

Flow Cytometric Method for Simultaneous Detection of Lymphocyte-K562 Conjugates and Immunophenotyping of the Conjugate Forming Cells¹

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A flow cytometric method for the simultaneous quantification and immunophenotyping of conjugates formed by human peripheral blood lymphocytes (PBL) and K562 cells has been developed. The method uses three fluorescent probes. One of the fluorescent probes (F-18) is used for labeling of PBL prior to incubation with K562 cells. After incubation the cells are treated with monoclonal antibodies labeled with phycoerythrin and Red613, respectively. The combination of F-18 fluorescence and light scattering signals enables identification and quantification of the conjugates while the fluorescence of the monoclonal antibodies provides information about the phe-

notype of the conjugate forming cells. Results obtained using different monoclonal antibodies are presented. The highest conjugate forming capacity has been found in the CD56+CD8+ population while the CD4+CD8- population has shown the lowest capacity to form conjugates. The influence of a washing step on the conjugate formation is discussed. The possibility to use the method in combination with a cytotoxicity assay is indicated. © 1993 Wiley-Liss, Inc.

Key terms: NK cells, conjugate formation, immunophenotyping, three fluorescent probes, flow cytometry

Natural killer (NK) cells represent a heterogeneous cell population that is able to destroy, in MHC-nonrestricted manner and without previous sensitization, different invaders of the body integrity (22). Their functional capability is usually assessed by cell mediated cytotoxicity assays, wherein the target cell death is used as a measure of the NK activity (4,12,14,17,18,25).

The capability of a cytotoxic cell to form a conjugate with a target cell is a prerequisite for the killing process to take place (3,5). The detection and quantification of conjugates have therefore been points of interest, resulting in the development of several flow cytometric techniques (2,6,11,13,20,26). The finding that distinct populations of peripheral blood cells can form conjugates with the NK-sensitive targets (10,15,16,19,24) made determination of the phenotype of the conjugate forming cells problematic. The conjugate identification techniques developed so far do not enable a direct characterization of conjugate forming cells but rather demand sorting, either prior to conjugate formation (enrichment for certain cell types) (20) or after conjugates have been detected (immunopheno-

typing of the conjugates). An exception is the work of Vitale et al. (26) wherein the conjugates formed by certain lymphocyte subpopulation (identified by means of monoclonal antibodies) and K562 cells could be detected. However, the conjugates that unlabeled lymphocyte subpopulations formed with K562 cells could not be resolved.

Here we report a three fluorescent probe technique that enables detection of all conjugates in combination with characterization of the conjugate forming cells using two monoclonal antibodies. PBL are labeled with the membrane probe F-18 (7,9,14) prior to incubation with K562 cells. After incubation the cells are stained with two monoclonal antibodies, labeled with phycoerythrin and Red613, respectively. The conjugates formed by F-18-labeled PBL and K562 cells can be identified as F-18 positive cells with large scattering

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signals while the monoclonal antibodies fluorescence enables further characterization of the conjugate forming cells.

MATERIALS AND METHODS

Cells

K562 cells were used as target cells for conjugate formation. Cells were grown as previously described (14). Mononuclear cells were isolated from the heparinized peripheral blood of healthy donors as described elsewhere (21). The monocytes were depleted by incubating the cells ($10^6/\text{ml}$ RPMI1640 + 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) in a plastic flask for 60' at 37°C. Non-adherent mononuclear cells were collected.

F-18 Staining

F-18 was prepared as previously described (7). The mononuclear cell fraction was incubated with the dye (final concentration 10–25 ng/ml) simultaneously with monocytes depletion, followed by additional incubation of PBL for 30' at 37°C. Simultaneous monocyte depletion and F-18 staining resulted in similar staining as the subsequent incubations and it was therefore chosen as a more convenient method. After incubation cells were washed twice with cold CM and resuspended in CM at a concentration of $2 \times 10^6/\text{ml}$.

Conjugate Formation and Labeling With Monoclonal Antibodies

Monoclonal antibodies were purchased from Becton Dickinson (Mountain View, CA). They included anti-CD3-PE, anti-CD4-PE, anti-CD16-PE, anti-CD56-PE, anti-CD8-biotin, and anti-CD3. Streptavidin-Red613 was purchased from Life Technologies, Inc. (Gaithersburg, MD).

K562 cells were mixed with F-18 labeled PBL in a ratio 1:1. Usually a total of 5×10^5 cells was used. The conjugate forming mixture was centrifuged for 5 min at 200g, gently resuspended, and incubated for 20 min at room temperature. After incubation the cells were washed once with cold PBS + 1% BSA, resuspended in 100 μl of cold PBS + 3% BSA, and incubated with 3 μl of monoclonal antibody (except for anti-CD56-PE where 10 μl was used) for 30' at 4°C. For double staining, 3 μl of both PE and biotin labeled monoclonal antibodies was used. After incubation the cells were washed twice with cold PBS + 1% BSA and resuspended in 100 μl of cold PBS + 3% BSA. Four microliters of streptavidin-Red613 was added to all samples containing anti-CD8-biotin and the samples were incubated for 30' at 4°C. The samples that did not contain biotin labeled monoclonal antibody were incubated in the same way but without addition of streptavidin-Red613. After incubation the samples were washed with cold PBS + 1% BSA, resuspended in cold PBS, and kept at 4°C until analyzed by flow cytometry. The

control samples (without monoclonal antibody labeling) were incubated in the same way but without addition of monoclonal antibody and streptavidin-Red613.

In the experiments where the influence of a washing step on conjugate formation was studied, anti-CD3 and anti-CD8-biotin were used. The cells were incubated with or without monoclonal antibodies for 30' at 4°C and analyzed before and after one washing step.

Flow Cytometer

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 (21). F-18 fluorescence was measured using the green fluorescence channel (510–550 nm), PE fluorescence was measured using the orange fluorescence channel (550–580 nm) and Red613 fluorescence was measured using the red fluorescence channel (> 610 nm).

From each sample 16,000 cells were measured three times: without gating, gated on the F-18 fluorescence (to measure PBL and conjugates containing PBL), and gated on the F-18 fluorescence + forward light scattering signal (to measure exclusively conjugates). Presented results are averages of these measurements (total 48,000 cells). Twice as many cells were measured in the experiments where the influence of the washing step on conjugate formation was studied.

RESULTS

Identification of Cell Populations

Conjugates formed by F-18 labeled PBL and K562 cells could easily be distinguished from the single cells on the basis of F-18 fluorescence and light scattering signals (Fig. 1a). Cells with relatively small scattering signals and positive for the F-18 fluorescence are PBL whereas nonfluorescent cells with relatively large scattering signals are K562 cells. Conjugates formed between PBL and K562 cells are positive for F-18 fluorescence and they exhibit large scattering signals. Addition of two monoclonal antibodies, labeled with phycoerythrin and Red613, respectively, enabled further division of the cells in different subtypes. This could be done for non-conjugated PBL (Fig. 1b) as well as for conjugated PBL (Fig. 1c).

Phenotypic Distribution

The method enabled the analysis of the phenotypic distribution of conjugated PBL. The results obtained using a number of monoclonal antibodies identifying NK cell and T cells are shown in Table 1. The relative occurrence of the specific subpopulations in the control (PBL incubated without K562 cells) is listed in column A. In columns B and C the phenotypical characteristics of the unbound and bound (conjugated) PBL

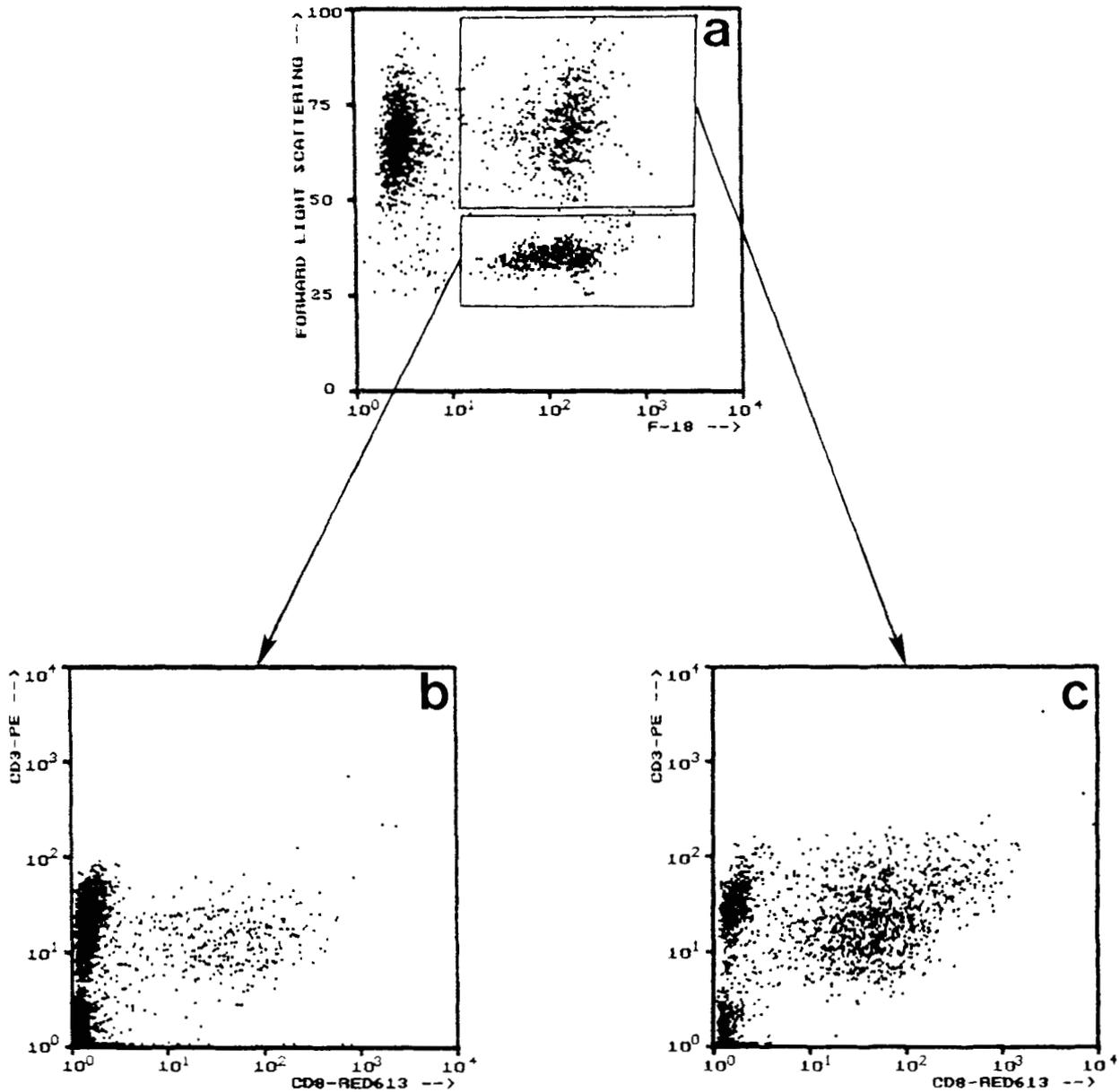


FIG. 1. Scatter plots showing identification of cell populations. F-18 labeled PBL were incubated with K562 cells to form conjugates and the sample was stained with monoclonal antibodies (anti-CD3-PE + anti-CD8-biotin/streptavidin-Red613). Single cells and conjugates were identified on the basis of F-18 fluorescence and light scattering signal (a). Phenotypic identification could be done for both non-conjugated PBL (b) and conjugated PBL (c). In each plot 2,500 cells are shown.

in the conjugate forming mixture are shown. In column D the relative occurrence of the specific subtypes of all PBL in the conjugate forming mixture is shown. All monoclonal antibodies used for double labeling were also tested separately (one monoclonal antibody labeling). As can be seen from the columns A and D, similar phenotypical distribution was obtained for control PBL and total PBL in the conjugate forming mixture.

A comparison between the free and bound PBL in the conjugate forming mixture (columns B and C, respectively) revealed the preference of some cell types (CD3+CD8+; CD4-CD8+; CD4+CD8+; CD16-CD8+; CD56-CD8+; CD56+CD8+) for conjugate formation. This can be seen more clearly in Figure 2 where the percentage of a subpopulation that forms a conjugate ("the conjugate forming capac-

Table 1
Phenotypic Distribution^a

	Control sample	Conjugate forming mixture		
	A (per 100 PBL)	B (per 100 unbound PBL)	C (per 100 bound PBL)	D (per 100 PBL)
CD3+	64.5 ± 9.9	57.6 ± 4.3	72.8 ± 11.4	60.8 ± 7.1
CD4+	38.9 ± 8.2	37.1 ± 10.5	42.6 ± 0.1	37.9 ± 8.6
CD8+	23.8 ± 5.7	9.9 ± 2.2	56.5 ± 11.9	20.5 ± 4.6
CD16+	5.4 ± 0.1	2.3 ± 0.8	12.3 ± 0.6	3.8 ± 1.3
CD56+	0.7 ± 0.3	0.6 ± 0.1	7.2 ± 2.9	1.7 ± 1.0
CD3-CD8-	32.7 ± 9.7	44.9 ± 9.7	23.5 ± 11.0	38.3 ± 5.0
CD3+CD8-	42.9 ± 6.5	45.5 ± 5.6	27.2 ± 4.3	39.6 ± 5.3
CD3-CD8+	3.6 ± 2.1	1.5 ± 0.4	1.6 ± 0.6	1.4 ± 0.5
CD3+CD8+	20.9 ± 7.7	8.2 ± 1.4	47.8 ± 11.1	20.8 ± 5.8
CD4-CD8-	41.8 ± 6.2	46.5 ± 7.4	40.2 ± 13.3	45.4 ± 3.4
CD4+CD8-	37.6 ± 7.6	42.2 ± 11.4	15.7 ± 3.5	36.5 ± 8.9
CD4-CD8+	18.9 ± 1.1	10.4 ± 3.6	37.6 ± 14.7	15.7 ± 4.8
CD4+CD8+	1.8 ± 0.2	1.1 ± 0.4	6.5 ± 2.1	2.6 ± 0.4
CD16-CD8-	72.0 ± 2.7	84.3 ± 5.4	46.6 ± 10.6	77.4 ± 5.7
CD16+CD8-	6.2 ± 0.4	3.9 ± 1.9	7.7 ± 1.3	4.8 ± 1.0
CD16-CD8+	18.4 ± 3.4	11.2 ± 3.7	42.5 ± 12.9	16.5 ± 4.7
CD16+CD8+	3.5 ± 0.4	0.7 ± 0.3	3.3 ± 1.0	1.5 ± 0.3
CD56-CD8-	70.0 ± 1.7	87.4 ± 0.4	35.8 ± 5.6	74.5 ± 2.0
CD56+CD8-	0.7 ± 0.4	0.6 ± 0.4	1.4 ± 0.4	1.1 ± 0.6
CD56-CD8+	29.2 ± 1.2	11.9 ± 0.6	60.3 ± 6.1	23.2 ± 1.0
CD56+CD8+	0.2 ± 0.1	0.3 ± 0.1	2.7 ± 0.1	1.3 ± 0.7

^aColumns A and D show the relative occurrence of the specific subtypes of all PBL in control sample (incubated without K562 cells) and in the conjugate forming mixture, respectively. Columns B and C show the phenotypic characteristics, respectively, of the unbound and bound PBL in the conjugate forming mixture. Data are expressed as means ± S.D. of 4 subjects (anti-CD3, anti-CD8, anti-CD3 + anti-CD8) or of 2 subjects (rest).

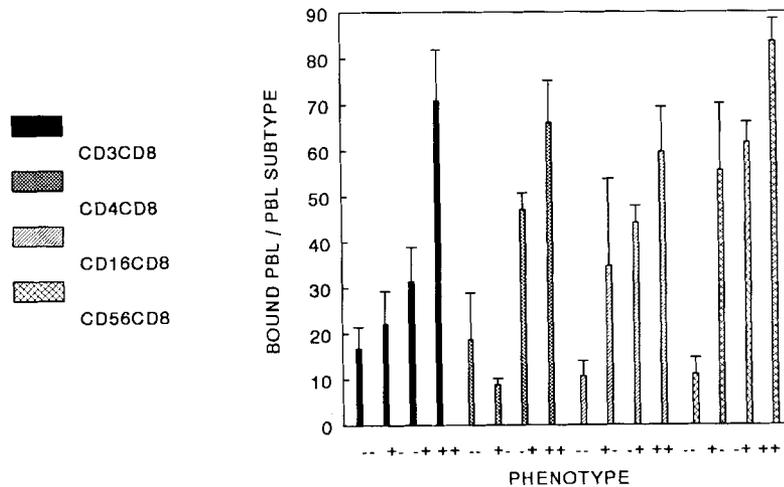


FIG. 2. Conjugate forming capacity of different PBL subpopulations. Different PBL subpopulations in a conjugate forming mixture were identified by double labeling with monoclonal antibodies. The percentage of a subpopulation that forms a conjugate was calculated. Data are expressed as means ± S.D. of 4 subjects (anti-CD3 + anti-CD8) or of 2 subjects (rest).

ity") is presented for different subpopulations. The highest conjugate forming capacity was exhibited by CD56+CD8+, PBL while CD4+CD8- PBL had the lowest capacity. CD3+CD8+, CD4+CD8+,

CD16+CD8+ and CD56-CD8+ PBL subpopulations showed a high conjugate forming capacity while CD3-CD8-, CD4-CD8-, CD16-CD8-, as well as CD56-CD8-, had a low conjugate forming capacity.

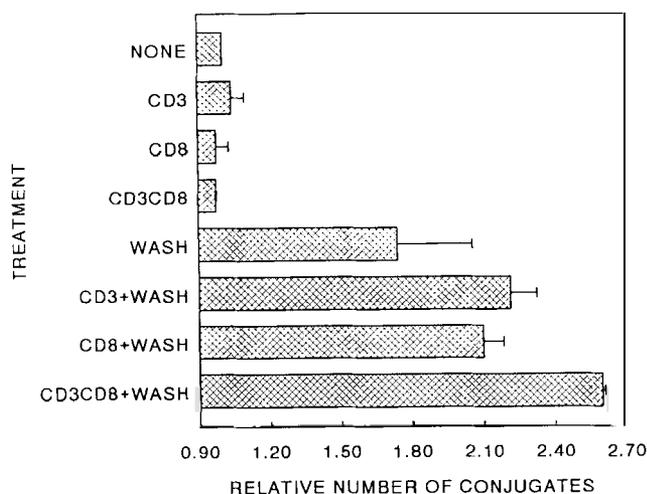


FIG. 3. Influence of washing step on conjugate formation. The percentage of PBL that forms a conjugate in the samples incubated with monoclonal antibodies and the control sample (incubated without monoclonal antibodies) before and after washing step was calculated. Data were normalized against the number of conjugates in control samples before washing step ("NONE"). Data are expressed as means \pm S.D. of 2 subjects.

Influence of Washing Step on Conjugate Formation

A detailed analysis of our data revealed a difference in the number of conjugates between samples incubated with monoclonal antibodies and the control sample (incubated without monoclonal antibodies) that was only observed if the cells were washed upon incubation (Fig. 3). A washing step caused an increase in the number of conjugates even in the absence of monoclonal antibodies (Fig. 3). A cumulative effect was found for anti-CD3 and anti-CD8 (Fig. 3). These results seem to indicate the activation of the conjugate forming ability of specific PBL subtypes but that conjugates are actually formed only if the cells are brought into close contact with target cell by centrifugation. Our results, however, do not exclude the possibility that Fc receptors present on K562 cells (8) play a role in the conjugate formation (1,23).

DISCUSSION

NK cells can be defined as a heterogeneous cell population able to kill tumor cells, immature cells, and virus-infected cells *in vitro* without prior sensitization or MHC-restriction (22). A first, necessary step in the killing process is the conjugate formation between a killer and a target cell (3,5).

In this study we have developed a three fluorescent probes flow cytometric assay that enables both quantification of all conjugates formed by human PBL and K562 cells and determination of the phenotypic characteristics of all conjugate forming cells.

In order to test our method we have done a series of

experiments using monoclonal antibodies that identify NK cells (anti-CD16, anti-CD56) and T lymphocytes (anti-CD3), both cytotoxic (anti-CD8) and helper cells (anti-CD4).

In agreement with the expectation, our results show that CD8⁺ PBL, including CD3⁺CD8⁺, CD56⁻CD8⁺, and CD56⁺CD8⁺, are predominant among the cells that form conjugates with K562 cells (Table 1). These PBL subtypes include both NK cells (CD56⁺) and cytotoxic lymphocytes (CD3⁺). As expected, CD3⁻CD8⁻ (mostly B lymphocytes), CD4⁺CD8⁻ (T helper lymphocytes), CD16⁻CD8⁻ and CD56⁻CD8⁻ (B lymphocytes, T helper lymphocytes) cells subtypes are predominant among unbound PBL. Analysis of the conjugate forming capacity (e.g., the percentage of a subpopulation that forms a conjugate) of PBL subtypes reveals the CD56⁺CD8⁺ (mostly NK cells) population as the cell type with the highest conjugate forming capacity (Fig. 2). Another subtype consisting mostly of the NK cells, the CD16⁺CD8⁺ population, shows also a high conjugate forming capacity while subtypes consisting mostly of T helper and B lymphocytes (CD4⁺CD8⁻, CD3,4,16,56⁻CD8⁻) have a low conjugate forming capacity.

An unexpected finding in our study is that labeling with monoclonal antibodies after the cells were incubated to form conjugates influences the conjugate formation. We found that this was due to the extra washing step (Fig. 3). Even without addition of monoclonal antibodies the washing step causes a significant increase in the number of conjugates. An even larger increase is observed if the cells are incubated with anti-CD3 and anti-CD8 that also show a cumulative effect (Fig. 3). These results could indicate that certain monoclonal antibodies can activate the conjugate formation but that the effect is observed only when the cells are brought into contact with target cells by additional centrifugation (washing). A possible involvement of the K562 cell Fc receptors (8) in the conjugate formation (1,23) is, however, not excluded.

The influence of the washing step on the conjugate formation can be avoided using directly labeled monoclonal antibodies (no washing step needed!) instead of indirect labeling.

There are at least two possibilities to combine this assay (identification of the conjugates and immunophenotyping) with a cytotoxicity assay. In the first case changes in the light scattering characteristics of a target cell could be used as the indicator of target cell death (25). Another possibility would be to use propidium iodide as target cell indicator. In this case only one monoclonal antibody could be used. With such a combined assay one could follow the kinetics of the cytotoxic process of different cell types.

In conclusion, our study demonstrates the possibility to identify and quantify conjugates simultaneously with immunophenotyping of the conjugate forming cells.

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