_i (0.2–0.3 pH units). TC do not reveal a significant change in pH_i .

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Key terms: Cytotoxic interaction, Fluo-3, SNARF-1, intracellular $[Ca^{2+}]_i$, intracellular pH

Recently many studies have been carried out to elucidate the intracellular signal transducing pathways involved in the cytotoxic process between natural killer (NK) cells and target cells (TC). Results from these studies show the involvement of the protein kinase C pathway in NK cells stimulated with appropriate TC, both from the increase in inositol phosphate generation (3,11) and from the increase in $[Ca^{2+}]_i$ (15). In contrast, Windebank et al. (15) and Whalen and Bankhurst (14) showed that activation of the cyclic AMP (cAMP)-dependent second messenger pathway inhibited the phosphoinositide hydrolysis as well as the cytotoxic activity of the NK cells. These results suggest that the cAMP-dependent pathway is acting as an inhibitory mechanism of the cytotoxic action.

The studies referred to are all based on overall effects of agents acting on specific sites of signal transducing pathways studied in mixtures of NK cells and TC without discriminating between single and conjugated NK cells or TC. For more detailed studies into the involvement of signal transduction pathways, one has to focus

on conjugated cells only. Edwards et al. (4) have described a simple method to detect conjugates in a mixture of 2 cell types using a flow cytometer. Using this technique in a suspension of NK cells and TC, conjugates can be distinguished from single cells. Distinction is based on the fact that the relatively big TC has a high forward light scatter signal as compared with the small NK cell. If the NK cells are stained with a fluorescent dye (e.g., a Ca^{2+} -specific dye), those events that have a fluorescence signal (from the NK cell), together with a high forward light scatter signal, will be the conjugates. However, this method has its limitations if one wants to study the fluorescently labeled parameters of the TC. Discrimination of conjugates is then impossible, as the forward light scatter signals

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from TC and TC-NK conjugates cannot be distinguished.

In the method presented here we have overcome this problem by using 2 fluorescent dyes which have different emission spectra. The dyes are separately loaded into NK cells and K562 TC. Using the Ca^{2+} -specific dye Fluo-3 (emission maximum: 525 nm) and the H^+ -specific dye SNARF-1 (emission maxima: 580 and 645 nm), we can clearly distinguish conjugated from non-conjugated cells. Moreover, this method enables us to monitor changes in $[\text{Ca}^{2+}]_i$ and pH_i in interacting cells during the cytotoxic process.

MATERIALS AND METHODS

Materials

Fluo-3-acetoxymethyl ester (Fluo-3-AM), seminaphthorhodafuor-1-acetoxymethylester (SNARF-1-AM), Pluronic F-127, 4-bromo A-23187 (Br-A23187), and nigericin were obtained from Molecular Probes Inc. (Eugene, OR). DMSO (analytical grade), L-glutamine, and NaHCO_3 were from Merck (Darmstadt, Germany). Leucoagglutinin, indomethacin, penicillin G, and streptomycin were all from Sigma Chemical Co. (St. Louis, MO). HEPES was obtained from Serva GmbH & Co. (Heidelberg, Germany)

Cell Cultures

As standard medium (SM) for cell cultures and experiments, RPMI 1640 without phenol red (Seramed, Berlin, Germany) was used with addition of penicillin G (100 U/ml), streptomycin (100 $\mu\text{g}/\text{ml}$), and L-glutamine (2 mM). All cell cultures were kept in an incubator in a humidified atmosphere at 37°C and 5% CO_2 . The NK cell clone was characterized as $\text{CD}2^+3^-4^-8^-16^+56^+$. The cells were maintained in U-shaped 96 well plates as described by Bolhuis et al. (1). In short, the NK cells were cultured together with 30 Gy irradiated feeder cells (a mixture of peripheral blood lymphocytes, and 2 Epstein Barr virus transfected B-cell lines APD and BSM) in SM with the following additions: HEPES (10 mM), bicarbonate (2 mM), recombinant interleukin-2 (25 U/ml, Cetus Inc. Amsterdam, The Netherlands; a gift from Dr. R.L.H. Bolhuis), indomethacin (1 $\mu\text{g}/\text{ml}$), leucoagglutinin (1 $\mu\text{g}/\text{ml}$), and human pooled serum (10%). Once a week the cells were harvested and kept in 24 well plates (1–2 ml per well, 5×10^5 cells/ml) until use (normally within 4 d). K562 cells were maintained in SM buffered with 2.4 mM bicarbonate and supplemented with 10% heat inactivated fetal calf serum (FCS; Gibco, Gaithersburg, MD). Experiments were carried out in complete medium (CM): SM buffered with HEPES (10 mM), bicarbonate (2 mM), and 10% FCS.

Loading Procedures

Intracellular calcium was determined with the calcium-sensitive fluorochrome Fluo-3 (7). Due to its excitation maximum (488 nm), this probe is suitable for use in our study carried out with an argon ion laser

based flow cytometer (9,12). Fluo-3 was loaded into the cells using the acetoxymethyl ester. Stock solution of Fluo-3-AM (1 mM in dry DMSO) was stored at -20°C . A 25% (w/w) stock solution of Pluronic F-127 was prepared in dry DMSO and liquified at 42°C before use. The working solution was prepared in CM by thoroughly mixing Pluronic F-127 (5 $\mu\text{l}/\text{ml}$) and Fluo-3-AM stock (final concentration 10 μM). For loading, NK or K562 cells ($10^7/\text{ml}$) were mixed 1:1 with the Fluo-3-AM working solution and incubated for 1 h at 37°C and 5% CO_2 . During incubation the cells were shaken every 15 min. After 1 h the cells were washed 3 times with cold CM and kept at 4°C before use. If desired, calibration of the cytosolic calcium concentration was carried out (6). As no ratio measurements can be carried out with this dye, changes in $[\text{Ca}^{2+}]_i$ are presented in this study as changes in Fluo-3 fluorescence.

SNARF-1 loading was also performed with the acetoxymethyl ester. A 1 mM SNARF-1-AM stock was prepared in ethanol and kept refrigerated. For loading, SNARF-1-AM was added directly to the cell suspension ($10^6/\text{ml}$) to a final concentration of 10 μM . Cells were loaded for 30 min at 37°C in an incubator and washed 3 times before use. For adequate loading of the K562 cells, serum was omitted from the CM during loading and the first 2 washes. The loaded cells were stored at 4°C . SNARF-1 fluorescence was detected linearly at 2 different emission wavelengths. With the analysis program, values obtained at 645 nm were divided by the 580 nm values. From the resulting histogram the peak value was used to indicate the pH_i value. Calibration of the pH_i was performed according to Rabinovich and June (8).

Conjugate Formation

During cytotoxic interaction conjugates are formed between an NK cell and a K562 target cell. In vitro conjugate formation is facilitated by centrifugation. Therefore, NK cells and K562 cells were mixed at a desired ratio and centrifuged for 1 min to a maximum of 200 g to allow the cells to form conjugates. Centrifugation was at 4°C . From previous studies it is known that at this temperature the cells form conjugates, whereas no cytotoxic action takes place. After centrifugation, the cells in the loose pellet were resuspended by careful shaking of the tube and were then handled as desired.

Flow Cytometry Measurements

The flow cytometer used was developed in our institute. It was equipped with an air-cooled 100 mW argon ion laser (model 5500A, Ion Laser Technology, Salt Lake City, UT) tuned to 488 nm and operating at 50 mW. Forward light scatter signals were detected with a photodiode (model Pin 10-D, United Detector Technology, Hawthorn, CA).

Orthogonal light scatter and fluorescence signals were detected with photomultipliers (type R928 from Hamamatsu Corporation, Bridgewater, NJ). Fluo-3

emission was measured using a 6-cavity bandfilter (510–550 nm; Pomfret Research Optics Inc. For simultaneous measurement of Fluo-3 and SNARF-1 emissions, a dichroic mirror of 550 nm (DC550LP, Omega Optical Inc., Brattleboro, VT) was placed at an angle of 45° to separate their emissions. SNARF-1 emission is further split by the 605 nm dichroic mirror (DC605LP, Omega Optical Inc.), also placed at an angle of 45° to allow detection at 580 nm (filtered by 580DF50, Omega Optical Inc.) and 645 nm (filtered by RG645-2, Schott, Tiel, The Netherlands). All data were acquired in the linear mode, except the Fluo-3 emission, which was acquired in the logarithmic mode. Data were stored in the list mode. Usually 4096 events were detected per measurement. Ratios of the SNARF-1 emissions were calculated afterwards by dividing the emission at 645 nm by the emission at 580 nm (Em_{645nm}/Em_{580nm}) for each event. Analysis of the samples was carried out at room temperature.

RESULTS

Dye Loading in NK Cells and K562 Cells

Fluo-3 loading. Both NK cells and K562 target cells show a substantial leakage (50% in about 60 min) of Fluo-3 during incubation of the cells at $37^\circ C$. No significant leakage was detected if the cells were kept at 4 or $25^\circ C$. Therefore, to minimize leakage, the cells are kept at $4^\circ C$ until use.

During the experiments Fluo-3 loaded cells were mixed with cells that were loaded with SNARF-1. In a mixture of Fluo-3 negative cells and Fluo-3 loaded cells, we tested whether the unloaded cells picked up the released Fluo-3. As was expected, regardless of which cell type was loaded with Fluo-3, no uptake of the free acid form of the released dye was detected in unloaded cells, even if a high ratio of Fluo-3 loaded to unloaded cells was used (data not shown).

SNARF-1 loading. From preliminary studies it was clear that leakage of this probe was negligible (data not shown), as was mentioned by Van Erp et al. (13). Calibration of the cytosolic pH has been performed using high K^+ buffers with different pHs and nigericin ($2 \mu g/ml$) as the proton ionophore. To establish a calibration curve, cells were incubated in the nigericin containing K^+ buffers for a maximum time of 10 min and measured. In Fig. 1 typical pH calibration curves (see also 8,13) of NK cells and K562 cells are shown. The discrepancy between the 2 curves can be explained by a difference in the protein composition of NK cells and TC, as was mentioned by Seksek et al. (10).

Detection of Conjugates

The purpose of our study is firstly to detect conjugates between an NK cell and a target cell in a mixture with single NK cells and TC, and secondly, to monitor cytosolic Ca^{2+} and H^+ concentrations in each cell type in these conjugates. Therefore, Fluo-3 loaded NK cells and SNARF-1 loaded TC, or SNARF-1 loaded NK cells

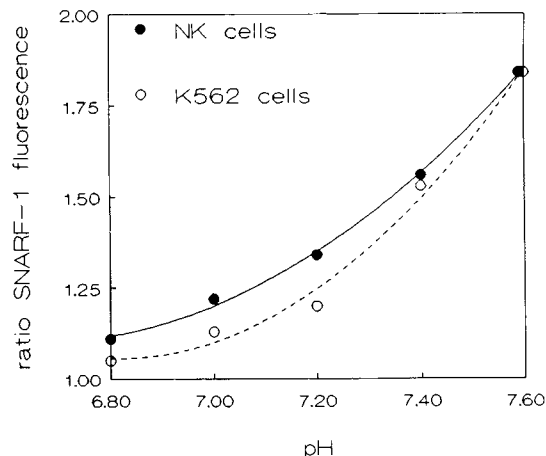


FIG. 1. pH calibration curves of SNARF-1 loaded NK cells (●) and K562 cells (○). The cells were incubated for a maximum of 10 min in nigericin ($2 \mu g/ml$) containing K^+ -buffers with different pH levels.

and Fluo-3 loaded TC, were mixed in a ratio 1:1. To facilitate conjugate formation, the mixture was centrifuged for a short period and the pelleted cells were incubated for 10 min at $4^\circ C$. Afterwards, the pellet was resuspended and analyzed. As we used 2 probes with different emission spectra loaded separately into the NK and K562 cells, we are able to identify the conjugates. In the dotplots, NK cells are indicated as NK, the K562 cells as TC, and the conjugates between an NK and a K562 cell as C.

Figure 2 shows the results of a typical experiment of Fluo-3 loaded NK cells and SNARF-1 loaded K562 cells. From the right angle, vs. the forward light scattering plot (Fig. 2a), it is clear that in a centrifuged mixture with 20% of conjugates present, a distinct population of conjugated cells cannot be identified. From the same sample, in Fig. 2b the dotplot of Fluo-3 vs. SNARF-1 fluorescence is shown. Three separate populations can be clearly distinguished. In addition to the Fluo-3 loaded NK cells and the SNARF-1 loaded K562 cells, a population of double positive events can be identified: the conjugates. Similar results were obtained when the staining procedure was reversed: Three distinct populations of conjugated and single cells can be observed (Fig. 3a,b). Notice that after centrifugation the number of conjugates has increased.

Cytotoxic Interaction

The following experiments serve to indicate the relevance of the method developed. The method enables us to monitor changes in $[Ca^{2+}]_i$ and pH_i in each cell type of conjugates between NK cells and K562 TC during the cytotoxic process. First, we focus on changes in $[Ca^{2+}]_i$. When NK cells were loaded with Fluo-3, we observed that the histogram of conjugated NK cells was shifted to higher Fluo-3 fluorescence values as compared to single NK cells (Fig. 4a). An artefactual increase was excluded, as was concluded from a similar experiment using EGTA (data not shown) to deplete

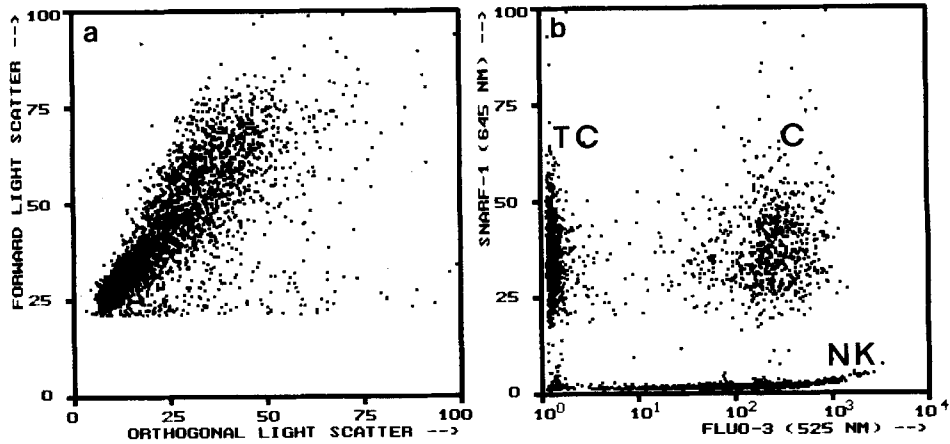


FIG. 2. Identification of conjugates (C) in a mixture of Fluo-3 loaded natural killer (NK) cells and SNARF-1 loaded K562 cells (TC). The cells were centrifuged for 1 min and incubated for 10 min at 4°C. **a:** Scatterplot of the forward vs. the orthogonal light scatter signals of the mixture of NK cells, TC, and conjugates. In this scatterplot, con-

jugates cannot be identified as a distinct population. **b:** Dotplot of the Fluo-3 vs. SNARF-1 fluorescence of NK cells and TC, respectively. Conjugates (20%), represented as double positive events, can be clearly distinguished from populations of single NK cells and TC.

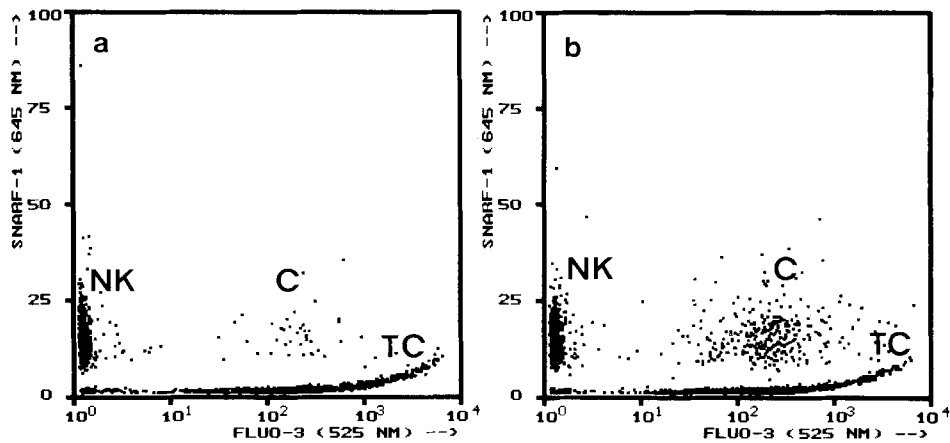


FIG. 3. Identification of conjugates (C) in a mixture of SNARF-1 loaded NK cells and Fluo-3 loaded K562 cells (TC). **a:** Fluo-3 vs. SNARF-1 fluorescence dotplot of a mixture of NK cells and TC. Separate populations of NK cells and TC can be distinguished as well as a low percentage of conjugates (1.6%). **b:** After centrifugation and incubation for 10 min at 4°C, the NK cell/TC mixture contains a higher percentage of conjugates (8.8%).

extracellular Ca^{2+} . In conjugates thus formed, Fluo-3 fluorescence of conjugated and single NK cells was identical. We could also exclude the possibility that this increase was due to spectral overlap, as Fig. 2b indicates that SNARF-1 fluorescence coming from the conjugated TC gave no signal in the Fluo-3 channel. Thus, the increased Fluo-3 fluorescence may be due to an increase in $[\text{Ca}^{2+}]_i$ upon binding to target cells. An alternative explanation was that this increase was due to the prevalence of NK cells with a relatively high $[\text{Ca}^{2+}]_i$ to bind to target cells. Therefore, the fluorescence histogram of Fluo-3 loaded NK cells mixed with K562 cells was compared with the histogram of the

same amount of Fluo-3 loaded NK cells in a control suspension. If NK cells with a relatively high $[\text{Ca}^{2+}]_i$ would bind to K562 cells, it was expected that both histograms would overlap completely. Figure 4b shows that the histogram of the mixed NK cells (solid line) is different from that of the control suspension in that it has a shoulder in the high Fluo-3 fluorescence region. Thus, the increase found in the Fluo-3 signal must be due to the effects occurring in the NK cell upon binding to a K562 target cell and, secondly, there was no prevalence of NK cells with relatively high Fluo-3 fluorescence for exclusive interaction with K562 cells. The same conclusion can be drawn from Fig. 5a for Fluo-3

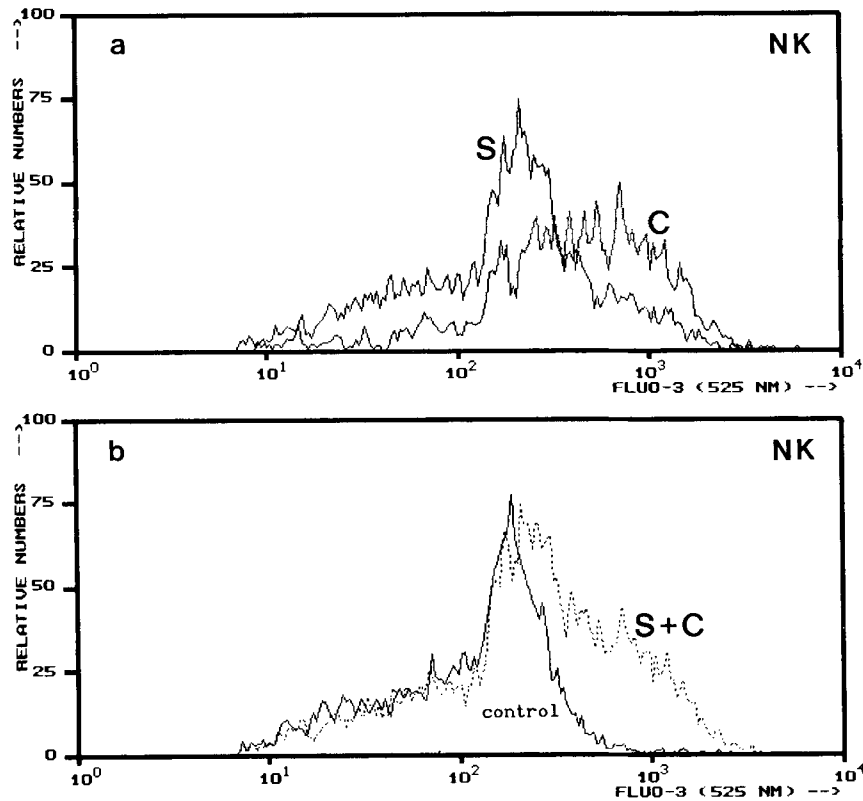


FIG. 4. Histograms of Fluo-3 loaded NK cells. **a:** To obtain conjugated NK cells, a mixture of Fluo-3 loaded NK cells and SNARF-1 loaded K562 cells was centrifuged and incubated in a water bath for 20 min at 25°C. Histograms of the Fluo-3 fluorescence of the gated populations of single (S) and conjugated NK cells (C) are presented. The histogram of the conjugated NK cells is shifted to higher fluorescence values. Note the logarithmic scale of the Fluo-3 fluorescence.

b: The histogram of the Fluo-3 fluorescence of the total NK cell population (S+C, ---) of the mixture described in a is compared with that of a control suspension of single NK cells (control, —), also incubated in a water bath for 20 min at 25°C. The shoulder of the former can be ascribed to the presence of conjugated NK cells and is located well outside the histogram of the control sample. Flow cytometric analysis of the cells was performed at room temperature.

loaded K562 cells. The conjugated cells have a higher mean Fluo-3 fluorescence than single K562 cells. Analysis indicates that here the increased Fluo-3 signal must be ascribed to an increase in $[Ca^{2+}]_i$ upon conjugate formation (Fig. 5b). Thus, we concluded that the intracellular $[Ca^{2+}]_i$ in both NK cells and K562 cells increases during the formation of a conjugate.

Comparing histograms of single cells in the mixtures (Figs. 4a and 5a) with that of control cells (Figs. 4b and 5b, respectively), the former shows a shoulder at higher $[Ca^{2+}]_i$. It is likely that in this case there are cells that already have interacted with, and subsequently detached from, the opposite cell type. Another possibility is that this shoulder records single cells that failed to form conjugates stable enough for flow cytometric analysis.

The increase in Fluo-3 fluorescence in conjugated NK cells is dependent on the incubation time and temperature (Fig. 6). If conjugates are kept at 4°C, the mean Fluo-3 fluorescence of conjugated NK cells is increased 2-fold more than single NK cells and remains at the same value during the period studied (35 min).

Higher Fluo-3 fluorescence values are observed if conjugates are incubated at 25 or 37°C. During incubation at 25°C, Fluo-3 fluorescence first increases (up to 20 min of incubation) and then decreases to a value which is still even higher than is observed at 4°C. If conjugates are kept at 37°C, the highest Fluo-3 fluorescence is observed 10 min after incubation. Then, the Fluo-3 fluorescence declines to a value in the range of the nonconjugated NK cells.

Concerning the pH measurements, an increase is found in the ratio of the SNARF-1 fluorescence in conjugated NK cells as compared with single NK cells, corresponding to 0.2–0.3 pH units (Fig. 7a). In K562 cells no significant difference is found in the ratio of SNARF-1 fluorescence in conjugated as well as in single cells (Fig. 7b).

DISCUSSION

The results show that with the method developed in a mixture of 2 different but interacting populations of cells, conjugates between 2 types of cells can be distinguished from noninteracting cells. Furthermore, this

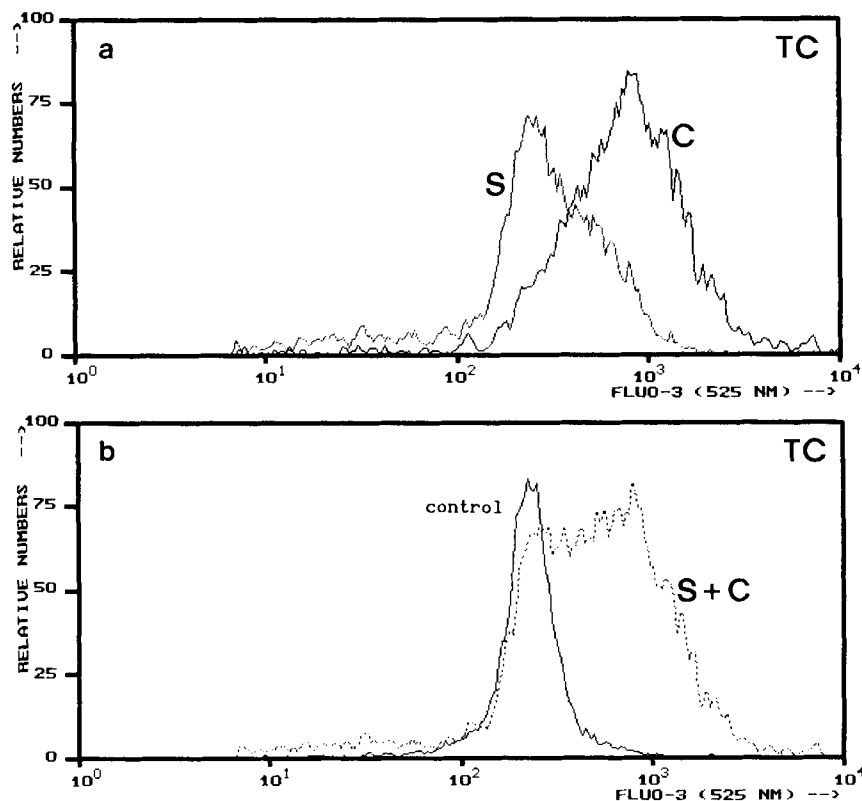


FIG. 5. Fluorescence histograms of Fluo-3 loaded K562 cells. **a:** Histograms of the conjugated (C) and single (S) Fluo-3 loaded K562 cells present in a centrifuged mixture with SNARF-1 loaded NK cells. The cells were measured at room temperature after 20 min incubation in a water bath of 25°C. The histogram of the conjugated K562 cells has

shifted to higher Fluo-3 fluorescence values as compared with that of single cells. **b:** The histogram of the total K562 population (S+C, ---) present in the mixture described in a shows a big shoulder at high Fluo-3 fluorescence values as compared with a control sample of K562 cells (control, —).

method enables us to study changes in $[Ca^{2+}]_i$ and pH_i in NK cells and K562 target cells during cytotoxic interaction. The noninteracting single cells can be considered as control cells. As they are included in the measurement, the problem of leakage correction in the case of the single excitation/single emission wavelength probe Fluo-3 has been reduced, which is a great advantage.

Callewaert et al. (2) have described a similar method for the identification of conjugates in a mixture of NK cells and TC. However, with the 2 fluorescent dyes they used, no information could be obtained about intracellular processes during cytotoxic interaction. Using our method, in agreement with Edwards et al. (4), we found an increase in the $[Ca^{2+}]_i$ in conjugated NK cells as compared with single NK cells present in the same mixture.

In conjugated NK cells, incubated at 4°C to preclude lethal hit delivery, the increase in $[Ca^{2+}]_i$ remained constant up to 35 min of incubation. Raising the temperature to 25 or 37°C, the initial increase was followed by a further enhancement of the $[Ca^{2+}]_i$. The increased calcium concentration had a transient character, with a temperature-dependent rate of increase and decrease,

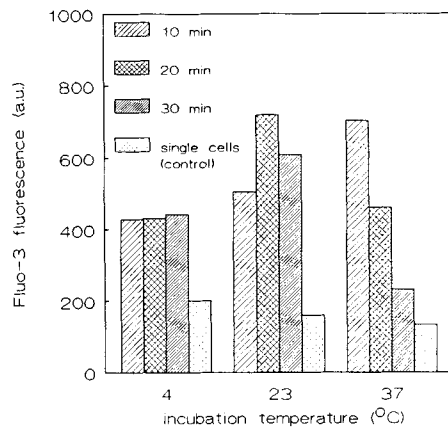


FIG. 6. Effect of different incubation times and temperatures on the $[Ca^{2+}]_i$ of conjugated NK cells in comparison with single NK cells. NK cells and TC were loaded with Fluo-3 and SNARF-1, respectively. The cells were mixed in a ratio of 1:1, centrifuged, and incubated in a water bath at the indicated temperatures and periods. Then the cells were aspirated and measured at room temperature with the flow cytometer. Average Fluo-3 fluorescence values of the conjugates are shown in the figure. Fluo-3 fluorescence values of the single cells in the same mixture serve as the control.

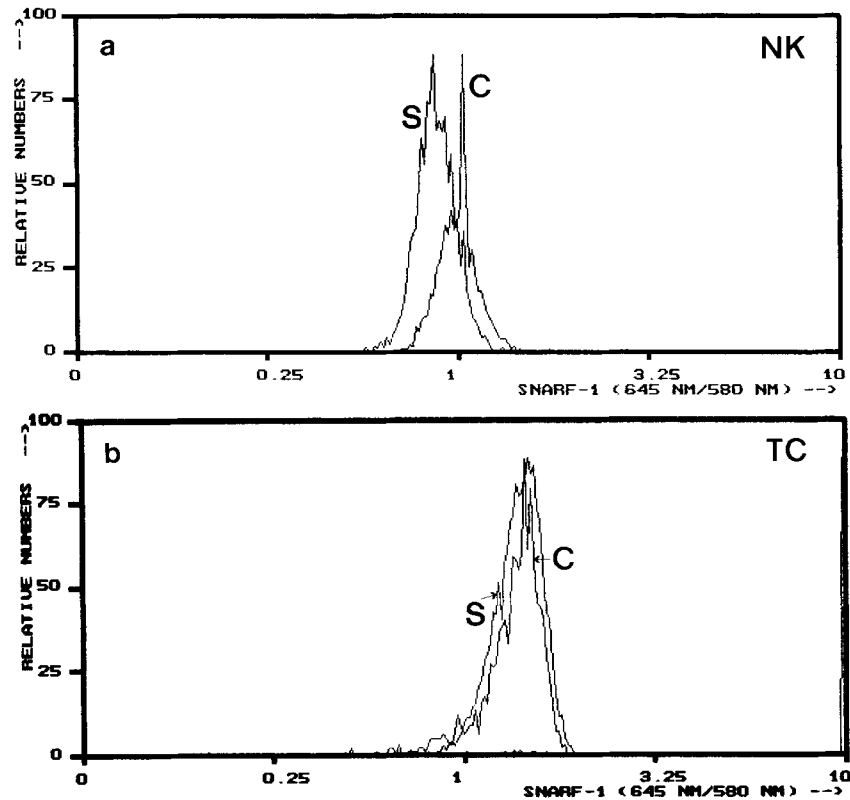


FIG. 7. Histograms of the ratio of 645 nm/580 nm fluorescence signals from SNARF-1 loaded cells. **a:** SNARF-1 loaded NK cells were centrifuged with Fluo-3 loaded K562 cells and incubated for 10 min at 25°C. The SNARF-1 ratio obtained from conjugated NK cells (C) is shifted to a higher value compared with that of single NK cells (S). **b:** When K562 cells were loaded with SNARF-1 and NK cells with Fluo-3, no significant shift in the SNARF-1 ratio of conjugated K562 cells (C) was observed compared with single K562 cells (S).

and a temperature-independent maximum level. As the cytotoxic action of NK cells is delayed or even prevented at 4°C, the results suggest that at this temperature in an NK cell, only binding to a TC leads to a 2-fold increase of the $[Ca^{2+}]_i$. As is well known, at 37°C—and to a lesser extent at 25°C—NK cells manifest their killing mechanism towards TC. At these temperatures in conjugated NK cells, the $[Ca^{2+}]_i$ rose to higher levels, as observed at 4°C. These higher levels for $[Ca^{2+}]_i$ at 25 and 37°C may be related to the cytotoxic action of the NK cells. Apparently, the intracellular Ca^{2+} level in the conjugated NK cell had to reach a certain threshold in order to exert its cytotoxic action.

In conjugated TC we also found an increase in the $[Ca^{2+}]_i$ as compared with single TC. McConkey et al. (5), who measured the overall effects in mixtures of single and conjugated Jurkat TC and NK cells, found a persistent increase in $[Ca^{2+}]_i$ of these TC up to 15 min. In contrast, in preliminary experiments we found a temporal increase in $[Ca^{2+}]_i$ in conjugated TC, which changed into a decrease in $[Ca^{2+}]_i$ after 10 min of incubation at 37°C. The pH_i is clearly increased to a more alkaline value in conjugated NK cells. No significant

changes in pH_i were observed in conjugated TC, although there may be a slight tendency for pH_i values to be lowered in the conjugated TC.

It can be concluded that with the method described here it is possible to detect conjugates in a mixture of NK cells and K562 TC, while simultaneously studying changes in $[Ca^{2+}]_i$ and pH_i in the conjugated cells. Using appropriate fluorescent dyes, other physiological phenomena can be studied as well in this biological system or in other systems in which the interaction between cells is of importance.

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