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# PRELYTIC STIMULATION OF TARGET AND EFFECTOR CELLS FOLLOWING CONJUGATION AS MEASURED BY INTRACELLULAR FLUORESCEIN FLUORESCENCE POLARIZATION

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## ABSTRACT

The aim of the present study was to detect prelytic intracellular changes induced in target and effector cells following their conjugation at room temperature. Changes in the cytoplasmic matrix were measured by means of intracellular fluorescein fluorescence polarization (IFFP) using the Cellscan apparatus. Both natural killer and lymphocyte activated killer cells were used as effector cells, while K562 and Daudi cell lines were used as targets. The results show that following their conjugation, both the effector and the target cells show significant reductions ( $>$ 10%) in IFFP values. Changes in IFFP were induced by specific interaction and only between viable cells. No evidence of fluorescein transfer from a stained cell to its nonstained counterpart was found. To the best of our knowledge, this is the first time that effector-target interaction is monitored on an individual cell basis within a population, by means of IFFP measurements. In addition, in order to explain the physical phenomena, measurements of physical parameters which might affect the IFFP, such as changes in osmolality and *p*H, were performed and discussed.  [S1083-3668(98)00103-8]

**Keywords** fluorescence polarization; stimulation; effector and target cells; Cellscan.

## 1 INTRODUCTION

Specific and nonspecific killing of viral infected and abnormally transformed cells by the immune system is carried out in cell mediated interaction throughout intensive physical contact, referred to as conjugation, between the target cells and effector lymphocytes, such as cytotoxic T-lymphocytes (CTL) or natural killer (NK) cells.[1]

Microscopic observations of such interactions reveal early effects of motile activation in both cells, followed by a final effect of membrane disruption of the target cell. Although maximum conjugation occurs at room temperature, virtually no lysis is detected.[1] Lethal activation of the target is enhanced by increasing the ambient temperature to 37 °C, ending in lysis, namely, membrane rupture and leakage of the cell content. This final lytic effect can be measured by the release of radioactive or fluorescent probes from the target cells.

The general approach to the killing mechanism considers membrane damage at the initial stage of the effector-target interaction.[2] An alternative approach considers lysis as the final effect of transmembrane stimulation which induces exhaustive metabolic activation of the target cell.[3,4] In this regard, prelytic target cell activation is reasonably temperature dependent. Therefore, the physiological route of the mutual effector-target stimulation during conjugation may be better resolved at room temperature, as carried out in the present study.

Transmembrane stimulation of lymphocytes and other cells at the $G_0$-$G_1$ resting phase results in a cascade of early events, well known in the stimulation of nerve and muscle cells. These include membrane potential depolarization associated with $Na^+$ influx and *p*H changes, followed by influx and/or internal release of $Ca^{2+}$ ions. These ionic changes activate various cytosolic ATPases and are regulated by multiple phosphorylation-dephosphorylation pathways. These early meta-

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bolic changes may be followed by later events such as cell differentiation, proliferation, or lysis.[5–7] Processes linking such early and late intracellular events following cell activation involves conformational changes of the cytosolic enzymes and/or their regulatory proteins, and their intracellular matrix reorganization.[1,8] These early structural changes may be monitored by fluorescence polarization (FP) of intracellular fluorescent probes.[9–14]

When fluorescence molecules are excited by polarized light, the emission from those molecules, which do not rotate while in the excited state, is more highly polarized than is the emission from those molecules which are more free to rotate while in the excited state. Measurements of emission polarized in planes parallel and perpendicular to that of the excitation beam can be combined to yield measures of FP. The more restricted the mobility of the fluorophores, the higher the FP.

FP measurements have been used to characterize various physical cell parameters.[15] Indeed, specific fluorescence probes such as diphenyl hexatriene (DPH) and fluorescein diacetate (FDA) have been utilized to measure membrane fluidity,[16,17] and changes in the cytoplasmic microviscosity,[9] respectively.

A relatively high degree of intracellular fluorescein fluorescence polarization (IFFP) is found in various cells in their $G_0$-$G_1$ resting state.[18] Following stimulation, a decrease in this IFFP value was observed.[12,14,19,20] IFFP was also measured during the following physical and physiological alteration of the cells: microviscosity changes induced by varying the osmolality;[15] physiological changes induced by stimulation;[10,12,13] progression through cell cycle phases;[18] and influence of stimulation inhibitors,[21] cytokines,[19] and viral infection.[22]

The biophysical aspects of FP and IFFP can be based on the fact that solvent effects can be dramatic, and a complete change of the spectroscopic nature of a fluorescent solute can occur with a change of a solvent. The cellular matrix is a heterogeneous and polyphasic media solvent, in which the physicochemical properties such as microviscosity, dielectric constants, polarity, and $p$H differ between its microdomains. Thus, the absorption and emission of the fluorescein molecule dissolved in such a media, can be redshifted or blueshifted, can have different decay time, and can be found in its neutral, cationic, and/or anionic molecular structure.[23] The fluorescein molecules in the living cells might therefore represent an ensemble of different fluorophores which probe different cellular regions. The steady-state IFFP is therefore an intensity weighted average parameter[24] which can be expressed as

$$\mathrm{IFFP}=\frac{\Sigma I_i P_j}{\Sigma I_j},$$

where $I_j$ and $P_j$ respectively are the local intensity and FP of specific cellular domain noted by $j$. Thus, IFFP is a submacroscopic measure rather than a microscopic one.

The relation between FP, the actual fluorescence lifetime (FLT-$\tau_F$) and the rotational freedom of globular fluorescent probe suspended in a homogeneous solution is given by the Perrin equation:[25]

$$\frac{1}{P}-\frac{1}{3}=\left(\frac{1}{P_0}-\frac{1}{3}\right)\left(1+\frac{RT}{\eta V}\cdot\tau_F\right),$$

where $V$ is the molar volume of the spherical assumed probe, $R$ is the gas constant, $T$ the absolute temperature, and $\eta$ the viscosity of the solvent. $(RT/\eta V)^{-1}$ is defined as $\tau_R$, the rotational correlation time of the probe. $P_0$ is the intrinsic polarization as measured in cases where $T/\eta \rightarrow 0$. Thus, as $\tau_R$ increases, FP decreases and vice versa. $\tau_R$ is the most dominant and common variable which reflects environmental changes such as temperature, viscosity, mobility, probe binding, etc., as monitored by FP.

The common way in which $\tau_F$ changes is due to nonradiative transitions, where the probe interacts with the phonon field and not with the photon field of the solvent (internal conversion, intersystem crossing, collisions, quenching, etc.) competing with fluorescence to depopulate the excited level. The actual excited state lifetime $\tau_F$ will therefore be shorter, yielding higher FP.

Phosphorescence is not relevant to this discussion, since the long lifetime of the triplet state (which decreases FP) makes it highly vulnerable to any quenching process which will remove energy. For this reason, phosphorescence is rarely observed in solutions.[26]

Most of the above mentioned IFFP measurements were performed by the Cellscan apparatus. The Cellscan is a static cytometer, which permits the repetitive spectroscopic measurement of individual cells within a population of many cells, while the location of each cell is preserved during various manipulations of the cells and/or their surrounding medium.[15] The present model of the Cellscan is uniquely designed to capture individual stained cells of one group (effector or target), and to carry out IFFP measurements upon topical application of a second, unstained cell population.

## 2 MATERIALS AND METHODS

### 2.1 ISOLATION OF ENRICHED NK CELLS FROM TOTAL POPULATION OF PERIPERAL BLOOD MONONUCLEAR CELLS (PBMC)

NK enrichment was performed as previously described.[27] Briefly, PBMC, obtained from healthy controls at the Department of Surgery B at the Beilinson Medical Center in Petach Tikva, were first isolated by layering 5 ml of heparinized whole

blood (30 units/ml) on top of 5 ml Histopaque in a 12 ml tube (16 mm OD) and centrifuged at 550 g for 30 min at room temperature. The PBMC layer between the density solution and plasma was collected and washed three times with 0.9% saline. These procedures were carried out for the separation of 60 ml whole blood.

For NK enrichment, all PBMC obtained were resuspended in 4 ml of 0.9% saline, loaded in a 12 ml tube (16 mm OD), on top of density solution of 0.320 osm/kg encompassing upper (1.0590 g/cc at 20°C) and lower (1.0670 g/cc at 30°C) density solutions, 4 ml each, centrifuged at 1100 g for 20 min at 20±1°C. The enriched NK population was collected from the interface between the two density solutions, washed twice with preservative 0.9% saline for injection, followed by a third wash in Dulbecco's complete phosphate buffered saline (PBS) and finally resuspended in 1 ml of PBS.

Under this separation procedure, approximately 70% of cells were found to be CD56 and CD16 positive by flow cytometer analysis, and showed substantial activity against NK sensitive K562 tumor cells in a 4 h $^{51}$Cr-release assay.[28,29]

## 2.2 LYMPHOCYTE ACTIVATED KILLER (LAK) CELL PREPARATION

PBMC at $2\times10^6$ cells/well were incubated in complete medium (CM) together with 1000 units/ml of Interleukin (IL)-2 (Cetus Incorporation, USA) in a 24 well tissue culture plate. After 72 h in culture, cells were washed twice in CM and brought to the desired cell concentration. CM consisted of RPMI 1640 and 10% heat-inactivated fetal calf serum (FCS) and antibiotic, as previously described.[30] For IFFP measurements, cells were harvested and washed three times with PBS and resuspended in PBS at a concentration of $4\times10^6$ cells/ml.

## 2.3 TARGET CELLS FOR NK AND LAK CELLS

The cell lines K562 (Sheba Medical Center, the Hematology Institute of Ramat-Gan) and K562R (Ariel Research Laboratories, R—for rapid proliferation)[28,29] were used as specific targets for NK and LAK cells. Daudi cell line (Sheba Medical Center, the Hematology Institute of Ramat-Gan) was used as a specific target for LAK cells.[31,32] For IFFP measurements, cells were harvested and washed three times with PBS and resuspended in PBS at a concentration of $1\times10^6$ cells/ml.

## 2.4 FLUORESCEIN SOLUTIONS

Fluorescein solutions (2 $\mu$M) were prepared by dissolving fluorescein (Sigma, St. Louis, MO) in PBS solutions having different *p*H and viscosities.

## 2.5 PHYTOHAEMAGGLUTININ (PHA)

Bottles containing PHA (HA15, Wellcome, England) were reconstituted in 15 ml distilled water and stored at −20 °C as stock in aliquots of 100 $\mu$l until use.

For stimulation, stock PHA and cell suspension was mixed at ratios of 1:10, respectively.

## 2.6 IFFP MEASUREMENT PROCEDURE WITH THE CELLSCAN

### 2.6.1 First and Second Group of Cells

In order to monitor stimulation of an effector cell by a target cell and vice versa, the two types of cells were brought into physical proximity on the cell carrier. This procedure was an integrated stage of the measurement procedure. The stained cells occupying the cell carrier are defined as the ''first group.'' The unstained ''second group'' of cells, which were loaded on top of the first group, were either another (control) or the same type of cells (used to detect possible auto-stimulation).

### 2.6.2 Cell Loading

50 $\mu$l of $10^6$ cells/ml, defined as the ''first group,'' were loaded on the cell carrier by means of a gentle subpressure maintained between the upper and lower surface of the carrier, causing rapid settling of the cells in their traps. The remaining uncaptured (free) cells were then washed away from the upper surface of the cell carrier by tangential rinsing with PBS.

### 2.6.3 Cell Staining

50 $\mu$l of 2 $\mu$M FDA (Sigma, St. Louis, MO, F7378) PBS+$Ca^{2+}$ were introduced onto the trapped cells. FDA is a nonfluorescent derivative of fluorescein, which upon penetration into cells, is hydrolyzed by internal esterases and converted into fluorescent fluorescein. Fluorescent fluorescein is then accumulated in the cell due to its extra negative charge. After 5 min of staining at room temperature, the cell carrier was extensively rinsed three times with PBS in order to remove extracellular FDA and fluorescein.

### 2.6.4 IFFP Control and Post Conjugation Measurement Procedure

Immediately after the initial measurement of $IFFP_0$ (control) of the first group of cells, 50 $\mu$l of cell suspension, at a concentration of $4\times10^6$ cells/ml (defined as the ''second group''), was loaded upon the first group for post conjugation(s) $IFFP_s$ measurements. The second group cell concentrations was $4\times10^6$ or $1\times10^6$ cells/ml of effector or target cells, respectively. All experiments were carried out at room temperature.

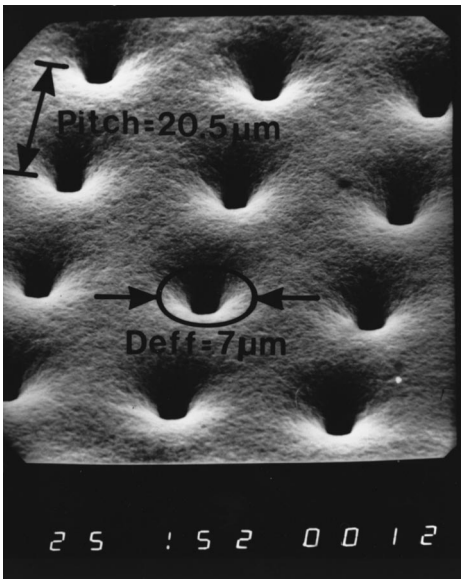


**Fig. 1** A section of the cell carrier.

### 2.6.5 Apparatus

The central feature of the Cellscan system[13] is the cell carrier (Figure 1), incorporating a 100 by 100 two-dimensional array of holes, each approximately 7 $\mu$m in diameter and approximately 20 $\mu$m apart, in which cells are trapped (Figure 2). The carrier is fixed to a holder which allows the introduction and extraction of liquids while cells are maintained in their individual traps, permitting observation of the cells while changes occur in the cellular microenvironment.

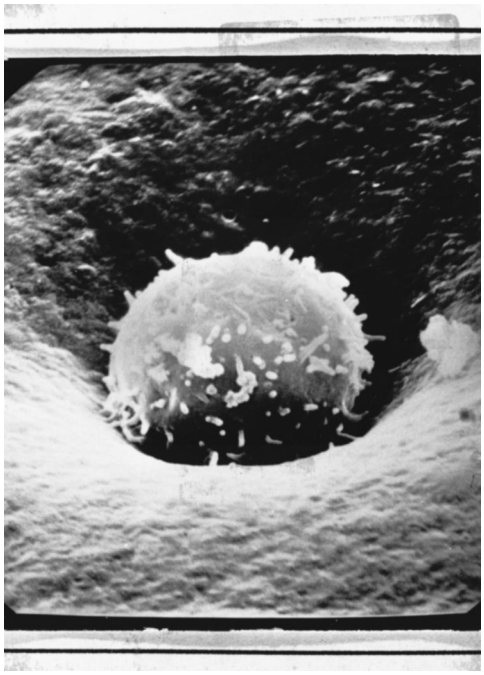

**Fig. 2** A lymphocyte after settling down in a trap.

A 50 $\mu$l aliquot of cell suspension is applied to a carrier; a negative pressure of approximately 1–5 mm $H_2O$ maintained between the upper and lower surfaces of the carrier causes rapid settling of the cells into traps as described elsewhere.[13]

Fluorescence measurements with the Cellscan employ a preset-count technique in which the same number of photons is counted for each cell, regardless of their fluorescence intensity (which is inversely proportional to the counting time), so that strongly emitting and weakly emitting cells are measured with the same precision. Measurement of background signals due to dark current, stray light, and/or autofluorescence can be made for each cell prior to its fluorescence measurement.

The cell carrier holder is fastened to a scanning table driven by three stepping motors; a motion in the $x$-$y$ plane will position any trap in the cell carrier in the center of the epi-illuminating laser beam and the microscope observation field, while focus is changed by the $z$-axis motor. Under the prescribed staining conditions and with illumination intensities of 1–5 $\mu$W at 441 nm, the sampling time for obtaining counts of 10,000 photons from a single fluorescein-loaded cell varies from 0.05 to 1 s. The coordinates of each cell, its fluorescent intensity (expressed in number of counts per unit time) at 527 $\pm$4 nm, the counting time, and the computed FP are recorded, as are the mean, standard deviation, coefficient of variation (CV) and number of good and bad (off limit) values for each parameter measured during the run.

### 2.6.6 Calibration of Polarization Measurements and Measurement Precision

Calibration was carried out as follows:[13] First, polarization measured by the Cellscan was compared with calculated polarization values using a white light source. Second, polarization values of various viscosity solutions of fluorescein in glycerin water mixtures obtained by the Cellscan were compared with those measured by a Perkin–Elmer MPF-44 and SLM-4800 polarization macro-spectrofluorometers. Third, polarization of fluorescent beads as measured by a fluorometer was compared with that measured by the Cellscan. Fourth, the previous procedure was repeated with living cells. All polarization measurements were carried out at a controlled room temperature of 22$\pm$1°C. Fading of the fluorescence is insignificant under the measurement conditions and, in any event, does not influence polarization.[33]

### 2.6.7 Calculation of Mean IFFP and Depolarization of FP (DFP)

Mean IFFP is determined after scanning 400 (20 $\times$20) trapped cells which are simultaneously measured at 527$\pm$4 nm.

DFP is expressed as the percentage of decrease in mean IFFP, relative to that of the control sample, and is defined as

$$\%\mathrm{DFP}=\left(\frac{\mathrm{IFFP}_0}{\mathrm{IFFP}_s}-1\right)\times 100,$$

where $\mathrm{IFFP}_0$ and $\mathrm{IFFP}_s$ are the mean FP of the first group, before and after adding the unstained second group of cells, respectively.

### 2.7 CHROMIUM RELEASE ASSAY

For radioactive labeling, K562 cell suspensions were incubated for 60 min in 1 ml of CM with 100 $\mu$Ci/100 $\mu$l of sodium-$^{51}$chromate (Rotem Industries, Israel), washed three times in PBS, resuspended in CM, counted and brought to $5\times10^4$ cells/ml. Aliquots of 100 $\mu$l of sodium-$^{51}$chromate-labeled K562 cells were transferred to individual wells of a 96-well microplate together with different numbers of effector cells (NK, LAK) in order to establish 40:1, 8:1, and 2:1 effector to K562 target cell ratios. After 4 h of incubation at 37 °C in a 5% $CO_2$ humidified incubator, 150 $\mu$l of cell culture supernatant was harvested from each well, and the amount of radioactivity in each sample was determined on a gamma-radioactive counter (Packard, USA). Values are presented as percent of lysis calculated as

$$\%\mathrm{Lysis}=\left(\frac{\mathrm{CPM}_s-\mathrm{CPM}_0}{\mathrm{CPM}_{\mathrm{HCl}}-\mathrm{CPM}_0}\right)\times 100,$$

where $\mathrm{CPM}_0$, $\mathrm{CPM}_s$, and $\mathrm{CPM}_{\mathrm{HCl}}$ are the radioactive counts per minute of K562 cells incubated at CM with no stimulant (intact cell control), with killer cells (stimulation), and with HCl (total lysis control), respectively.

### 2.8 ABSORPTION, DECAY TIME AND POLARIZATION MEASUREMENTS

A Perkin–Elmer Lambda 19 spectrofluorometer (Norwalk, CT) was used for the absorption measurements.

Fluorescence decay time and polarization measurements of fluorescein homogeneous solutions were carried out using the multifrequency cross-correlation phase and modulation fluorometer (ISS-K2, North Mattis Ave., Champaign, IL) at stabilized room temperature. For excitation, a 488 nm argon ion laser 85 mW (Omnichrome model No. 543-AP) was used. Emission was measured at $535\pm30$ nm. Decay time measurements were carried out using the demodulation ratio and the frequency synthesizers in the 5–200 MHz range.

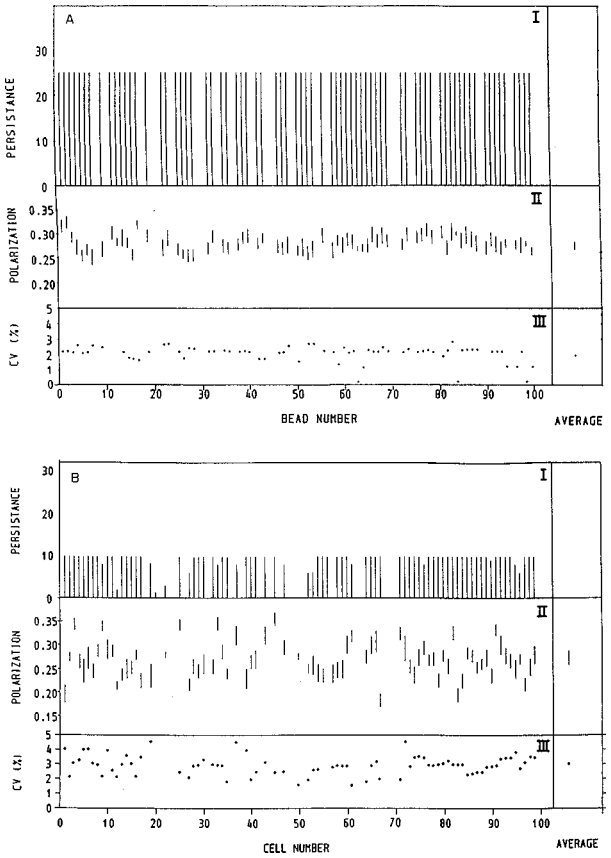


**Fig. 3** IFFP distribution and persistence. (I) Persistence of particles; (II) spread of polarization values; (III) spread of polarization on CV. (A) For 25 consecutive scans of fluorescent beads, (B) for 10 consecutive scans of fluorescent cells. Larger spaces between the lines indicate an unoccupied trap.

## 3 RESULTS

### 3.1 PERSISTENCE AND REPRODUCIBILITY MEASUREMENTS

Persistence and reproducibility were tested by 25 routine cell scans of a field of $10\times10$ traps for beads and by 10 scans for cells. For each bead or cell, the persistence on the carrier and spread of polarization, as well as the CV, were determined. The results are presented in Figure 3, parts I, II, and III, respectively. Although the particles were rinsed throughout the scans, no bead was lost (the lengths of all the persistence lines are equal and indicate 25 scans) and the majority of the cells also survived the ten measurements. The spread of polarization values is shown by the length of the line. The CVs for individual beads never exceeded 3% and some were much lower. The distribution of polarization values for cells was appreciably wider due to the fact that they are living cells, as well as to the fact that these cells were separated from PBMC and must therefore contain various subsets of cells. The differences between cells were also considerably greater than those between beads. Nevertheless, the

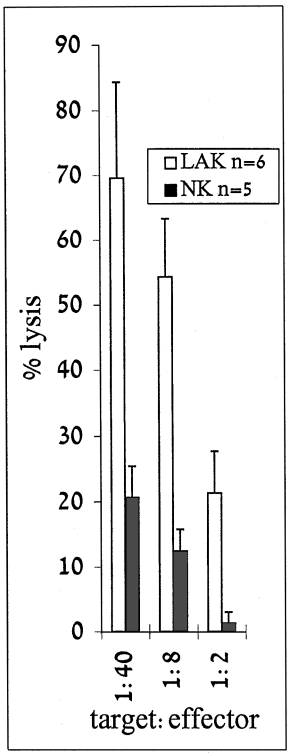


**Fig. 4** Lysis of K562 cells by LAK and NK cells as measured by $^{51}$Chromium release assay in different target:effector ratios. *n*—number of repeated measurements.

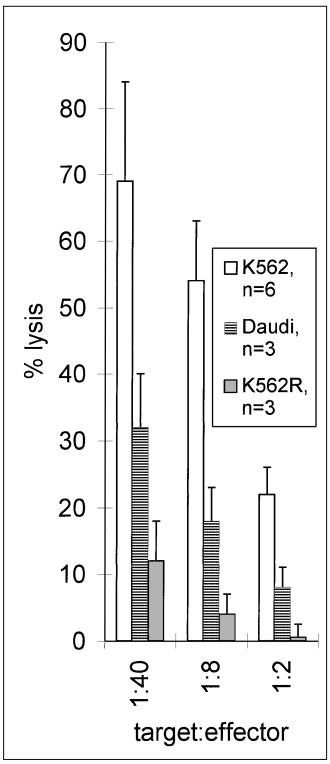


**Fig. 5** The effect of LAK cells on the sensitivity lysis of K562, Daudi, and K562R cells as measured by $^{51}$Chromium release assay in different target: effector ratios. *n*—number of repeated measurements.

average CV for the individual cells did not exceed 2.75%.

### 3.2 CONTROL CHROMIUM RELEASE MEASUREMENTS OF KILLING

The killing of K562 by NK and LAK cells was tested both by chromium release assay (Figure 4) and by IFFP measurements. The first monitors late effects ($>4$ h) and the latter monitors early stages of the cytolytic interaction between the cells. Peripheral blood lymphocytes (PBL) were used as a negative control since they are not sensitive to K562. Moreover, K562R cells were used as negative control against both NK and LAK cells, while Daudi cells were used as a positive control for LAK cells. The results obtained for all groups of cells involved in this study are presented in Figures 4 and 5 and summarized in Table 1. The results demonstrate that both NK (sensitive to K562) and LAK (sensitive to K562 and Daudi) cells were effective in lysing their tumor target cells. It was also found that such an effect depends on effector to target ratio.

### 3.3 IFFP RESULTS

#### 3.3.1 K562 IFFP Measurements

Fresh K562 cells from culture medium, serving as the ''first group'' on the cell carrier, were washed three times in PBS and kept at room temperature for 2 h prior to IFFP measurements. After loading, staining, and $IFFP_0$ measurement of K562, NK/LAK serving as the ''second group,'' were loaded on top of the ''first group.'' The control groups consisted of either PBL or K562 as the ''second group.''

The results shown in Table 2 indicate that conjugation of K562 with NK/LAK cells yielded an average decrease in IFFP of 14% and 22%, respectively. No change was observed in control groups with either PBL of K562 cells as the ''second group.''

A similar experiment was carried out using two types of K562 cells, K562, and K562R, yielding 22%, and 0% DFP, correspondingly, as presented in Table 3. This finding is in agreement with the results obtained using $^{51}$Chromium release assay, as shown in Figure 5. It is also consistent with phenotypic analysis of both K562 populations, indicating that 94% of K562 cells were ICAM-1 positive, while only 27% of K562R expressed ICAM-1 (unpublished results). Since ICAM was shown to serve as an adhesion molecule regulating the binding of NK to their target, our results further strengthen the role of ICAM in the IFFP changes induced during NK and target interaction.

#### 3.3.2 Killer Cells (NK/LAK) IFFP Measurements

Possible mutual stimulations (effector stimulation of target cells and vice versa), between effector (NK/LAK) and their K562 target cells were also

**Table 1** Assessment of target cell killing by effector cells as demonstrated by $^{51}Cr$ release assay. Effector cells were incubated with sodium $^{51}Chromate$ labeled effector cells in different effector to target (E/T) ratios, for 4 h at 37 °C in 5% $CO_2$ humidified incubator. In order to assess killing efficacy, each cell mixture supernatant was collected, and its radioactivity was measured by gamma counter, indicating % of lysis. The first row of the table presents the relevant effector cells, and the first left column presents the relevant target cells. *n*—number of repeated experiments.

| % of lysis | E/T | NK | LAK | PBL |
|---|---|---|---|---|
| K562 cells | 40:1 | 20.67±4.9 | 69.58±14.7 | 4.15±1.2 |
| | 8:1 | 12.48±3.3 | 54.28±9 | 3.5±2.1 |
| | 2:1 | 1.3±1.7 | 21.36±6.4 | 0 |
| | 1:2 | - | 7.31±1.63 | 0 |
| | *n* | 5 | 6 | 2 |
| K562R cells | 40:1 | 0 | 12.65±8.3 | |
| | 8:1 | 0 | 3.1±1.41 | |
| | 2:1 | 0 | 0.8±0.6 | |
| | 1:2 | - | - | |
| | *n* | 1 | 3 | 0 |
| Daudi cells | 40:1 | | 31.25±8.1 | |
| | 8:1 | | 18.55±4.2 | |
| | 2:1 | | 8.5±1.7 | |
| | 1:2 | | 1.5±0.7 | |
| | *n* | 0 | 3 | 0 |

tested. In this set of experiments, NK/LAK cells were used as the first group, while K562 were used as the second group. The results presented in Table 4 show 14% and 15% DFP in NK and LAK killer cells, respectively, indicating that both effectors (NK and LAK cells) are activated by their K562 target cells. These results suggest that under the described conditions, a mutual activation, associated with structural changes of the intracellular matrix, occurs between effector and target cells during their interaction.

**Table 2** Induction of fluorescence depolarization (% DFP) by cell conjugation in stained K562 cells. For cell conjugation, different unstained cells (left column) were loaded on top of the ''first'' group and IFFP of K562 cells was measured. The results indicate that DFP occurs only when conjugation takes place between K562 and either NK or LAK cells. *n*—number of repeated experiments.

| | *n* | % DFP of K562 cells |
|---|---|---|
| NK | 15 | 13.95±0.92 |
| LAK | 18 | 22.48±1.1 |
| K562 | 4 | 0 |
| PBL | 3 | 0 |

**Table 3** Sensitivity of fluorescence depolarization (% DFP) to target cell phenotype. Two types of K562 target cells (K562 and K562R) were brought to proximity on the cell carrier, with NK and LAK cells. The ''first'' stained group consists of K562 and K562R listed in the upper row of the table. The ''second'' unstained group is listed in the left column and consists of NK and LAK cells. The results indicate that DFP occurs only when conjugation takes place between K562 and either NK or LAK cells, in agreement with results obtained using $^{51}Cr$ release assay and phenotype analysis. *n*-number of repeated experiments.

| | | K562 | K562R |
|---|---|---|---|
| NK | DFP | 13.95±0.92 | 0 |
| | *n* | 15 | 10 |
| LAK | DFP | 22.48±1.1 | 0 |
| | *n* | 18 | 12 |

As in the previous section, control measurements were carried out using NK, LAK, and PBL cells as a second group. Using these cells no DFP was induced (Table 4).

### 3.3.3 Specificity of Effector-target Conjugation Assessed by IFFP Measurement

In order to test the specificity of DFP due to effector-target conjugation, NK and LAK were used as effector cells against LAK sensitive Daudi cells, as well as NK and LAK resistant PBL.

**Table 4** Stimulation of effector cells by conjugation with target cells. Possible mutual stimulation between effector (NK, LAK) and target (K562) cells was investigated. This time, the ''first'' stained group consists of NK and LAK cells which are listed in the upper row of the table. The ''second'' unstained group is listed in the left column and consist of K562 as a target and NK, LAK, and PBL for control measurements. The results indicate that DFP occurs only when conjugation takes place between NK or LAK cells with K562 cells and that following conjugation with target cells, effector cells are mutually stimulated. *n*—number of experiments.

| | | NK | LAK |
|---|---|---|---|
| K562 | DFP | 14.3±1.9 | 15.3±1.8 |
| | *n* | 8 | 4 |
| NK | DFP | 0 | |
| | *n* | 5 | 0 |
| LAK | DFP | | 0 |
| | *n* | 0 | 1 |
| PBL | DFP | 0 | 0 |
| | *n* | 2 | 2 |

**Table 5** Specificity of effector-target conjugation assessed by IFFP measurement. Specificity of DFP due to effector-target conjugation was tested by employing NK/LAK cells against Daudi cells. The ''first'' stained group is listed in the upper row and the ''second'' unstained group is listed in the left column of the table. The results indicate that DFP occurs only when conjugation takes place between relevant cells (Daudi with LAK cells). *n*—number of experiments.

| | | Daudi | PBL | LAK | NK |
|---|---|---|---|---|---|
| LAK | %DFP | 12.01±0.87 | 0 | 0 | |
| | *n* | 5 | 3 | 1 | 0 |
| NK | %DFP | 0 | 0 | | 0 |
| | *n* | 2 | 3 | 0 | 5 |
| PBL | %DFP | | 0 | 0 | 0 |
| | *n* | 0 | 4 | 2 | 2 |
| Daudi | %DFP | 0 | | 11.3±2.2 | 0 |
| | *n* | 1 | 0 | 3 | 1 |

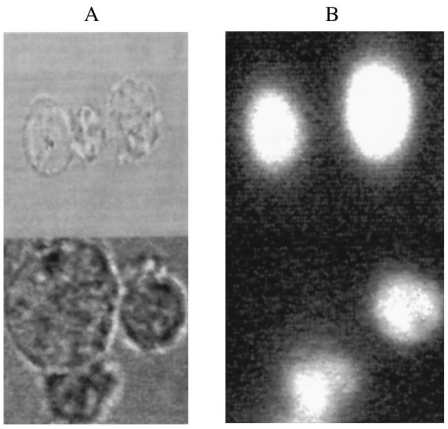


**Fig. 6** Computerized photographs indicating the persistence of differential staining with fluorescein of either K562 (upper panel) or NK (lower panel) cells during their conjugation, as observed using transparent light microscope (A), and epi fluorescence microscopy (B). The same field was periodically observed along 45 min. K562 are the bigger cells and NK are the smaller cells.

IFFP experiments were carried out as described in previous experiments (Sec. 3.3, paragraphs 1 and 2). Both Daudi and PBL were used as a first and second group. The results shown in Table 5 indicate a 12% DFP when Daudi and LAK cells were employed as either the first or second group, whereas 0% DFP was noted with NK being used as the second group. When Daudi cells was used as the second group, 11% and 0% DFP was observed with LAK and NK cells as the first group, respectively.

These results are in correlation with the cytotoxic activity observed in combinations of Daudi/NK and Daudi/LAK cells (Figure 5).

As shown in Table 5, the use of PBL as nontarget cells for both NK and LAK cells yielded 0% DFP in both, whether they were employed as the first or second group.

### 3.4 POSSIBILITY OF FLUORESCEIN TRANSFER FROM ''FIRST GROUP'' TO ''SECOND GROUP'' CELLS

Possible transition of fluorescein from the first stained group to the second nonstained group, would ruin $IFFP_s$ measurements due to the fact that a fluorescence signal would be obtained from both groups.[16] In order to test this possibility, 100 $\mu$l of $10^6$ cell/ml were mixed with an 0.5 ml FDA+PBS staining solution for 5 min at room temperature. To this suspension 7 ml of PBS were added in order to terminate the staining process by diluting FDA concentration. To that suspension $8\times10^5$ cells/100 $\mu$l of nonstained cells were added, gently mixed using a Pasteur pipette and recentrifuged in order to induce conjugation in the pellet. After 10 min, a sample of the pellet was examined under an epifluorescent microscope. The computerized photographs of the samples shown in Figure 6 indicate no evidence of detectable level of transferred fluorescein, whether employing K562 or NK as stained cells. This might suggest that during the formation of effector and target conjugates, fluorescent signals were measured only from the first stained group.

Further information overrules possible transport of fluorescein between the first and second groups was obtained from polarization measurements. The IFFP of K562R was found to be 0.33±0.03, distinguishing it from NK and LAK cells which exhibited IFFP of 0.265±0.02. When K562R was used as that first group and NK/LAK cells as the second group, IFFP remained 0.33. When LAK/NK and K562R were used as the first and second group, respectively, IFFP remained 0.265.

### 3.5 DEPENDENCE OF CONJUGATION INDUCED DFP UPON CELL VIABILITY

The process of conjugation involves the interaction between specific sites in the membranes of the conjugated cells. Such an interaction is not dependent on cell viability.[34] Therefore, the question arises whether DFP measurements require the viability of both cells involved as first and second group.

For this purpose, $IFFP_s$ of NK serving as the first group, and dead K562 cells, serving as the second group, and vice versa, were measured. Cell mortality was confirmed by Trypan blue staining and by the inability to hydrolize FDA. The results shown in Table 6 indicate that viability of both target and effector cells is required for the reciprocal stimulation upon target-effector interaction, as monitored by IFFP measurements.

**Table 6** Influence of conjugation between viable cells and nonviable cells on DFP. (a) K562 serve as the "first" living stained group, (b) NK serve as the "first" living stained group. *V*-viable cells, *D*-nonviable cells. The results indicate that DFP measurements require the cell viability of both cells involved in conjugation.

| (a) | | | K562 |
|---|---|---|---|
| NK | *V* | DFP | 13.95±0.92 |
| | | *n* | 15 |
| | *D* | DFP | 0 |
| | | *n* | 4 |
| (b) | | | NK |
| K562 | *V* | DFP | 14.3±1.9 |
| | | *n* | 8 |
| | *D* | DFP | 0 |
| | | *n* | 2 |

## 3.6 POSSIBLE CANDIDATES FOR THE INDUCTION OF DFP

### 3.6.1 DFP Dependence on Microviscosity Induced by Varying Osmolarity

IFFP may be related to the microviscosity of the cells. This is demonstrated by a change in osmolarity causing a change in the water content of the cells. Changing the osmolarity from 0.344 to 0.230 os/kg by periodic rinsing of the cells on the carrier, induced an immediate (and reversible) decrease in IFFP from 0.2 to 0.168. The overall duration of the experiment was 100 min. An example of individual cell behavior is illustrated in Figure 7.

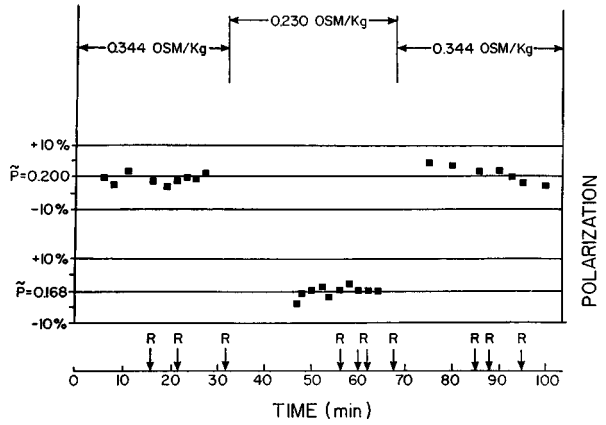


**Fig. 7** Modulating of intracellular microviscosity by various osmolarities as monitored by IFFP (*R*=rinsing).

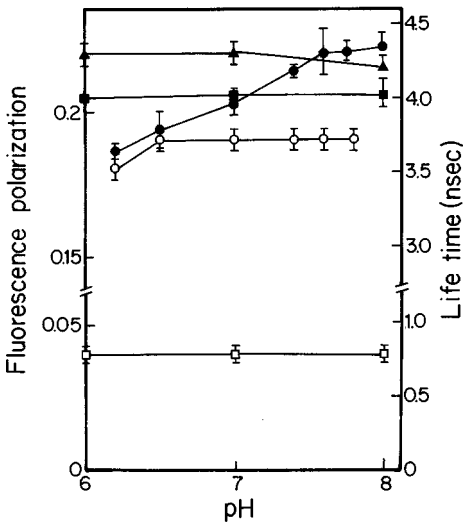


**Fig. 8** Fluorescein fluorescence lifetime and polarization of 2 $\mu$m fluorescein solutions, as a function of *p*H. FFLT (■) and FFP (□) of 2$\mu$M fluorescein in PBS solutions as well as FFP of 2 $\mu$M fluorescein 60% (v/v) glycerol in PBS solution (▲) and IFFP of PHA stimulated cells (○) show no *p*H dependence at the 6–8 range at room temperature, while IFFP of control cells (●) show moderate *p*H dependence, gradually reaching a plateau, increasing from 0.182 to 0.220 when *p*H is elevated from 6.2 to 7.6. Further elevation results in IFFP approaching a limit of 0.220. Each point and its corresponding bar represents the average and standard deviation obtained from at least six measurements.

### 3.6.2 Fluorescein Fluorescence Lifetime (FFLT) Dependence on *p*H

One might suspect that the variation of IFFP due to stimulation, reflects changes of intracellular *p*H ($pH_i$), resulting in changes in the intrinsic FFLT of the excited energy level ($\tau_0$), as well as changes in the actual decay time ($\tau_F$), due to solute/solvent interaction, both influencing FP.

In order to investigate the $\tau_F$ and FP dependence on *p*H, free of perturbations arising in heterogeneous medium such as living cells, three fluorescein (2 $\mu$M) aqueous solutions in PBS with *p*H values of 6, 7, and 8 were prepared. Lifetime was measured for each solution at least in triplicate. Each experiment lasted 5 min, using 5–200 MHz modulation frequencies. For all solutions, 1 component of FLT was found, and equaled 4.01±0.01 ns. The results indicate that FFLT is not *p*H dependent in the range of 6–8 *p*H units in a nonviscous aqueous solution at room temperature (Figure 8, full squares). Moreover, FP (open squares, lower line in Figure 8) was found to be 0.035±0.007 and constant.

### 3.6.3 Fluorescence Polarization of Viscous Solution of Various *p*H

In order to directly measure the influence of *p*H upon FP, three solutions having *p*H units of 6, 7, and 8, and the same glycerol concentration of 60% (V/V) in PBS, were prepared. The results are shown in Figure 8 (full triangles, upper line).

The FP of all three solutions was found to be the same, and equaled $0.218 \pm 0.002$. These results are in agreement with those of the previous section, and point toward the conclusion that in homogeneous solutions at room temperature, there is no indication of FFLT nor FP dependence on $p$H in the relevant range.

### 3.6.4 IFFP Dependence on $p$H

IFFP of HPBL suspensions incubated with and without PHA, was measured at different $p\mathrm{H}_i$ units. $p\mathrm{H}_i$ was changed by controlling extracellular $p$H ($p\mathrm{H}_{ex}$), namely, the FDA-PBS suspending staining solutions.

PHA, a mitogen which induces cell proliferation, was used in this experiment in order to achieve stimulated cell populations. The cells were exposed to various $p$H levels. The IFFP of the control group, those incubated without PHA, increased from 0.182 to 0.220 when $p$H was elevated from 6.2 to 7.6. Further elevation of $p\mathrm{H}_{ex}$ resulted in IFFP approaching a limit value of 0.220.

With PHA stimulated cells, $\mathrm{IFFP}_{PHA}$ was 0.176, yielding DFP of ~20%. Nevertheless, $\mathrm{IFFP}_{PHA}$ was found to be non-dependent on $p$H in the range 6.5–7.8, despite the fact that $p\mathrm{H}_i$ changes.[35] IFFP results of control and stimulated cells are presented in Figure 8 by full and open circles correspondingly.

### 3.6.5 Dependence of Fluorescein Absorption Spectra on $p$H

IFFP measurements were carried out at two main excitation wavelengths, 470 and 442 nm. In both cases fluorescence depolarization was observed following stimulation. Since $p\mathrm{H}_i$ changes due to stimulation, the absorption spectra of fluorescein (2 $\mu$M) in PBS solutions having $p$H units of 6, 7, and 8 were measured (Figure 9) in order to examine possible correlation between $p\mathrm{H}_i$ and IFFP as a function of excitation wavelength. The results indicate that above 465 nm the absorption power increases with $p$H, while below 465 nm, the absorption power decreases with $p$H. At 465 nm, absorption power is independent of $p$H.

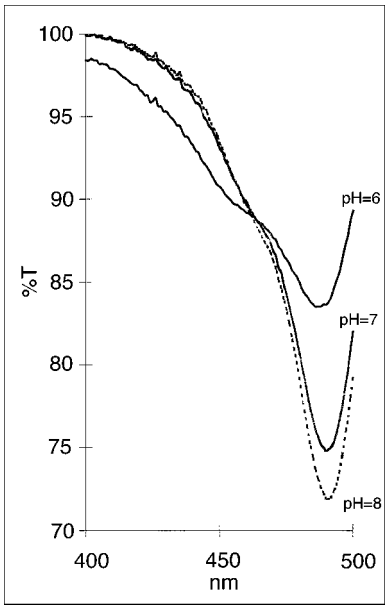


**Fig. 9** Absorbtion spectra of fluorescein (2 $\mu$M) in PBS having $p$H levels of 6, 7, and 8. %*T* means the percentage of the excitation intensity which passed the sample.

## 4 DISCUSSION

### 4.1 PRELYTIC MUTUAL STIMULATION OF E/T AS MONITORED BY IFFP

The present IFFP results indicate early and specific stimulation responses of K562 cells in conjugation with NK/LAK cells, but not with K562R cells (Table 3). This is in agreement with the results obtained using $^{51}$Chromium release assay, indicating an enhanced killing activity of NK and LAK cells against K562, compared with a modest activity against K562R cells (Table 1). Furthermore, DFP of K562 was found to be greater when conjugated with LAK than when conjugated with NK cells (Panels a and b of Table 2) in correlation with $^{51}$Chromium release results (Table 1). These two findings might be due to the fact that the cytolytic efficiency of LAK cells is greater than that of NK cells.[36] Moreover, these findings are in agreement with phenotypic analysis of both K562 and K562R populations indicating that 94% of K562 cells were ICAM-1 positive, while only 27% of K562R expressed ICAM-1 (unpublished results). Since ICAM-1 was shown to serve as an adhesion molecule regulating the binding of NK to their target, our results further assess the role of ICAM in the IFFP changes induced during NK and target interaction.

The results of the present study, showing a decrease of IFFP in both E/T cells due to conjugation, are consistent with the reciprocal mode of effector-target stimulation in the early stages of interaction between NK and K562 cells.

In this work, the IFFP measurements following killer-target interaction at room temperature revealed, within minutes, a decrease in both cells. These changes in IFFP were observed only in conjugation between the relevant viable cells, and in time scales consistent with early increase in cytosolic $Ca^{2+}$ and changes in $p\mathrm{H}_i$.

### 4.2 CELLSCAN IFFP MEASUREMENTS REPRESENT STIMULATION STATUS

Our previous findings indicate clear correlation between PBL cellular response to mitogens,[14,21] as well as to antigens[37] or phorbol esters,[20] and a decrease in IFFP occurring minutes after exposure to the stimulant. In addition, modulating the cytoskeleton activity by cytochalasin B (which regulates the

microfilament structure), and vinblastine and colchicine (which affect the microtubules), completely abolishes the changes induced in PBL IFFP by both PHA and anti-CD3. This indicates the involvement of the cytoskeleton polymerization processes in cellular activation which can be monitored by IFFP changes. Using the Cellscan-IFFP technique, it was also shown that: (i) ethyl-$\alpha$-D-mannopyranoside ($\alpha$MM) is able to reverse the depolarizing effect induced by ConA as long as the cells are not committed to proliferation; (ii) H7 and staurosporin, both inhibitors of protein kinase C (PKC), inhibit the PHA induced depolarization; and (iii) the mitogen-induced depolarization is dependent on metabolic energy.[21]

We also reported that IFFP levels decrease monotonically in cells undergoing transition from the $G_0$ phase to the $S$ phase.[18]

All these previous results strongly suggest that changes in IFFP values following cellular stimulation, as monitored by the Cellscan, reflect an early effect associated with lymphocyte activation. Thus, the Cellscan-IFFP measurements provide a sensitive means for the rapid detection of biological response to stimulation, and might be adapted to trace its time dependent nature.

## 4.3 BIOPHYSICAL ASPECTS

### 4.3.1 Independence of IFFP on $p\mathrm{H}_i$

$p\mathrm{H}_i$ is one of the prominent factors which has been observed to change during early stages of the surface receptor-mediated activation processes following cell stimulation. $p$H influences the spectroscopic features of fluorescent probes, a fact which is very well established and used for $p\mathrm{H}_i$ determination.[38–43] Regarding fluorescein, variations of fluorescence intensity (FI) and color as a function of $p$H are commonly related to the extent of fluorescein dissociation and ionization. Nevertheless, one might suspect that changes of $p\mathrm{H}_i$ following stimulation,[35] as well as in cells equilibrated with buffers of increasing pH,[38] will be manifested by changes of FI absorbing power and of FI. These two parameters are related to the fluorescence quantum yield $\phi_F$ which is proportional to the lifetime ratio $\tau_F / \tau_0$ and where $\tau_0$ is the intrinsic radiative lifetime. Thus, changes in FP and IFFP could be physically induced by $p$H changes through changes in $\tau_F = \tau_0 \cdot \phi_F$. However, the fluorescein fluorescence polarization (FFP) measured in glycerine-PBS solutions was found to be $p$H independent in the range 6–8 at room temperature (Figure 8). Moreover, various correlations between changes in IFFP and $p\mathrm{H}_i$ (Figure 8), as measured in control and stimulated cells under control of $p\mathrm{H}_{ex}$, indicate that these two variables probe different physiological effects of cellular stimulation, rather than being effected one by the other. This conclusion is based on the following arguments:

(1) $p\mathrm{H}_i$ of T-lymphocytes was found to decrease upon PHA stimulation, while an increase in $p\mathrm{H}_i$ was observed upon phorbol ester stimulation.[44] In both cases, however, IFFP was found to decrease.[14,21]

(2) Upon increasing $p\mathrm{H}_{ex}$ from 6 to 7.4, an increase in IFFP in resting T cells indeed occurs, but not in PHA stimulated cells (Figure 8), despite intracellular changes in $p\mathrm{H}_i$, induced by changes in $p\mathrm{H}_{ex}$ between 6 and 8.[35,38]

(3) At the $p\mathrm{H}_i$ buffered region, external alkalinization from 7 to 7.4 still induces IFFP increase in resting cells.

(4) $p\mathrm{H}_{in}$ increases above $p\mathrm{H}_{ex}$ 7.4. Nevertheless, no change is observed in IFFP of resting cells in that region.

(5) As seen in Figure 9, if changes in FI are $p$H related to FFLT, one should expect a decrease in IFFP when measured at 470 nm, and an increase in IFFP when measured at 442 nm—following PHA stimulation. However, stimulating PBL by PHA resulted in a decrease in IFFP, in both excitation wavelengths.[14,18] Again, this contradicts direct correlation between FP and $p$H in IFFP measurements.

### 4.3.2 Decrease of IFFP due to Increasing Fluidity as a Direct Result of Lysis

Decrease of IFFP following conjugation caused by dilution of the cell content as a direct result of lysis, has been ruled out for the following reasons. (1) Lysis is associated with dye leakage out of the cells which in turn is indicated by a marked decrease in the IFF intensity; no such decrease has been observed. (2) Lysis does not occur at room temperature but at 37°C (Ref. 1). (3) Had the FP occurred due to lysis, only the target cell would have exhibited a change in polarization. We observe that changes in FP take place in both target and effector cells.

Finally, our previous work with 2′, 7′-bis(carboxyethyl)-5-(6′)-carboxy-fluorescein (BCECF)[45] showed that following cell disruption FP at the red region was increased compared to that of IFFP. We then suggested that this might be due to renewed binding of free BCECF to new exposed cellular components due to disruption. Such a phenomena did not occur when fluorescein or carboxyfluorescein (CF) was used, ruling out the possibility of increased fluorescein binding during the lytic process.

### 4.3.3 The Dependence of IFFP upon Quenching

In general, fluorescence quenching will decrease $\tau_F$, leading to an increase of FP. This will also be associated to decrease of FI. Moreover, such a dependence of IFFP upon quenching will define the stimulated status as less quenched since its FP is lower than that of nonstimulated cells.

In the present work no significant evidence to FI decrease was observed following E/T conjugation,

indicating that FP decrease is not related to quenching.

#### 4.3.4 FP Changes due to IF Binding and Viscosity

The rather high degree of IFFP in cells indicates a somewhat strong interaction between the probe and its intracellular environment. Different mechanisms which might explain this behavior were considered; namely, caging, attachment of dye molecules to macromolecules, or restricted orientational motion due to ''wobbling-in-cone.'' [46–48] If fluorescein is tightly trapped in a cage of the surrounding molecules, its motions will be limited although not bound to them. In this case, the embedding of fluorescein is assumed to be strongly dependent on the structure of the surrounding medium. On the other hand, if some kind of binding of fluorescein to the surrounding macromolecules (e.g., H bonding, charge attraction, or even covalent bonding) exists, the motion of each macromolecule is more relevant to the degree of FP than to the viscosity effect of the fluid domains formed by the cytoplasmic matrix structure.

In our previous studies we have shown that FP of ultrasonically disrupted cell suspension was the same as in PBS ($\sim$0.02) when FDA and carboxyfluorescein diacetate (CFDA) were used for cell staining,[48] in contrast to a rather high level of FP ($\sim$0.2) when BCECF/AM was used.[45] Intracellular fluorescein and CF, correspondingly hold one [??] and two[49] surplus negative charges. This seems to be insufficient in order to secure a permanent binding to the macromolecules, which is strengthened by the fact that both intracellular fluorescein and CF relatively easily leak out of the cell.[48,50]

In contrast, the leakage of intracellular BCECF, which holds 4 to 5 negative surplus charges, is null and reveals FP of $\sim$10% in PBS (five times greater than fluorescein) and FP of $\sim$0.2 following disruption. This suggests that while at least part of the intracellular BCECF might bind to sites on macromolecules or be strongly associated to them, fluorescein and CF seem to be trapped within the cytosol. Nevertheless, the fact that the intracellular fluorescein, CF, and BCECF fluorescence are redshifted, might point towards a temporary binding or preferably—a dynamic balance of binding, which completely reverses after cell lysis.

As for the intracellular viscosity, the values found by FP methods (6.3 and 14 cp at 27°C for Euglene and yeast cells, correspondingly)[51] are within the range found for protoplasmatic viscosity[52] and for Ascites cells[53] by methods such as Brownian motion. For such low viscosity levels, changes in IFFP following stimulation are probably due to changes in the rotation correlation time ($\tau_R$), associated with intracellular viscosity rather than to conformational alteration of the probe, resulting in changes of both $P_0$ and $\tau_0$ occurring at higher viscosities.[54]

#### 4.3.5 Electrostatic Solvent Effect

In light of the arguments listed in the previous paragraph, it is believed that rather than tight binding, the parameter contributing to the redshift phenomenon is solvent polarity.

In most polar aromatic molecules whose lowest single states are ($\pi,\pi^*$), the excited state is more polar than the ground state.[55] Interaction of the solute probe with the solvent molecules may occur prior to emission. Therefore, the excited molecule will tend to interact with a polar solvent so as to align the solvent dipoles. This alignment decreases the energy of the excited state and causes the emission spectra to shift towards the red. This generally holds true when both solvent and solute are polar, and even when both are not polar, since excitation induces electrostatic polarization. When excitation causes depolarization of the probe, a blueshift might also occur.[56]

Interestingly enough, in the case of fluorescein, the redshift increases as solvent polarity decreases.[57] This might explain that the redshift of intracellular fluorescein compared to that of fluorescein in PBS, is due to a lower polarity of the cellular content, in respect to that of the suspending PBS solution, rather than to intracellular binding. The fact that PHA stimulation caused a decrease in the redshift of FI (unpublished data) might suggest that reorganization of the intracellular matrix due to stimulation is associated with increasing intracellular polarity.

#### 4.3.6 Fluorescein Transition between Conjugate Cells

We have excluded possible transport of fluorescein between first stained cell to second nonstained cells during conjugation. Generally speaking, there is a slight probability for such transition to occur since fluorescein is a charged molecule having extra negative charge (when dissolved at physiological solutions of 7.4 *p*H). Thus, the membrane permeability to fluorescein is very poor.[58] In particular, when using the Cellscan, the possibility of measuring a dimly fluorescent cell which can yield FP is nil, since the average intensity of stained cells was 20 kHz, in comparison to less than 400 counts per second for unstained cells (autofluorescence) including scattering signals. Nevertheless, in order to avoid recording of fluorescent debris or aqueous fluorescent regions, FP measurements of each individual cell are limited by predefined setup of intensity and FP thresholds.

## 5 CONCLUSIONS

The mutual stimulation of effector and target cells can be demonstrated by the Cellscan-IFFP measurement on individual cell basis. Most of the intracellular fluorescein, from which fluorescence is observed in the IFFP measurement, exists in the form of an anion, presumably attached by dynamic bal-

ance to intracellular proteins. Possible candidates of such attaching sites are $Ca^{2+}$-regulated proteins like the troponin-tropomyosin complex, which might change their flexibility upon binding of $Ca^{2+}$ ions. An IFFP decrease may also reflect some combination of an increase in intracellular mobility, i.e., a transition of the cytoplasm from a more ''gel-like'' to a more ''sol-like'' state. An IFFP decrease may also be the result of a change in the ratio of free to bound probes reflecting changes in cytosolic *p*H.

By comparing the IFFP decrease to lifetime and energy-transfer measurements, and by aiming towards reconstitution experiments with purified proteins, ongoing attempts are being made to address the physical and biochemical basis of the phenomena causing the decrease in IFFP following cellular stimulation.

Kinetic IFFP measurements on the effector and target cells are presently being conducted in order to investigate the dynamic nature of stimulation due to conjugation.

The investigation of killer-target interaction and of antigenic stimulation of lymphocytes, at the single cell level, might contribute to basic research of transmembrane signaling and the mechanism of cell-mediated killing.

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