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Occurrence and a Possible Mechanism of Penetration of Natural Killer Cells Into K562 Target Cells During the Cytotoxic Interaction

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The cytotoxic interaction between cloned human Natural Killer (NK) cells and K562 target cells was studied using confocal laser scanning microscopy (CLSM) and conventional fluorescence microscopy. We observed, using fixed as well as living cells, the occurrence of (pseudo)emperipolesis during the interaction. About 30% of *conjugated* NK cells penetrated, partly or completely, into the target cells (*in-conjugation*). Virtually all in-conjugated target cells exhibited polymerized actin. Killer cells of in-conjugates were frequently seen approaching the target cell nucleus or aligning along it. If the cytotoxic process was inhibited by the absence of calcium neither actin polymerization nor in-conjugation were observed. A kinetic study showed that in-conjugation starts somewhat later than actin polymerization but still within a few minutes after addition of calcium to conjugates previously formed in the absence of calcium. The presence of cytochalasin D (an inhibitor of actin polymerization) completely inhibited in-conjugation and partly reduced the cytotoxic activity. Zinc ions (endonuclease inhibition) inhibited in-conjugation and decreased the total number of target cells with polymerized actin in a concentration dependent manner. Cytotoxic activity was also reduced but not as efficiently as in-conjugation.

Our study demonstrates that in-conjugation represents a *significant fraction* of the cytotoxic interaction. The results indicate that it may be a *consequence* of an actin polymerization and endonuclease activity dependent part of a cytotoxic mechanism. © 1995 Wiley-Liss, Inc.

Key terms: Natural Killer cell, emperipolesis, actin polymerization, endonuclease activity

Elucidation of the killing mechanism(s) involved in cell mediated cytotoxic processes has been the subject of numerous studies (6,9,16,23,32). In spite of great efforts of different research groups, the exact cause of target cell death has not been resolved yet. In general, two hypotheses dominate the cytotoxicity literature. According to the "colloid osmosis" mechanism, target cells die due to an osmotic imbalance/shock caused by incorporation of killer cell granule material into the target cell membrane (9,30,32). The "internal disintegration" model, on the other hand, implies that interaction with a killer cell activates the self-destroying pathway in the target cell which results in DNA fragmentation and cell death (6,16,23). These two mechanisms, however, need not to be mutually exclusive (33). It is also not clear how much each of them participates in the actual killing process and/or what is the crucial change that ultimately leads to target cell death. It remains to be elucidated whether the changes observed in the target cell during the cytotoxic process, such as pore formation and DNA fragmentation, represent the ultimate *cause* or simply the *consequence* of approaching cell death.

One of the crucial steps during cell mediated cytotoxic interaction is formation of a close contact (conjugate formation) between killer and target cells (1,9,28). The attachment enables the killer cell to recognize whether a target cell has to be killed or not. The formation of a closed "protected" pocket between the two cells may ensure an effective action of the environment sensitive killer molecules.

Our confocal microscopy study of actin reorganization during the cytotoxic interaction showed not only that during the attack of a Natural Killer (NK) cell polymerization of actin takes place in a fraction of the target cells (20) but it also revealed the occurrence of a peculiar type of conjugate formed between NK and K562 target

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cells. Killer cells did not just firmly attach to target cells but some also deeply penetrated into the cytoplasm of the target cells, occasionally entering the cell completely. The phenomenon wherein one cell *completely* enters the cytoplasm of another cell is called emperipolesis (gr. "inside round about wandering" (10)) and it was observed for the first time by Fischer and Dolschansky in 1929 (7). After that time there have been numerous descriptions of this aspect of lymphocyte behavior occurring with a variety of "host" cells (10,14,15,17,29,31). Ultrastructural characterization of in vitro emperipolesis revealed NK cells (i.e., large granular FcR+ (CD16+) cells) as the emperopoletic cells (2,11,21,26). Deeply penetrating projections/protrusions of cytotoxic T lymphocytes (CTL) into their target cells were also observed using electron microscopy (22,25). The demonstration that emperipolesis also occurs in vivo (2) makes the phenomenon even more interesting to study. The mechanism, however, and the relevance of this type of interaction for the cytotoxic process have not been resolved.

In this study we report the occurrence of *in-conjugation* (penetration of killer cell, *completely* or *partly*, into the target cell) during interaction between cloned human NK cells and K562 target cells as observed using confocal laser scanning microscopy on fixed and unfixed (living) cells. A possible mechanism for the in-conjugation phenomenon is proposed.

MATERIALS AND METHODS

Chemicals

RPMI-1640 was obtained from Seramed (Berlin, Germany), fetal calf serum from Gibco (Gaithersburg, MD), and rIL-2 from PromoCell (Heidelberg, Germany). The antibiotics, L-glutamine, leucoagglutinin, Triton X-100, cytochalasin D, DABCO (1,4-diazobicyclo[2.2.2]octane), and poly-L-lysine hydrobromide (PLL; Mw 70–150 kDa) were purchased from Sigma Chemical Co. (St. Louis, MO). Hepes, paraformaldehyde, Na_2EDTA and $ZnSO_4$ were obtained from Merck (Darmstadt, Germany). Pluronic F-127 and fluorescent probes (rhodamine phalloidin; Hoechst 33258; Calcium Green-1-AM; ("DiO"/ $DiOC_{18}(3)$) 3,3′-dioctadecyloxacarbocyanine perchlorate; carboxy SNARF-1-AM acetate; ("DiI"/ $DiIC_{18}(3)$) 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; propidium iodide) were purchased from Molecular Probes (Eugene, OR). F-18 (octadecylamine-fluorescein isothiocyanate) was prepared as described elsewhere (13).

Stock solutions of cytochalasin D and $ZnSO_4$ were prepared in DMSO (5 mM) and double distilled water (500 mM), respectively.

Cells

NK cells, clone NK76 (generous gift from Dr. R.L.H. Bolhuis, Den Hoed Cancer Center, Rotterdam), phenotype CD2+3–16+56+, were cultured in 96-well-plates in RPMI-1640 medium, supplemented with 25 mM Hepes, 2 mM L-glutamine, 10% pooled human serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 μg/ml indomethacin, 1 μg/ml leucoagglutinin (PHA-L), and 25 U/ml rIL-2. Cells were subcultured every seventh day on a layer of 30 Gy-irradiated feeder cells (Epstein-Barr-virus-transformed B cell lines APD and BSM, and PBL).

K562 cells were maintained in exponential growth phase in RPMI-1640 medium, supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (further referred to as a complete medium).

Conjugate Formation

Equal volumes of NK and K562 cells (2×10^6 cells/ml complete medium, total volume 100 μl) were mixed and centrifuged for 5 min at 200*g*. The cell suspension was gently resuspended and incubated at 37°C for the indicated time. Control samples (consisting of NK and K562 cells, respectively, incubated alone) were treated in the same way. After incubation cells were plated on PLL-coated cover glasses (0.01% PLL/PBS, overnight) and allowed to attach for 15 min at room temperature. The control samples and the test sample, consisting of an NK and K562 cell mixture, were plated on the same cover glass, divided by thin layers of nail polish into three parts.

For the experiments with calcium-free medium and the experiments with living cells, NK and K562 cells were resuspended in complete medium containing 2 mM EDTA prior to mixing. After mixing and centrifugation, cells were attached on PLL-coated cover glasses as described above. The cover glasses were rinsed with PBS, transferred to the incubation medium and incubated at 37°C for the indicated time.

For the experiments with living cells, the cover glasses were mounted in a holder (that enables the cells to be kept in the incubation medium).

Rhodamine Phalloidin and Hoechst Staining

Cover glasses with attached cells were rinsed with PBS and the cells were fixed with paraformaldehyde (3–4% in PBS) for 15 min at room temperature. After fixation, the cells were permeabilized with 0.1% Triton X-100/PBS for 5 min at room temperature, followed by rhodamine phalloidin staining (1 U per cover glass, 40 min at room temperature). Optional, after washing with PBS, the cover glasses were incubated in PBS containing 5 μg/ml Hoechst 33258 for 15 min at room temperature. The rinsed cover glasses were mounted on the object glasses in PBS + 50% glycerol + 100 mg/ml DABCO (antibleaching reagent) and sealed with nail polish.

Calcium Green, DiO, SNARF, and DiI Staining

K562 cells (10^6/ml complete medium) were incubated with 5 μg/ml DiO for 90 min at 37°C, washed twice and resuspended in complete medium at a concentration of 10^6/ml. An equal volume of the Calcium Green working solution (2 μM Calcium Green, 0.1% Pluronic F-127 in complete medium) was added to the cell suspension and incubated for 60 min at 37°C. The cells were washed twice and resuspended in complete medium at a concentration of 2×10^6/ml. Until use, the cells were kept at 4°C.

NK cells (10^6/ml complete medium) were incubated with 4 μg/ml DiI for 90 min at 37°C, washed twice, and resuspended in complete medium at a concentration of 10^6/ml. SNARF was added (final concentration 10 μM) and the cell suspension was incubated for 60 min at 37°C. The cells were washed twice and resuspended in complete medium at a concentration of 2×10^6/ml. Until use, the cells were kept at 4°C.

Fluorescence Microscopy

The confocal laser scanning microscope (CLSM) (Leica Lasertechnik, Wetzlar, Germany) was equipped with an inverted fluorescence microscope (Leica Fluovert FU), an air-cooled argon-krypton laser, and a water-cooled argon laser. For excitation of Hoechst, the water-cooled Ar laser (lines 351 and 364 nm) was used. Rhodamine phalloidin was excited using the Ar-Kr laser (line 568 nm). Calcium Green/DiO and SNARF/DiI were simultaneously excited using 488 and 568 nm lines of the Ar-Kr laser. A 535 nm band pass filter and a 580 nm long pass filter were used in the respective fluorescence detection paths. The emitted fluorescence light was collected using a 63× oil-immersion objective, N.A. 1.40 (PL Apo, Leica). Series of two times optically zoomed confocal sections, 1 μm apart, were scanned, each image averaged by 8 or 16 line scans. Examining optical sections with CLSM is very time consuming and therefore not a very practical method for obtaining an insight into the relative occurrence of the in-conjugates. Therefore, we investigated whether it would be possible to recognize the in-conjugates using rhodamine phalloidin staining and conventional fluorescence microscopy. Indeed, after some training period this appeared to be possible. More than 95% of the conjugates scored as in-conjugates using fluorescence microscopy were indeed in-conjugates as determined by CLSM, using optical sectioning and 3D-reconstruction. In this way a practical method was obtained that could be used to determine the relative occurrence of in-conjugates under different conditions.

An inverted fluorescence microscope (IMT-2, Olympus Optical, Tokyo, Japan) was used for conventional fluorescence microscopy. A filter block, consisting of a 530 nm long pass filter + 545 nm band pass filter (excitation), 580 nm dichroic mirror, and 590 nm long pass filter (emission) was used for rhodamine fluorescence (green excitation). A filter block consisting of a 375 nm band pass filter (excitation), 400 nm dichroic mirror, and 420 nm long pass filter (emission) was used for Hoechst fluorescence (UV excitation). The fluorescence light was collected using a 100× oil-immersion objective, N.A. 1.30 (DPlanApo 100 UV, Olympus Optical). For each sample at least 200 conjugates were analyzed.

Flow Cytometric Cytotoxicity Assay

The cytotoxic activity of NK cells was tested using the cytotoxicity assay developed in our laboratory (19) with slight modifications. Briefly, equal volumes of F-18 (cell membrane marker) labeled K562 cells (2×10^6/ml and 2×10^5/ml, respectively, for killer cell:target cell ratio 1:1 and 10:1) and unlabeled NK cells (2×10^6/ml) in complete medium or complete medium + 2 mM EDTA were mixed (total volume 100 μl), centrifuged for 5 min at 200*g* and incubated at 37°C for the desired period of time. The control sample consisted of F-18 labeled K562 cells incubated without NK cells. The samples were diluted with PBS, stained with propidium iodide (PI, dead cell indicator) and analyzed by flow cytometer. The samples were measured in triplicate and at least 4,000 F-18+ cells were analyzed per measurement. The percentage of cytotoxicity was calculated as follows:

$$((cont - test)/cont) \times 100 = \%$$

cytotoxicity with:

cont = number of F-18+, PI-cells (live K562 cells) divided by total number of F-18+ cells (total number of K562 cells) in the control sample;

test = number of F-18+, PI-cells (live K562 cells) divided by total number of F-18+ cells (total number of K562 cells) in the test sample.

Statistical Analysis

The data are presented as means ± S.D. Significance of differences was evaluated using the two-tailed t test.

RESULTS

Morphology of Fixed Cells

Interacting NK and K562 cells, fixed and stained with rhodamine phalloidin (F-actin) and Hoechst 33258 (DNA) (optional), were studied using CLSM. Since NK cells stained more intensely with rhodamine phalloidin as compared to the staining of K562 cells, F-actin of NK cell could be well distinguished from F-actin of target cell (20). Using the optical sectioning capability of the CLSM, we frequently observed that NK cells penetrated deeply into the K562 cells (in-conjugation). Examples of in-conjugation are shown in Figure 1.

Figure 1a shows a series of optical sections through a rhodamine phalloidin stained in-conjugate consisting of one K562 cell and two NK cells (arrows). As can be seen from the figure, the bottom NK cell completely entered the cytoplasm of the target cell. Figure 1b shows four optical sections, 1 μm apart, through the middle of an in-conjugate wherein the killer cell penetrated deeply into the target cell with a finger-like projection. In Figure 1c one optical section through a rhodamine phalloidin and Hoechst stained in-conjugate is shown. The nucleus of the target cell seems to be deformed by the F-actin rich protrusion of the killer cell. Actin filaments, that appear in the fraction of target cells during the cytotoxic process (20), were observed in virtually all in-conjugated K562 cells (>90%). They were occasionally seen to spread across the target cell nucleus (arrow), forming indentations. However, we do not know whether the target cell filaments deformed the nucleus or they were polymerized over already existing nuclear grooves.

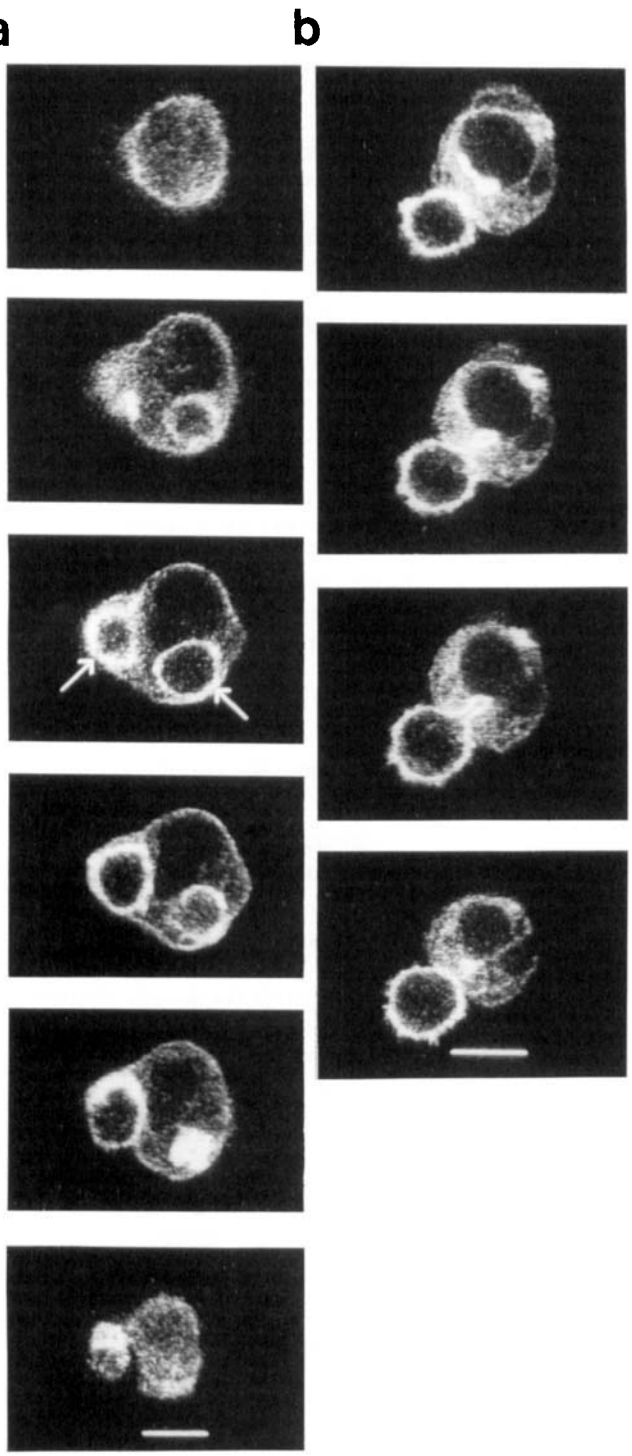

FIG. 1. Morphology of fixed cells. The bars represent 10 μm. **a:** Six optical sections (3 μm apart) through a rhodamine phalloidin labeled in-conjugate, containing two NK cells (arrows). The bottom NK cell completely entered the cytoplasm of the target cell. **b:** Four optical sections (1 μm apart) through the middle of a rhodamine phalloidin labeled in-conjugate, containing NK cell with a finger-like projection. **c** (see facing page): One optical section through a rhodamine phalloidin and Hoechst 33258 labeled in-conjugate. Green: Rhodamine phalloidin labeled F-actin; red: Hoechst 33258 labeled DNA. F-actin filaments (arrow) spread across the target cell nucleus which seems to be deformed by the killer cell protrusion.

Morphology of Living Cells

In order to confirm that the observed in-conjugation phenomena were not artifacts due to the fixation or staining procedures, we have done CLSM measurements using living interacting NK and K562 cells. Instead of phalloidin, which does not penetrate into living cells, we applied a vital staining using four different fluorescent probes. Prior to coincubating NK and K562 cells, both cells were labeled each with a combination of two dyes. NK cells were labeled with DiI (membrane probe) and SNARF (pH probe) (both are red fluorescing dyes); K562 cells were labeled with DiO (membrane probe) and Calcium Green (Ca^{2+} probe) (these are both green fluorescing dyes). Using this double-double labeling, we obtained two fluorescent signals that were distinguishable from each other and strong enough to perform confocal microscopy while the cells stay alive. Figure 2 shows CLSM images of two living in-conjugates, illustrating that in-conjugation indeed occurs during the cytotoxic interaction.

Relative Occurrence and Calcium Dependence of In-Conjugation

Using rhodamine phalloidin staining and conventional fluorescence microscopy, we have studied the relative occurrence and calcium dependence of in-conjugation. Since all in-conjugated K562 cells exhibited actin filaments, we have also determined the relative occurrence of conjugates that were *not* in-conjugates (thus, out-conjugates), but contained K562 cells exhibiting actin filaments (further referred to as *filamentous out-conjugates*). NK and K562 cells, resuspended in calcium-rich or calcium-free medium, were mixed, centrifuged, and incubated at 37°C. After different incubation times, the cell suspension was plated on PLL-coated cover glasses, fixed and stained with rhodamine phalloidin. The cytotoxic activities in the presence and absence of calcium were determined separately using the flow cytometric cytotoxicity assay as described in Materials and Methods. As can be seen from Table 1, the relative number of both in-conjugates and filamentous out-conjugates increased as a function of incubation time in calcium-rich medium (up to about 25 and 60%, respectively) but not in calcium-free medium. Similar effect was observed for the cytotoxic activity, as shown in Table 2[a].

Kinetics of In-Conjugation and the Effect of Cytochalasin D

Conjugates between NK and K562 cells were formed in calcium-free medium, allowed to attach on the PLL-coated cover glasses, transferred to the calcium-rich medium and incubated at 37°C. At the indicated time, cells were fixed, stained with rhodamine phalloidin, and the relative numbers of in-conjugates and filamentous out-conjugates were determined.

As can be seen from Figure 3, formation of in-conjugates was somewhat slower than formation of filamentous out-conjugates. While a significant number of fila-

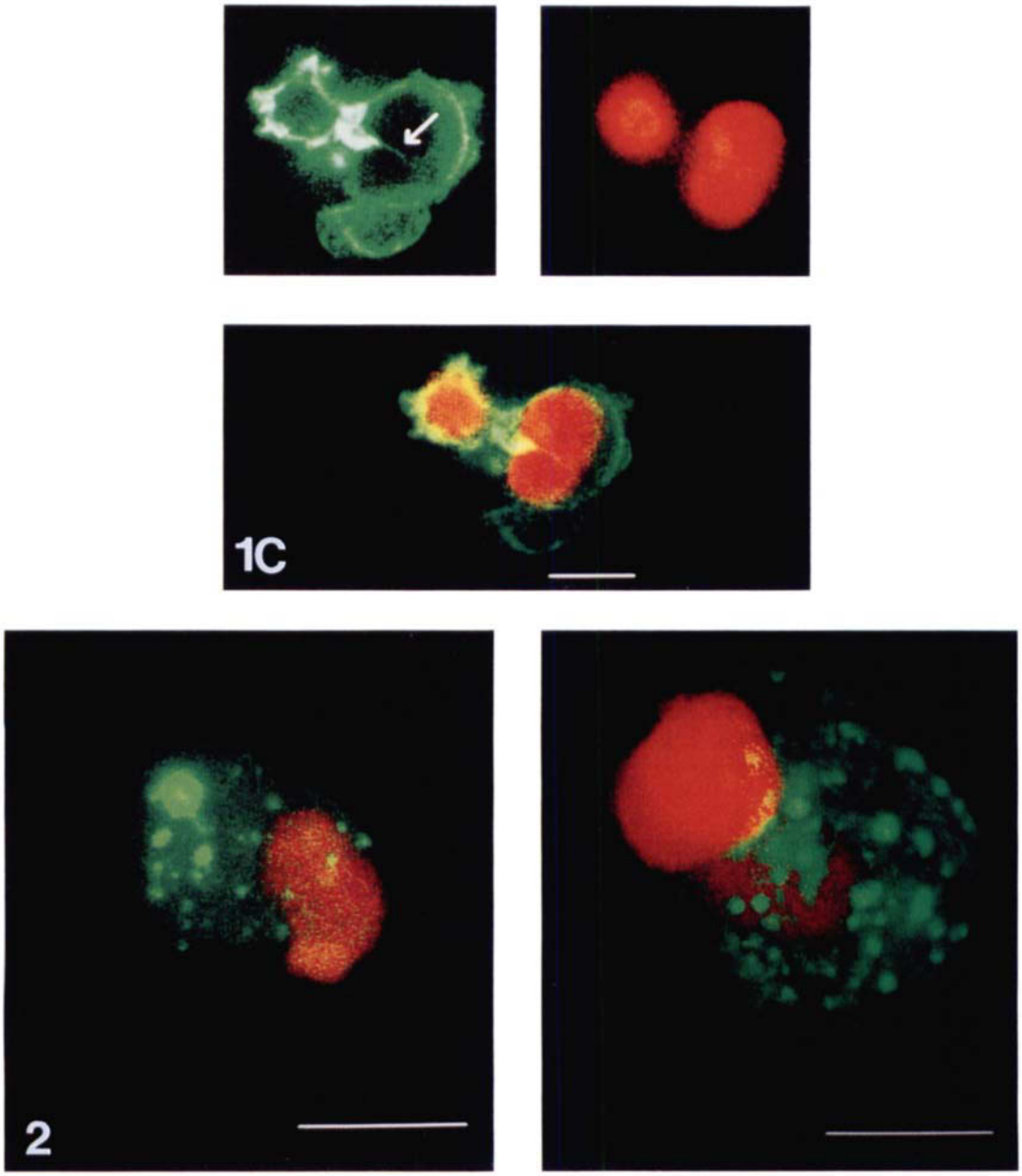

FIG. 2. Morphology of living cells: 3D-reconstructed CLSM images of two in-conjugates. Red: DiI/SNARF labeled NK cells; green: DiO/Calcium Green labeled K562 cells. The bars represent 10 μm.

mentous out-conjugates was observed already after 2 min incubation at 37°C, reaching a maximum in less than 10 min, in-conjugates started to form after about 4 min incubation and reached their maximum number after about 14 min.

Since virtually all in-conjugated K562 cells exhibited actin filaments, it was interesting to test whether actin polymerization was *necessary* for the in-conjugation to take place. Therefore, simultaneously with kinetics experiments as described above, the incubation of cells was also performed in calcium-rich medium containing 1 μM cytochalasin D [an actin polymerization inhibitor (4)]. Cytotoxic activities in the absence and presence of cytochalasin D were determined using the flow cytometric cytotoxicity assay. Presence of cytochalasin D completely inhibited formation of in-conjugates (less than 3% in-conjugates was detected), and reduced the cytotoxic activity (Table 2[b]).

Effect of $ZnSO_4$ on In-Conjugation

In order to investigate whether an endonuclease activity plays a role in the process of in-conjugation, we tested

Table 1
Effect of Calcium on In-Conjugation[a]

Incubation at 37°C	In-Conjugates (%)		Filamentous Out-Conjugates (%)	
	+Ca	−Ca	+Ca	−Ca
0′	2.2	<1	36.4	<2
15′	9.8	<1	59.7	<2
30′	14.0	<1	60.1	<2
45′	21.5	<1	58.9	<2
60′	26.3	<1	51.5	<2

[a]Conjugates were formed in calcium-rich (+Ca) or calcium-free (−Ca) medium and incubated at 37°C. After incubation cells were attached on PLL-cover glasses. A representative result, out of three similar experiments, is shown.

whether zinc ions [an endonuclease inhibitor (6)] influence in-conjugation. NK and K562 cells, resuspended in calcium-free medium at different concentrations of $ZnSO_4$, were mixed, centrifuged, and plated on PLL-cover glasses. The cover glasses were transferred to calcium-rich medium containing different concentrations of $ZnSO_4$ and incubated at 37°C. After 30 min incubation the samples were fixed, stained with rhodamine phalloidin, and the relative numbers of in-conjugates and filamentous out-conjugates were determined. The cytotoxic activity was determined under the same conditions using the flow cytometric cytotoxicity assay. Figure 4 shows the effect of $ZnSO_4$ on the occurrence of in-conjugates (black bars) and filamentous out-conjugates (shaded bars) as a function of $ZnSO_4$ concentration. As can be seen from the figure, there was a concentration dependent decrease in the relative number of both in-conjugates and filamentous out-conjugates. However, in-conjugates appeared to be more sensitive to $ZnSO_4$ than filamentous out-conjugates, being completely inhibited at concentrations of $ZnSO_4$ higher than 0.25 mM.

As can be seen from Table 3, $ZnSO_4$ partly inhibited the cytotoxic activity. The lowest concentration causing detectable inhibition of the cytotoxic activity was 0.25 mM.

DISCUSSION

In this study, we report on the appearance of a (pseudo)emperipoletic interaction (in-conjugation) between cloned human NK cells and K562 target cells as revealed by confocal microscopy on fixed (Fig. 1) as well as on living cells (Fig. 2). Although emperipolesis has been observed previously, its occurrence was believed to be very rare (18). However, our study and that of others (2,26) demonstrate that penetration of killer cells into their target cells during the cytotoxic interaction is far from being rare and should not be neglected. We show, using cloned human NK cells, that a significant part of the conjugated killer cells (about 30%) penetrates, partly or completely, into the target cells. It has been shown previously, by ultrastructural studies of biopsies of primary human malignant melanoma (2), that in-conjugation also occurs in vivo. This further necessitates more studies of this interesting phenomenon.

In order to get an insight into the factors affecting in-conjugation, we studied parameters known to influence the cytotoxic interaction. Since the majority of in-conjugated target cells (>90%) exhibited actin filaments [a phenomenon observed in the fraction of target cells during the cytotoxic process (20)], in all experiments we also considered effects on the relative number of filamentous out-conjugates (thus, out-conjugates with target cells exhibiting actin filaments).

Actin polymerization and in-conjugation are closely associated with the cytotoxic process. In the absence of calcium, neither of the processes takes place (Tables 1, 2[a]). Immediately after addition of calcium to conjugates, actin starts to polymerize in the targets cells and a few minutes later the first in-conjugates can be detected (Fig. 3). Whether in-conjugation develops in those conjugates that already exhibit actin filaments (filamentous out-conjugates) or is preceded by new actin polymerization is not clear. It is clear, however, that actin polymerization, although is not necessary to obtain a partial cytotoxic activity (i.e., cytotoxic activity is not completely blocked by cytochalasin D) (Table 2[b]), it is absolutely needed for in-conjugation to take place (less than 3% in-conjugates is detected in the presence of cytochalasin D). When analyzing the effect of cytochalasin D on the cytotoxic activity and in-conjugation, however, different aspects [such as involvement of actin in the recycling of killer cells (12) or endocytosis of killer cell material by target cells (5,11,27), as well as complex regulatory mechanisms of actin polymerization (3,8)] should be considered.

Our results on the effects of calcium and cytochalasin on in-conjugation are in disagreement with the results of Rosen et al. (22). These authors reported that the absence of calcium or presence of *cytochalasin A* in the incubation medium did not reduce the formation of *CTL projections*, as detected using *electron microscopy*, while it did reduce the cytotoxic activity. The discrepancy with our results is probably due to different experimental procedures, including the definition of the terms "projections" and "in-conjugation".

The concentration dependent inhibitory effect of zinc ions on in-conjugation (Fig. 4) is another interesting finding. Since it has been reported previously that zinc ions suppress an endonuclease activity and so inhibit target cell DNA fragmentation (6), this result may indirectly indicate a relationship between in-conjugation and DNA damage. Besides completely inhibiting in-conjugation, $ZnSO_4$ also decreases the number of filamentous out-conjugates, although in a less sensitive way than observed for in-conjugates (Fig. 4). Thus, all in-conjugates and a *fraction* of filamentous out-conjugates are affected by zinc ions, presumably indicating that in these target cells an endonuclease dependent process takes place. The inhibition of in-conjugation by $ZnSO_4$ closely resembles the reported concentration dependent inhibitory effect of $ZnSO_4$ on DNA fragmentation (6). The kinetics of in-conjugation are also similar to the kinetics of CTL-induced nuclear lesions as reported by Russel et al. (24). It

Table 2
Effect of Calcium and Cytochalasin D on Cytotoxicity

	Cytotoxicity (%)					
	Calcium[a]		Cytochalasin D[b]			
	Ratio 1:1		Ratio 1:1		Ratio 10:1	
Incubation at 37°C	+	−	−	+	−	+
0′	4.4 ± 0.6	0.4 ± 0.1*	1.1 ± 0.1	1.0 ± 0.3	12.1 ± 4.7	4.1 ± 0.7
15′	12.3 ± 1.0	1.2 ± 0.3*	4.6 ± 1.8	1.9 ± 0.7**	20.1 ± 3.0	12.5 ± 4.3
30′	17.6 ± 1.1	1.8 ± 0.6*	7.7 ± 2.1	2.4 ± 0.8*	26.5 ± 2.1	20.9 ± 5.9
45′	22.6 ± 2.1	1.6 ± 0.6*	9.3 ± 1.8	2.6 ± 1.8*	30.0 ± 0.5	21.1 ± 5.7
60′	27.8 ± 2.5	1.0 ± 0.7*	13.7 ± 2.5	3.1 ± 1.4*	38.3 ± 2.2	19.9 ± 2.8*

[a]Conjugates were formed in calcium-rich (+) or calcium-free medium (−) at a ratio 1:1 and incubated at 37°C. After incubation, cells were measured in the flow cytometer. The result of two separate experiments is shown [*, significantly different from the control (+calcium); $P < 0.001$.]

[b]Conjugates were formed in calcium-free medium at ratios 1:1 and 10:1, centrifuged and resuspended in calcium-rich medium with (+) or without (−) 1 μM cytochalasin D. After incubation, cells were measured in the flow cytometer. The result of two separate experiments is shown. [*,**, significantly different from the control (−cytochalasin D): *$P < 0.001$; **$P < 0.01$.]

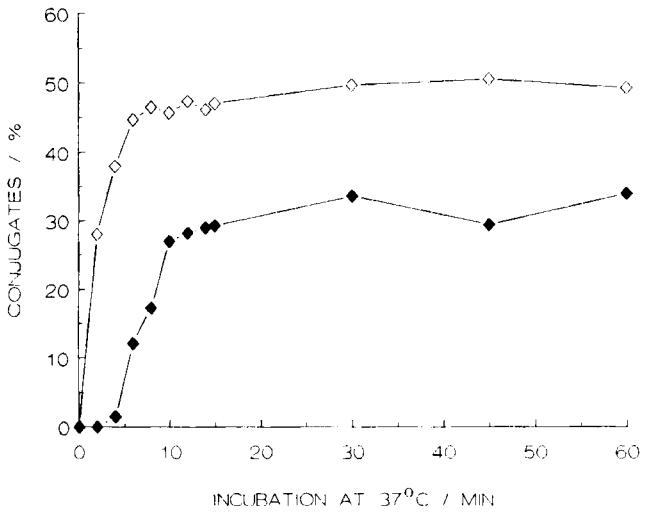

FIG. 3. Kinetics of in-conjugation. Conjugates were formed in calcium-free medium and, after attachment to PLL-cover glasses, transferred to calcium-rich medium and incubated at 37°C. The averaged results of two experiments are shown. Closed symbols: in-conjugates/conjugates (%); open symbols: (filamentous out-conjugates) conjugates (%).

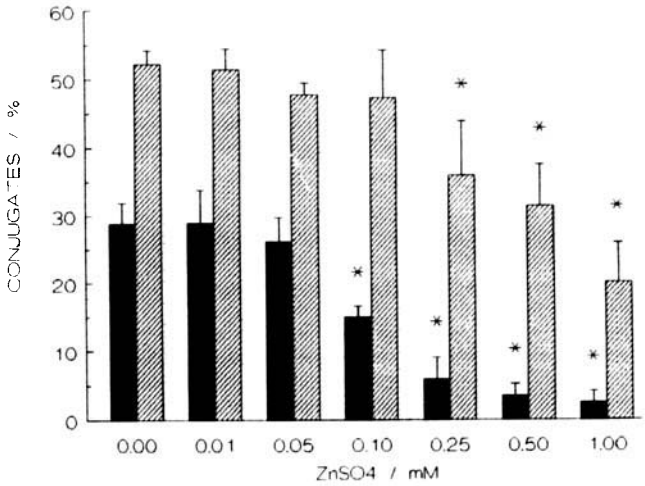

FIG. 4. Concentration dependence of in-conjugation on $ZnSO_4$. Conjugates were formed in calcium-free medium with or without $ZnSO_4$. After attachment to PLL-cover glasses (in-conjugation test) cells were transferred to calcium-rich medium with or without $ZnSO_4$ and incubated at 37°C for 30 min. The result of four different experiments is shown. Black bars: in-conjugates/conjugates (%); shaded bars: (filamentous out-conjugates) conjugates (%). [*, significantly different from the control (0 mM $ZnSO_4$), $P < 0.001$.]

is interesting to mention that killer cells often approach the target cell nucleus (Fig. 1). This striking phenomenon could easily be placed in a hypothesis about relationship between in-conjugation and DNA damage. However, we have no *direct* evidence that DNA damage indeed takes place in the in-conjugated target cells.

The relationship between endonuclease activity (i.e., an activity that can be inhibited by zinc ions), actin polymerization, and in-conjugation is completely unclear. However, a sequence of events can *indirectly* be deduced from the results. The inhibition experiments show that both activity of endonuclease and intact actin polymerization are necessary for in-conjugation, thus, they apparently precede it. The treatment with $ZnSO_4$ decreases the total number of cells with polymerized actin, indicating that the activity of endonuclease precedes actin polymerization. Thus, the activity of endonuclease, followed by the polymerization of actin, may result in the formation of in-conjugates. According to this scheme, in-conjugation could be an actin polymerization dependent *consequence* of the cytotoxic mechanism that is affected by zinc ions, thus, presumably target cell DNA damage.

The inhibitory effects on the cytotoxic activity are not completely complementary to the inhibitory effects on in-conjugation. While the cytotoxicity assay indicates *complete* cytotoxic activity, in-conjugation probably only reflects a part of a certain killing mechanism. However, since our evidence is primarily indirect, we can not exclude other possible causes of the in-conjugation.

In conclusion, we have demonstrated the significant

Table 3
Effect of $ZnSO_4$ on Cytotoxicity[a]

	Cytotoxicity (%)	
$ZnSO_4$ (mM)	Ratio 1:1	Ratio 10:1
0.00	9.8 ± 1.0	34.2 ± 1.3
0.01	9.3 ± 1.4	37.8 ± 3.8
0.05	9.0 ± 1.2	36.0 ± 3.0
0.10	9.3 ± 0.2	33.8 ± 1.2
0.25	4.8 ± 0.8**	29.5 ± 2.1
0.50	2.7 ± 2.3*	15.1 ± 3.6*
1.00	3.3 ± 4.7	17.4 ± 3.5*

[a]Conjugates were formed in calcium-free medium with or without $ZnSO_4$. After centrifugation, cells were resuspended in calcium-rich medium with or without $ZnSO_4$ and incubated at 37°C for 30 min. A representative result, out of two separate experiments, is shown. [*,**, significantly different from control (0 mM $ZnSO_4$): $^*P < 0.001$; $^{**}P < 0.01$.]

occurrence of in-conjugation during the cytotoxic interaction between human NK cells and K562 target cells. Our results demonstrate the dependence of in-conjugation on the presence of calcium, actin polymerization, and an endonuclease activity, indicating that in-conjugation may represent an actin polymerization dependent consequence of target cell DNA damage.

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