Journal of Immunological Methods, 161 (1993) 119–128

JIM 06675

A flow cytometric study of the membrane potential of natural killer and K562 cells during the cytotoxic process

Katarina Radošević, Tom C. Bakker Schut, Marja van Graft, Bart G. de Grooth and Jan Greve

University of Twente, Department of Applied Physics, Applied Optics Group, Enschede, Netherlands

(Received 14 September 1992, revised received 9 December 1992, accepted 5 January 1993)

This study demonstrates that it is possible to investigate the membrane potential of interacting cells during the cytotoxic process using flow cytometry. Changes in the membrane potential of NK and K562 cells, involved in a cell-mediated cytotoxic process, were studied by standard and slit-scan flow cytometry, using the membrane potential sensitive fluorescent probe $DiBAC_4(3)$. The NK cells were labeled with a membrane marker (TR-18 or DiI) prior to incubation with K562 cells and the conjugates that were formed could be identified on the basis of the membrane marker fluorescence and light scattering signals. With a slit-scan technique we measured the membrane potential of each cell in a conjugate separately. The results show that depolarization of the K562 cell occurs as a consequence of the cytotoxic activity of the NK cell. This depolarization appears to be an early sign of cell damage because the cell membrane still remains impermeable to propidium iodide. Our data also indicate that depolarization of the NK cell occurs as a result of its cytotoxic activity.

Key words: Natural killer cell; Cytotoxicity; Membrane potential; Flow cytometry

Introduction

The most prominent functional characteristic of NK cells is their ability to kill tumor cells, immature cells and virus-infected cells in vitro without prior sensitization and in an MHC-unrestricted fashion (Trinchieri, 1989). Close contact between the NK cell and its target cell (so-called conjugate formation) is a prerequisite for the killing process to take place (Bonavida et al., 1983; Henkart, 1985). Using light microscopy, it can be shown that active NK cells move towards the target cells and then form large contact regions with the target cells. It has generally been accepted that during the killing process the NK cell secretes toxic molecules that damage the target cell membrane. The disturbance of target cell membrane integrity then causes impaired ion fluxes which eventually result in target cell death.

The changes in cell physiology during the cytotoxic process have been the subject of numerous studies (reviewed by Young, 1989). The use of fluorescent probes specific for some of the physiological parameters, such as intracellular calcium (Gray et al., 1988; Edwards et al., 1989; McConkey et al., 1990), pH (Van Graft et al., 1993)

Correspondence to: J. Greve, University of Twente, Dept. of Applied Physics, P.O. Box 217, 7500 AE Enschede, Netherlands. Tel.: 31-53-893157; Fax: 31-53-309549.

Abbreviations: $DiBAC_4(3)$, bis-(1,3-dibutylbarbituric acid)trimethine oxonol; DiI, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate; NK, natural killer; TR-18, octadecylamine-tetramethylrhodamine isothiocyanate.

and membrane fluidity (Roozemond and Bonavida, 1985), have provided a better insight into the process.

Here we report a study of the membrane potential changes of NK and K562 cells during the cytotoxic process. The aim of the study was to determine by means of flow cytometry if there are changes in the membrane potential of the interacting cells which reflect the activation of the killing mechanism in the NK cell and/or the damage of the K562 cell.

As a membrane potential indicator we used $DiBAC_4(3)$, an anionic probe whose distribution across the cell membrane depends on membrane potential (Wilson and Chused, 1985). Upon depolarization, when the inside of the cell becomes more positively charged, more dye enters the cell resulting in an increase in cellular fluorescence. Upon hyperpolarization, the dye is extruded from the cell resulting in a decrease in fluorescence.

Identification of the conjugates formed by NK and K562 cells was done using an additional probe. This was necessary because the scattering signals of conjugates overlap with the scattering signals of single K562 cells. By labeling the NK cells with a membrane marker (TR-18 or DiI) prior to incubation with the K562 cells it was possible to identify single NK cells (membrane marker positive, small scattering signal), single K562 cells (membrane marker negative, large scattering signal) and conjugates formed by NK and K562 cells (membrane marker positive, large scattering signal). In order to monitor the membrane potential of each cell in a conjugate separately we have used a recently developed slit-scan technique (Bakker Schut et al., 1993). Our results demonstrate that it is possible to monitor the membrane potential of interacting cells during the cytotoxic process by means of flow cytometry.

Materials and methods

Cells

K562 cells, for use as targets, were grown as previously described (Radošević et al., 1990). The NK cell-clone NK76 was generously provided by Dr. R.L.H. Bolhuis and the cells maintained as described elsewhere (Bolhuis et al., 1984).

Staining procedures

TR-18 was prepared as previously described (Koolwijk et al., 1988). A DiI ($DiIC_{18}(3)$) (Molecular Probes, Eugene, OR) stock solution was prepared in DMSO (concentration 4 mg/ml). NK cells were resuspended in RPMI 1640 + Hepes + 10% fetal calf serum + 2 mM L-glutamine + antibiotics (100 IU penicillin-G + 100 μg streptomycin–sulfate/ml) (further referred to as a complete medium, CM) at a concentration of 10^6/ml. TR-18 was added to the cell suspension at a final concentration of 15–30 ng/ml. If DiI was used, it was added to the cell suspension at a final concentration of 2 μg/ml. The cell suspension was incubated at 37°C under 5% CO_2 for 1 h in the dark with occasional mixing. After incubation the cells were washed twice with an excess volume of cold CM and resuspended in CM at a concentration of 2.5×10^6/ml.

A 5 mM $DiBAC_4(3)$ (Molecular Probes Inc., Eugene, OR) stock solution was prepared in ethanol. Cells were incubated with $DiBAC_4(3)$ (concentration 200 nM) in PBS at a concentration of 2.5×10^5/ml for 3–5 min at room temperature and, without additional washing, analyzed by flow cytometry.

Propidium iodide stock solution was prepared by dissolving 0.01 mg propidium iodide in 1 ml 10% ethanol. Stock solution was added to the cell suspension to a final concentration of 0.1 μg/ml.

Conjugate formation

Equal volumes of labeled NK cells and K562 cells (concentration 2.5×10^6 cells/ml) were mixed and divided into aliquots of 0.1–0.2 ml. The cells were centrifuged for 1 min at $200 \times g$, the pellet was gently resuspended and the cell suspension was incubated at 37°C. The samples were stained with $DiBAC_4(3)$ in PBS with or without addition of propidium iodide and analyzed by means of flow cytometry.

Flow cytometry

Experiments were performed with a home built flow cytometer equipped with an argon ion laser (model 2020, Spectra-Physics, Mountain View, CA) tuned to 488 nm. The instrument is comparable to commercially available instruments and has been described elsewhere (Terstappen et al.,

1986). $DiBAC_4(3)$ fluorescence was measured using the green fluorescence channel (510–550 nm), TR-18 or DiI fluorescence was measured using the orange fluorescence channel (550–580 nm) and propidium iodide fluorescence was measured using the red fluorescence channel (> 640 nm).

The slit-scan flow cytometer has been described elsewhere (Bakker Schut et al., 1993). The principle of the measuring technique is as follows. If two adherent cells have similar sizes, the cells will be oriented along the direction of flow by the hydrodynamic forces in a typical flow cell. When illuminated with a small slit (slit-scan) one can resolve the individual cells of a conjugate. Typical slit-scan signals of the conjugates measured are represented in Fig. 1. The pulse form of the forward scattering signal shows two peaks when a conjugate passes the slit (Fig. 1*a*). Two peak detectors were used to measure the membrane marker fluorescence for each cell in a conjugate separately (Fig. 1*b*). In order to activate these peak detectors, the first derivative of the forward scattering signal was employed and the zero crossing points of this derivative were used to determine the contact region. The intensity of the $DiBAC_4(3)$ fluorescence was correlated with the TR-18 or DiI membrane marker fluorescence for each cell (Fig. 1*c*).

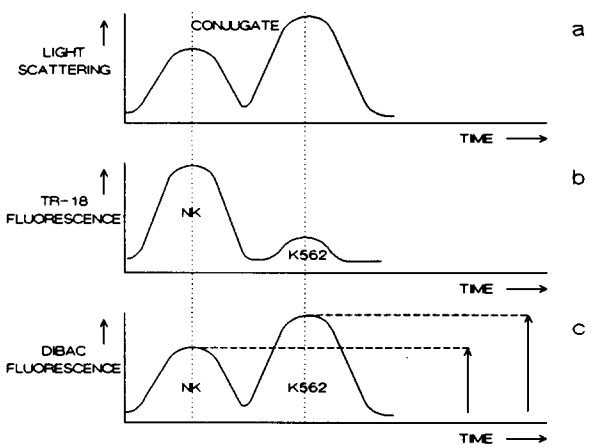

Fig. 1. Principle of the slit-scan technique used to monitor $DiBAC_4(3)$ fluorescence of each cell in a conjugate separately. The conjugates were detected on the basis of the pulse form of the scattering signal (*a*). Cells in the conjugate were identified on the basis of TR-18 fluorescence (*b*) and intensity of their $DiBAC_4(3)$ fluorescence was determined (*c*).

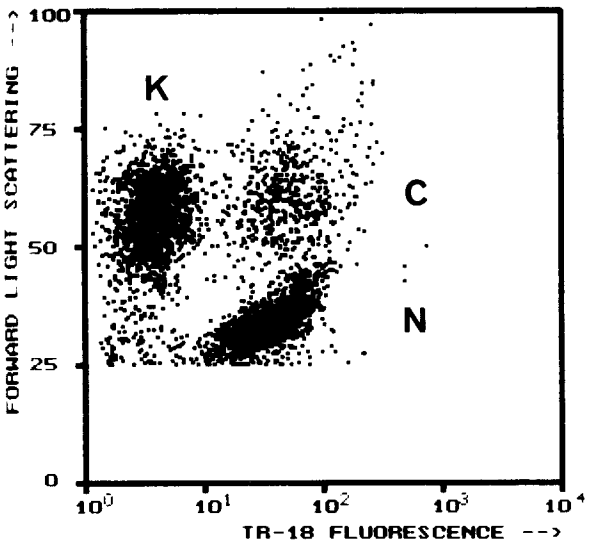

Fig. 2. Forward light scattering versus TR-18 fluorescence scatter plot showing single and conjugated NK and K562 cells. TR-18-labeled NK cells were incubated with K562 cells at 37°C for 20 min. The figure illustrates that single K562 cells, single NK cells and conjugates could be distiguished. C, conjugates; K, K562 cells; N, NK cells.

The data were analyzed by Analysis, a software program developed in our lab. In the histograms presented cell populations are scaled to an arbitrary maximum and not to each other.

Results

Conjugate discrimination

Conjugates formed by TR-18 labeled NK cells and K562 cells could be distinguished from single cells on the basis of TR-18 fluorescence and forward light scattering signals (Fig. 2). Similar results were obtained using DiI instead of TR-18 to label the NK cells. During incubation at 37°C, the number of conjugates increased, reaching a maximum after about 20 min. The maximum number of conjugated NK cells varied between 20–40% in different experiments.

Potential changes during incubation

In Fig. 3 the $DiBAC_4(3)$ fluorescence histograms of single NK cells, single K562 cells and conjugates are shown for a sample analyzed immediately after conjugate formation. The average fluorescence intensity of single K562 cells was

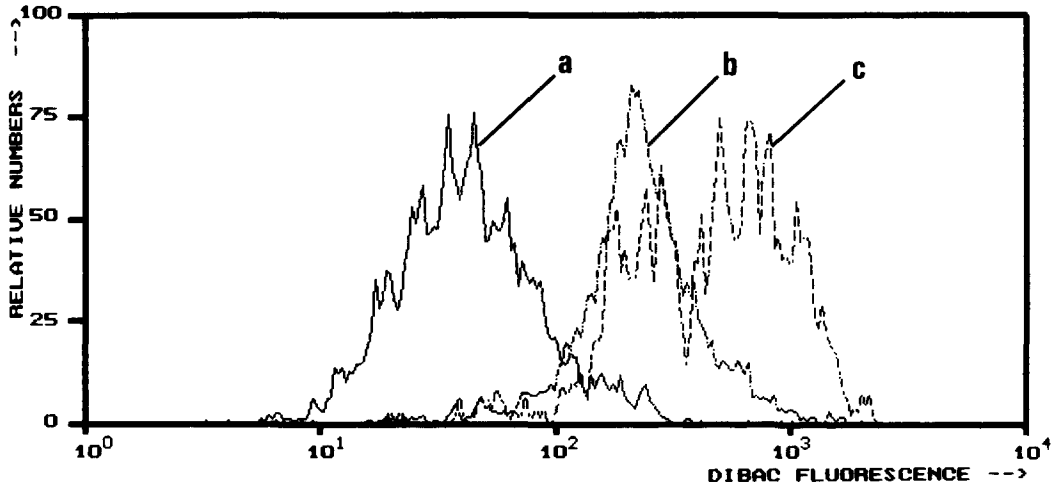

Fig. 3. $DiBAC_4(3)$ fluorescence histograms of a mixture of K562 and NK cells obtained immediately after conjugate formation. The populations were identified using the principle illustrated in Fig. 2 except that the NK cells were labeled with DiI instead of TR-18. a, single NK cells; b, single K562 cells; c, conjugates.

about five times higher than the average fluorescence intensity of single NK cells. This was presumably due to the difference in the volume of both cells. Conjugates formed by NK and K562 cells showed a bimodal $DiBAC_4(3)$ distribution: part of the conjugates exhibited a $DiBAC_4(3)$ fluorescence comparable to that of single K562 cells while another part was shown to have an intensity about three times higher. Most (> 90%) of the conjugates with the high $DiBAC_4(3)$ fluorescence did not stain with propidium iodide.

During incubation of NK and K562 cells at 37°C a population of depolarized K562 cells and a population of depolarized NK cells appeared.

Fig. 4 illustrates $DiBAC_4(3)$ fluorescence histograms for single K562 cells incubated with (*a*)

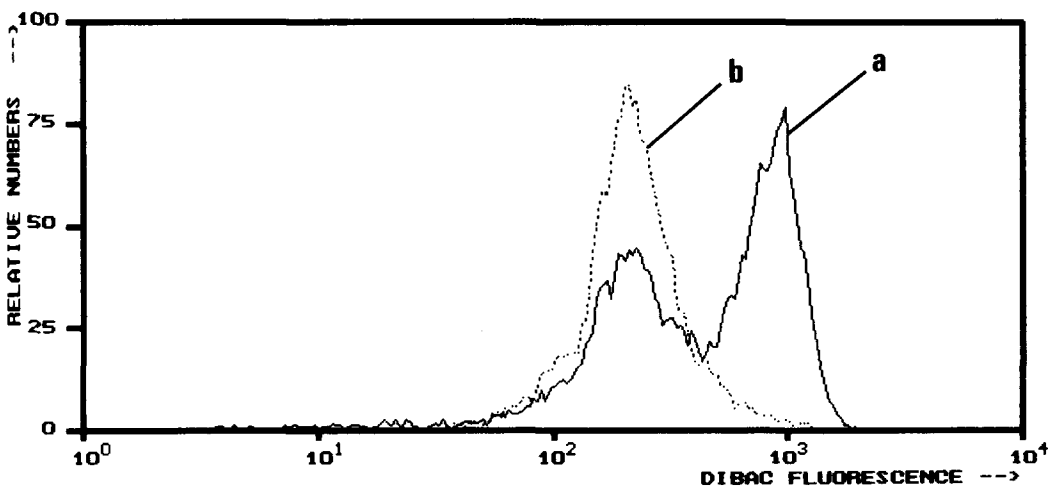

Fig. 4. $DiBAC_4(3)$ fluorescence histograms showing depolarization of the single K562 cells during the cytotoxic process. K562 cells were incubated at 37°C for 35 min in the presence of NK cells (*a*) or without NK cells (*b*). The $DiBAC_4(3)$ fluorescence histograms of propidium iodide negative K562 cells are shown.

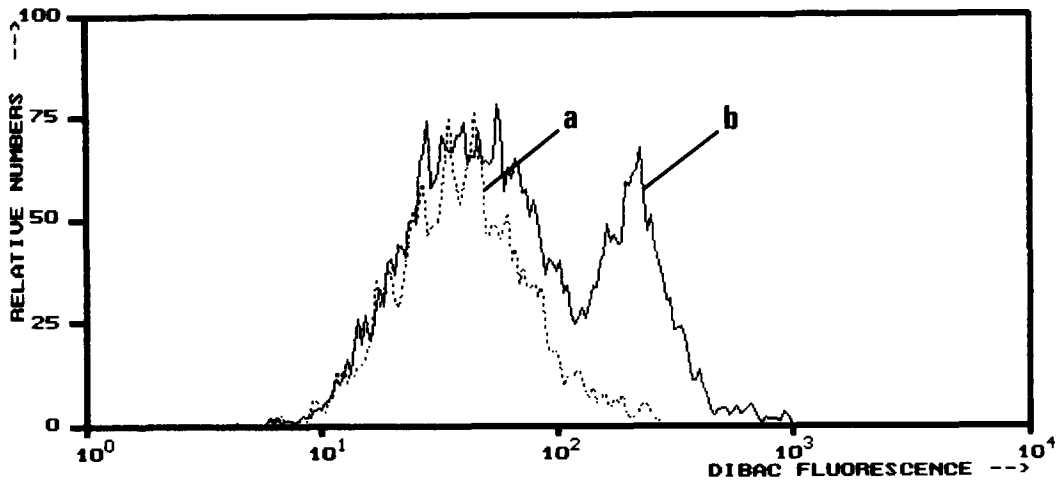

Fig. 5. $DiBAC_4(3)$ fluorescence histograms showing depolarization of single NK cells during the cytotoxic process. DiI-labeled NK cells were incubated with K562 cells for 0 min (*a*) or 35 min (*b*) at 37°C. The $DiBAC_4(3)$ fluorescence histograms of propidium iodide negative NK cells are shown.

and without (*b*) NK cells at 37°C for 35 min. Incubation with the NK cells resulted in a fraction of single K562 cells with higher $DiBAC_4(3)$ fluorescence intensity than the control cells.

Fig. 5 shows $DiBAC_4(3)$ fluorescence histograms for single NK cells in the sample analyzed immediately after conjugate formation (*a*) and after an additional 35 min incubation at 37°C (*b*). The $DiBAC_4(3)$ fluorescence intensity of a fraction of single NK cells increased about five times during incubation with K562 cells at 37°C. In the control sample (NK cells incubated alone) the increase in the number of depolarized cells did not exceed 5% of all NK cells after a 1 h incubation.

Most (> 90%) of the NK and K562 cells that depolarized during incubation also remained impermeable to propidium iodide.

The fraction of depolarized conjugates varied between 20–50% in different experiments and remained relatively constant during incubation up to 1 h (Fig. 6). On the other hand, the fraction of depolarized single NK and K562 cells increased during incubation and reached a maximum at about 35 min (Fig. 6).

Slit-scan flow cytometry

The above results suggest that in conjugates, formed by NK and K562 cells, a depolarization was occurring during incubation at 37°C. However, because the $DiBAC_4(3)$ fluorescence signal of the conjugate is the sum of the $DiBAC_4(3)$ fluorescence signals of the cells that form the conjugate, it is not clear in which of the conjugate forming cells the change in membrane potential occurs. In order to clarify this point we used the slit-scan analysis technique developed in our lab

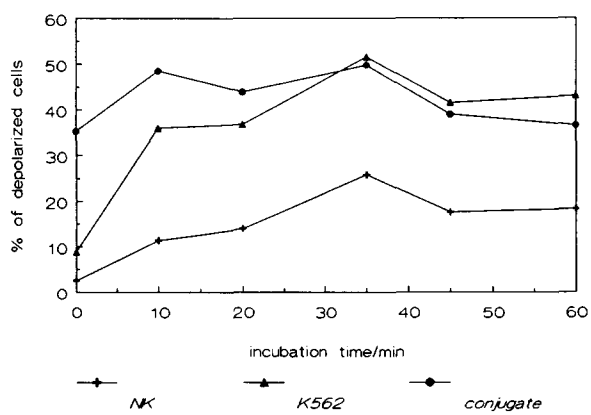

Fig. 6. Fractions of depolarized cells as a function of incubation time. DiI-labeled NK cells were incubated with K562 cells at 37°C, stained with $DiBAC_4(3)$ and analyzed by the flow cytometer. The percentage of cells with high $DiBAC_4(3)$ signal was determined for each cell type. + ——— +, single depolarized NK cells; △ ——— △, single depolarized K562 cells; ● ——— ●, depolarized conjugates.

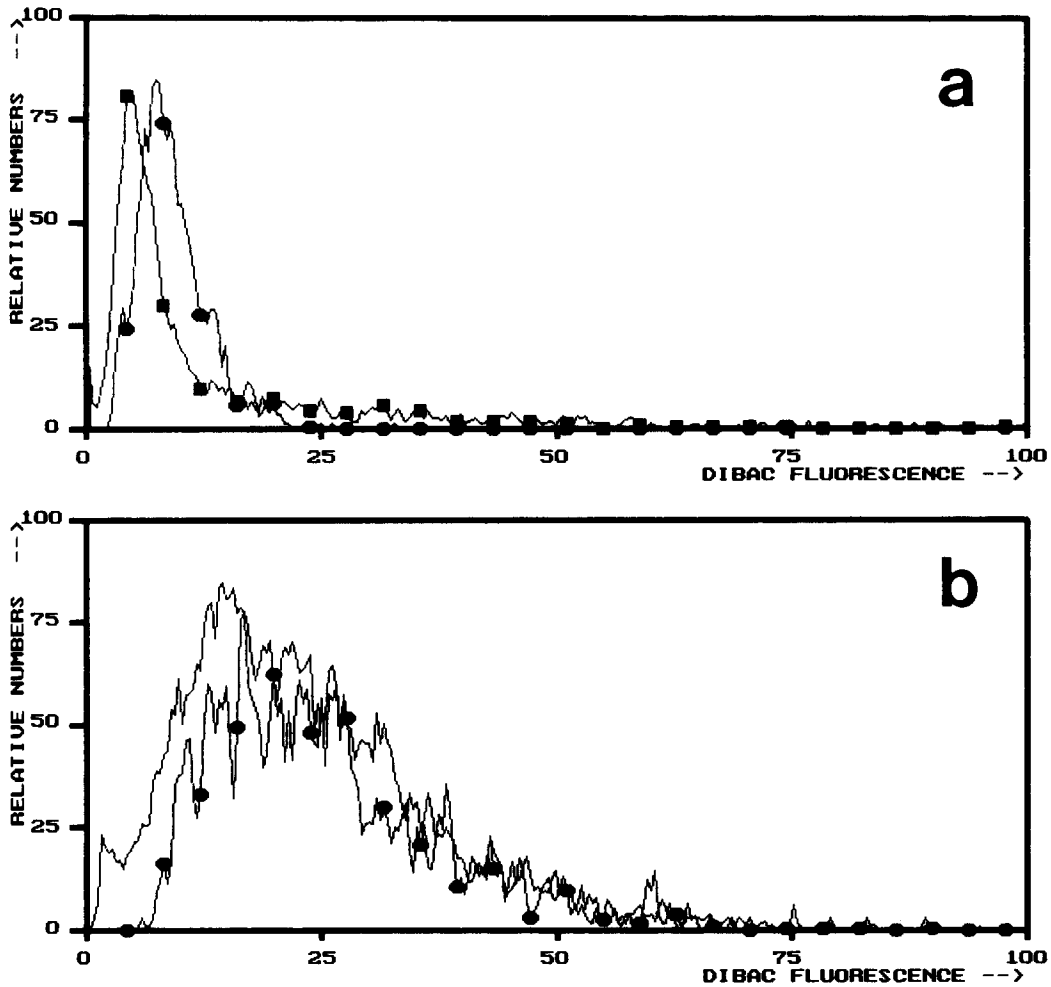

Fig. 7 (*continued on next pages*).

(Bakker Schut et al., 1993) by means of which we can measure the $DiBAC_4(3)$ fluorescence and a membrane marker fluorescence for each cell in a conjugate separately (Fig. 1). Fig. 7 presents the results obtained with K562 and NK cells prior to incubation at 37°C (*a,b*), after 10 min (*c,d*) and 20 min (*e,f*) incubation at 37°C. As can be seen from Fig. 7*b,d,f*, incubation of NK and K562 cells at 37°C led to the depolarization of a fraction of the conjugated K562 cells. Therefore, we conclude that the increase in the $DiBAC_4(3)$ fluorescence of conjugates, observed with the standard flow cytometer (Fig. 3), was due to depolarization of a fraction of conjugated K562 cells. Conjugated NK cells also exhibited a somewhat higher $DiBAC_4(3)$ fluorescence than single NK cells although this did not appear to depend on the incubation time (Fig. 7*a,c,e*). The appearance of single depolarized NK cells and single depolarized K562 cells during incubation at 37°C (Fig. 7*a–f*) should be noted. Depolarization of the cells was not observed in the control samples (Fig. 7*g,h*).

Discussion

In cell functioning the membrane potential has an important role by controlling, either directly or indirectly, ion fluxes across the cell membrane.

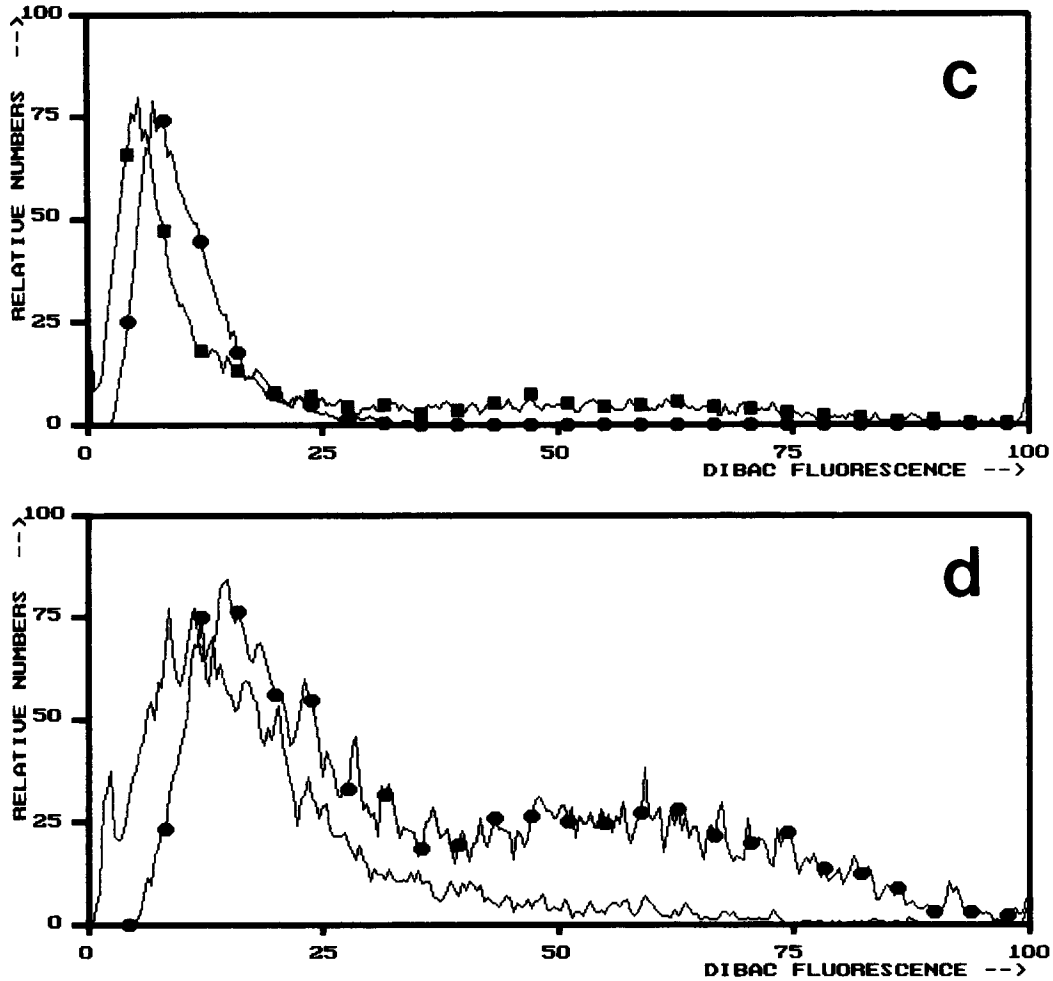

Fig. 7 (*continued on next pages*).

Signal transduction as well as osmotic balance of the cell are both influenced by the potential across the cell membrane. In our study we have tried to detect whether there are changes in the membrane potential of the NK cells, due to activation of the cytotoxic process, and in the membrane potential of target cells (K562 cells), due to cell damage inflicted during the cytotoxic process. As an indicator of membrane potential we have used $DiBAC_4(3)$, an oxonol dye that has proved to be the most sensitive to manipulations of membrane potential (Rink et al., 1980; Brauner et al., 1984; Wilson and Chused, 1985; Civitelli et al., 1987).

During incubation at 37°C we observed a depolarization of a fraction of the conjugates (Fig. 3). This depolarization is mainly due to the depolarization of conjugated K562 cells as identified by the slit-scan flow cytometry (Fig. 7). The cell membrane of depolarized cells remains impermeable for the cell death indicator, propidium iodide, for incubation periods up to at least one hour. Depolarization of the conjugated K562 cells could be an early sign of cell damage caused by NK cell cytotoxic activity. It has been reported previously that the resting membrane potential of target cells depolarizes after the cells are treated with a pore forming protein (perforin) (Young et

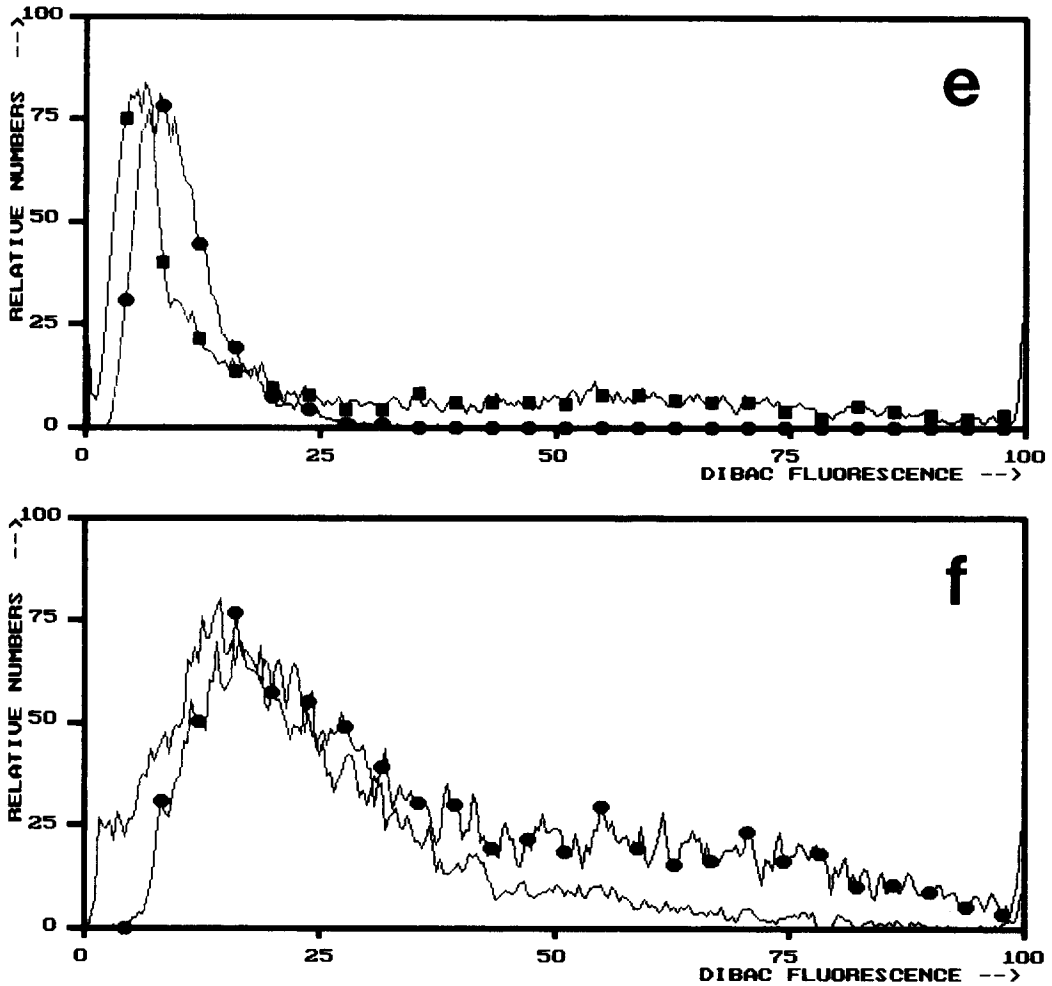

Fig. 7 (*continued on next page*).

al., 1986). The appearance of the single depolarized K562 cells (Figs. 4, 7*d*,*f*), whose number reaches a maximum after about 35 min incubation (Fig. 6), may be due to detachment of the depolarized K562 cells from conjugates after the cytotoxic process has occurred.

We do not know whether the observed depolarization of conjugated NK cells (Fig. 7*a*,*c*,*e*) is real or the result of 'leakage' of the relatively stronger $DiBAC_4(3)$ fluorescence of the conjugated K562 cell into the NK cell channel. However, we can speculate that the single depolarized NK cells (Figs. 5, 7*c*,*e*), whose number also reaches a maximum after about 35 min incubation (Fig. 6), represent cells that were conjugated with K562 cells and, after expression of their cytotoxic activity, became detached. Depolarization of the NK cells could be due to activation of voltage-dependent Na^+ channels. It has been reported that activation of such channels not only depolarizes the NK cells but also reduces their in vitro cytotoxic activity (Mandler et al., 1990). The reported inactivation of the cytotoxic activity of NK cells after target cell killing (Abrams and Brahmi, 1988) could, at least in part, be caused by depolarization of the NK cell due to activated Na^+ channels. However, this assumption remains to be tested.

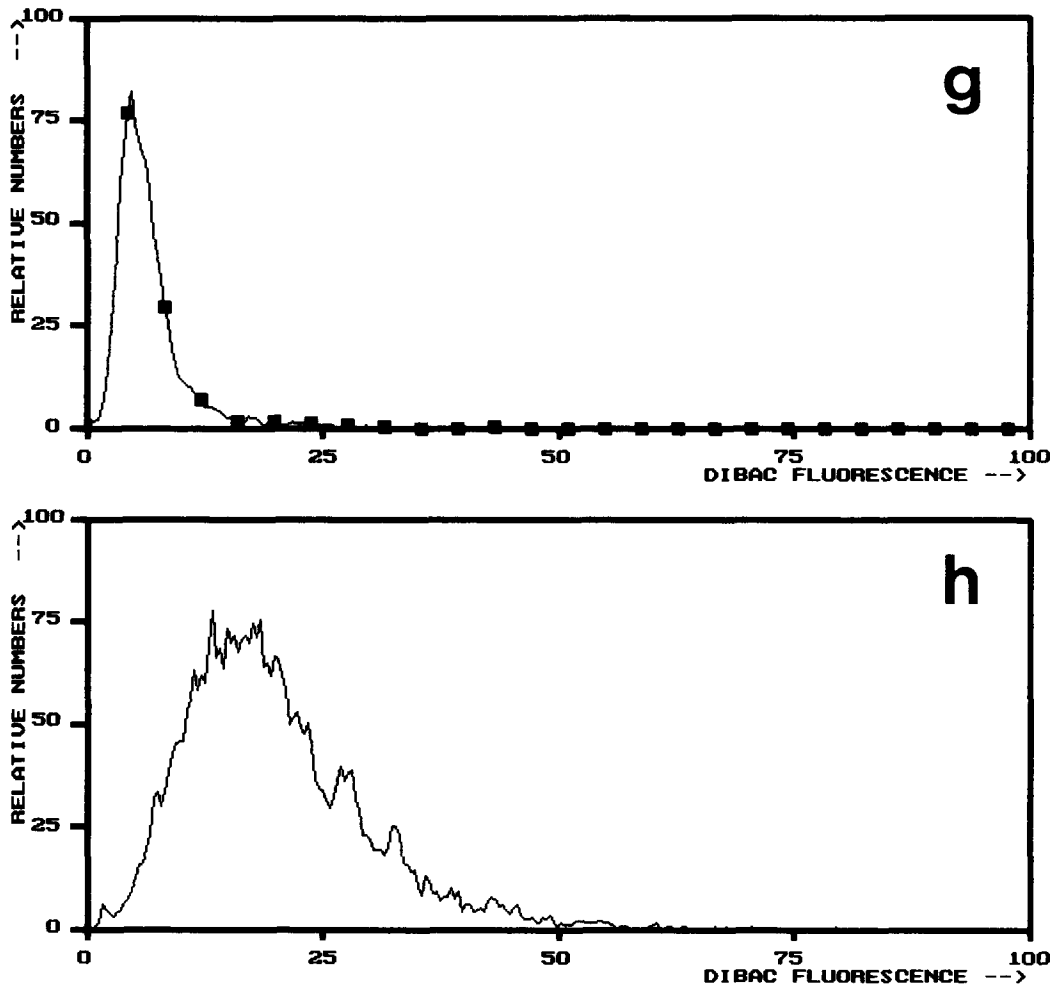

Fig. 7. $DiBAC_4(3)$ fluorescence histograms of conjugated and single NK and K562 cells obtained with the slit-scan flow cytometer. TR-18-labeled NK cells were incubated with K562 cells for 0 min (*a,b*), 10 min (*c,d*) and 20 min (*e,f*) at 37°C, stained with $DiBAC_4(3)$ and analyzed by the slit-scan flow cytometer. *a*, *c*, *e*, histograms of NK cells (■———■, single; ●———●, conjugated); *b*, *d*, *f*, histograms of K562 cells (———, single; ●———●, conjugated); *g*, *h* histograms of control samples (NK cells and K562 cells, respectively, incubated at 37°C for 20 min).

It has been suggested that hyperpolarization of the cytotoxic cell could contribute to the signal transduction associated with cytotoxic activity (Gray et al., 1988). Hyperpolarization accompanies mitogen (Tsien et al., 1982; Tatham et al., 1986) or antigen (Gray et al., 1987) induced increases in the intracellular Ca^{2+} and it is likely to be due to an activation of the Ca^{2+}-dependent K^+ channels (Tsien et al., 1982; Tatham et al., 1986; Gray et al., 1987). However, we have not observed hyperpolarization of the NK cell possibly because such changes would probably occur very early in the cytotoxic process, during activation of the cytotoxic cell for lytic activity. The activation of voltage-dependent Na^+ channels leading to NK cell depolarization could occur later in the cytotoxic process.

In conclusion, our study demonstrates that it is possible to investigate the membrane potential of interacting cells using fluorescent probes and flow cytometry. We have shown that depolarization of the target cell occurs as a result of the cytotoxic activity of the NK cell and the depolarization occurs before the target cell membrane becomes

permeable to propidium iodide. The results also suggest that cytotoxic activity leads to depolarization of the NK cells and the latter may mark the termination of cytotoxic activity.

Acknowledgements

We thank G.M.J. Segers and Y. Kraan for excellent technical assistance, and Dr. C. Figdor and Dr. G.J. Puppels for critical reading of the manuscript.

This work was supported by the E.C., Project No. SC1 0358-C (EDB).

References

Abrams, S.I. and Brahmi, Z. (1988) Target cell directed NK inactivation. Concomitant loss of NK and antibody-dependent cellular cytotoxicity activities. J. Immunol. 140, 2090.

Bakker Schut, T.C., Florians, A., Radošević, K., De Grooth, B.G. and Greve, J. (1993) Signal processing in slit scan flow cytometry of cell conjugates. Cytometry 14, in press.

Bolhuis, R.L.H., Van Griend, R.J. and Ronteltap, C.P.M. (1984) Clonal expansion of human B73.1-positive natural killer cells or large granular lymphocytes exerting strong antibody-dependent and -independent cytotoxicity and occasionally lectin-dependent cytotoxicity. Nat. Immun. Cell. Growth. Regul. 3, 61.

Bonavida B., Bradley, T.P., Grimm, E.A. (1983) The single cell assay in cell-mediated cytotoxity. Immunol. Today 4, 196.

Brauner, T., Hülser, D.F. and Strasser, R.J. (1984) Comparative measurements of membrane potentials with microelectrodes and voltage-sensitive dyes. Biochim. Biophys. Acta 771, 208.

Civitelli, R., Reid, I.R., Halstead, L.R., Avioli, L.V. and Hruska, K.A. (1987) Membrane potential and cation content of osteoblast-like cells (UMR 106) assessed by fluorescent dyes. J. Cell Physiol. 131, 434.

Edwards, B.S., Nolla, H.A. and Hoffman, R.R. (1989) Relationship between target cell recognition and temporal fluctuations in intracellular Ca^{2+} of human NK cells. J. Immunol. 143, 1058.

Gray, L.S., Gnarra, J.R., Russell, J.H. and Engelhard, V.H. (1987) The role of K^+ in the regulation of the increase in intracellular Ca^{2+} mediated by the T lymphocyte antigen receptor. Cell 50, 119.

Gray, L.S., Gnarra, J.R., Sullivan, J.A., Mandell, G.L. and Engelhard, V.H. (1988) Spatial and temporal characteristics of the increase in intracellular Ca^{2+} induced in cytotoxic T lymphocytes by cellular antigen. J. Immunol. 141, 2424.

Henkart P.A. (1985) Mechanism of lymphocyte-mediated cytotoxity. Annu. Rev. Immunol. 3, 31.

Koolwijk, P., Roozemuller, E., Stad, R.K., De Lau, W.B.M. and Bast, B.J.E.G. (1988) Enrichment and selection of hybrid hybridomas by Percoll density gradient centrifugation and fluorescent-activated cell sorting. Hybridoma 7, 217.

Mandler, R.N., Seamer, L.C., Whitlinger, D., Lennon, M., Rosenberg, E. and Bankhurst, A.D. (1990) Human natural killer cells express Na^+ channels. A pharmacologic flow cytometric study. J. Immunol. 144, 2365.

McConkey, D.J., Chow, S.C., Orrenius, S. and Jondal, M. (1990) NK cell-induced cytotoxicity is dependent on a Ca^{2+} increase in the target. FASEB J. 4, 2661.

Radošević, K., Garritsen, H.S.P., Van Graft, M., De Grooth, B.G. and Greve, J. (1990) A simple and sensitive flow cytometric assay for the determination of the cytotoxic activity of human natural killer cells. J. Immunol. Methods 135, 81.

Rink, T.J., Montecucco, C., Hesketh, T.R. and Tsien, R.Y. Lymphocyte membrane potential assessed with fluorescent probes. Biochim. Biophys. Acta 595, 15.

Roozemond, R.C. and Bonavida, B. (1985) Effect of altered membrane fluidity on NK cell-mediated cytotoxicity. 1. Selective inhibition of the recognition or post recognition events in the cytolytic pathway of NK cells. J. Immunol. 134, 2209.

Tatham, P.E., O'Flynn, K. and Linch, D.C. (1986) The relationship between mitogen-induced membrane potential changes and intracellular free calcium in human T-lymphocytes. Biochim. Biophys. Acta 856, 202.

Terstappen, L.W.M.M., De Grooth, B.G., Nolten, G.M.J., Ten Napel, C.H.H., Van Berkel, W. and Greve, J. (1986) Physical discrimination between human T lymphocyte subpopulations by means of light scattering, revealing two populations of T8 positive cells. Cytometry 7, 178.

Trinchieri, G. (1989) Biology of natural killer cells. Adv. Immunol. 47, 187.

Tsien, R.Y., Pozzan, T. and Rink, T.J. (1982) T-cell mitogens cause early changes in cytoplasmic free Ca^{2+} and membrane potential in lymphocytes. Nature 295, 68.

Van Graft, M., Kraan, Y., Segers, G.M.J., Radošević, K., De Grooth, B.G. and Greve, J. (1993) Flow cytometric measurement of $[Ca^{2+}]_i$ and pH_i in conjugated natural killer cells and K562 target cells during the cytotoxic process. Cytometry 14, in press.

Wilson, H.A. and Chused, T.M. (1985) Lymphocyte membrane potential and Ca^{2+} sensitive potassium channels described by oxonol dye fluorescence measurements. J. Cell. Physiol. 125, 72.

Young, J.D.-E., Podack, E.R. and Cohn, Z.A. (1986) Properties of a purified pore-forming protein (perforin 1) isolated from H2 restricted cytotoxic T cell granules. J. Exp. Med. 164, 144.

Young, J.D.-E. (1989) Killing of target cells by lymphocytes: a mechanistic view. Physiol. Rev. 69, 250.