Assays of Natural Killer (NK) Cell Ligation to Target Cells

BASIC PROTOCOL

The formation of a conjugate between an effector lymphocyte and a target cell is the first step in cellular cytotoxicity reactions. This protocol, previously known as the "binding assay," measures the ability of different lymphoid cell populations to adhere to a tumor target cell. In particular, it is used to monitor the formation of conjugates in vitro between natural killer (NK) cells and susceptible target cells (e.g., K562 cells for human NK, YAC cells for mouse NK) based on side-scatter signals, and it allows discrimination between effector and target cells based on detection of effector cell–specific antigens using fluorochrome-conjugated monoclonal antibodies.

Most commercially available flow cytometers can be used to perform this assay. This protocol requires expertise in basic flow cytometric techniques (Chapter 1, *UNITS 5.1 & 6.2*).

Materials

Carbonyl iron (Sigma)
Heparinized human blood (*UNIT 5.1*)
Phosphate-buffered saline (PBS), pH 7.4 (*APPENDIX 2A*)
Ficoll-Hypaque (Seromed) or Percoll (Sigma; S = 1.077)
RPMI-10 medium (see recipe)
K562 chronic myeloid leukemia cells grown in suspension in RPMI-10 to logarithmic phase
Propidium iodide (PI) stock solution (see recipe)
Fluorochrome-conjugated monoclonal antibodies (MAbs; e.g., PE-conjugated anti-CD16, FITC-conjugated anti-CD3, and PerCP-conjugated anti-CD8)

37°C water bath
15- and 50-ml polypropylene centrifuge tubes (Falcon)
Beckman GS-15R centrifuge (or equivalent)
Flow cytometer with 488-nm light source (e.g., Becton Dickinson, Coulter, or Ortho)
Software for multiparametric analysis

Additional reagents and equipment for removing monocytes from blood (*UNIT 5.1*), cell culture (*APPENDIX 3B*), and counting cells (*APPENDIX 3A*)

NOTE: All solutions and equipment coming into contact with cells must be sterile and proper sterile technique should be used accordingly.

NOTE: All culture incubations are performed in a humidified 37°C, 5% CO_2 incubator unless otherwise specified.

Prepare effector cells

1. In order to deplete monocytes, add carbonyl iron to a final concentration of 10 mg/ml to heparinized human blood, and resuspend the mixture with a 10-ml pipet. Incubate 1 hr at 37°C, gently agitating the tube every 10 min.

   Heparinized blood (10 ml) or buffy coat cells (50 ml) can be used as a source of effector cells.

   Monocytes phagocytose iron particles so they become heavier than other cells. The majority of the monocyte population will be at the bottom of the tube after gradient separation.

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2. Dilute the blood cell suspension 1:1 with PBS, pH 7.4.

   Dilute buffy coat material 1:5 with PBS, pH 7.4.

3. Layer 10 ml diluted cells on a 3-ml Ficoll or Percoll cushion in a 15-ml polypropylene centrifuge tube. Centrifuge 20 min at 700 × *g*, room temperature.

4. Using a pipet, gently remove 3 to 5 ml of plasma, then collect the white ring of mononuclear cells. (*UNIT 5.1*).

   The mononuclear cells are found in the interface between Ficoll or Percoll cushion and the plasma.

5. Wash the mononuclear cells twice by resuspending the cells in RPMI-10 and centrifuging 5 min at 400 × *g*, room temperature.

   The mononuclear fraction is a heterologous population containing B and T lymphocytes and NK cells.

6. Resuspend the pellet in 10 ml RPMI-10 and transfer to a 75-cm^2 flask. Incubate 30 min at 37°C to deplete residual monocytes by adhesion to the plastic flask (*UNIT 5.1*).

   At this point, it is a good idea to immunophenotype the cells to determine the distribution of lymphocyte subpopulations, e.g., $CD16^+$, $CD\ 56^+$, $CD16^+8^+$, and $CD57^+$.

Prepare target cells

7. Harvest logarithmic phase culture of K562 target cells.

   If the cultures are harvested during the logarithmic phase, there are a minimum of dead target cells.

8. Count both peripheral blood lymphocytes (PBL; step 6) and K562 cells using a hemocytometer (*APPENDIX 3A*).

   Alternatively, the cells can be counted using a Neubauer chamber.

   It is important to assess the number of dead cells in the target population before starting each experiment (UNIT 9.2). Determine the percentage of dead cells by trypan blue exclusion (APPENDIX 3A). In order to obtain good results, the proportion of dead cells should not exceed 5%, not only in this ligation protocol but also in the killing protocol.

9. Mix effector and target cells in an effector-to-target (E:T) ratio of 1:1 and check their proportion using the cytometer.

   Manual counting can lead to an error in the relative proportion between target and effector cells. A good method to check the real E:T ratio is to draw two different gates in the scatter contour plot as shown in Figure 9.10.1. Using this plot it is also possible to check for the presence of a residual monocytic population that can alter the assay result.

Form conjugates

10. Transfer a total of 2×10^5 cells to each 15-ml tube. Add PI stock solution to a final concentration of 0.5 µg PI/ml.

    CAUTION: *Propidium iodide is potentially hazardous: it is known to be a tumorigenic agent.*

    E:T ratios of 2:1 and 4:1 could also be used in order to enhance the relative number of conjugates; higher E:T ratios will not affect the determination, but they will allow a more precise definition of the level of conjugation for each lymphocyte subpopulation.

    Propidium iodide is used in this case for detecting dead cells. Dead cells will appear bright orange-red in the fluorescence diagram and can easily be gated out.

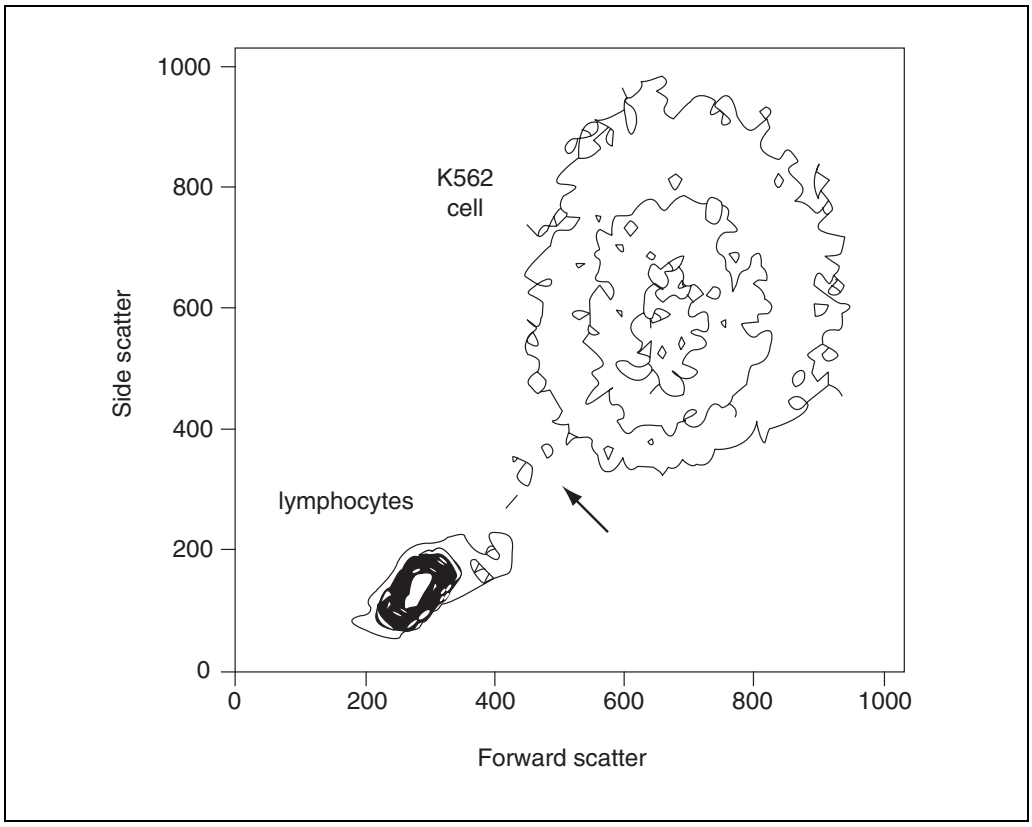

Figure 9.10.1 Scatter-contour plot of lymphocytes and K562 cells mixed in a 1:1 ratio. Note the difference in size between the lymphocytes and the big tumor cells. Using this graph, it is possible to verify both the ratio between effector and target cells and the presence of monocytes, which occupy the intermediate area and which could be confused with small target cells. The arrow indicates the region where monocytes are found if they are not depleted.

11. Centrifuge the tube 7 min at 250 × *g*, room temperature. Incubate 10 min in a 37°C water bath to promote conjugate formation. Then incubate 30 min at 4°C to prevent lytic activation.

    It is preferable to use a water bath with mild agitation because that will help the sample reach working temperature in a shorter period of time.

    Times must be strictly adhered to because activation of the lytic process can reduce the number of bound effectors due to their detachment from the target cells just after the release of lysosomal products.

Stain samples

12. Gently resuspend samples. Add fluorochrome-conjugated MAbs to a concentration of 20 µg/ml per 10^6 effector cells and incubate 20 min at 4°C. At the same time incubate 10^6 mononuclear cells with the same combination of MAbs for instrument standardization. Incubate 10^5 target cells alone to check for autofluorescence.

    For the experiment described here, only two MAbs (PE-conjugated anti-CD16 for NK cells and FITC-conjugated anti-CD3 for T lymphocytes) are used, to simplify the description. The MAbs should be directly labeled with fluorochromes such as FITC, PE, PerCP, PECy5, or APC (UNIT 6.2). The MAbs should recognize antigens that are not present on the target cell surface and do not interfere with the specific binding of effector cells. Experiments can also be performed to investigate the presence of surface antigens that are present in the binding site (Papa et al., 1994; Zamai et al., 1994).

13. Wash the cells once with PBS.

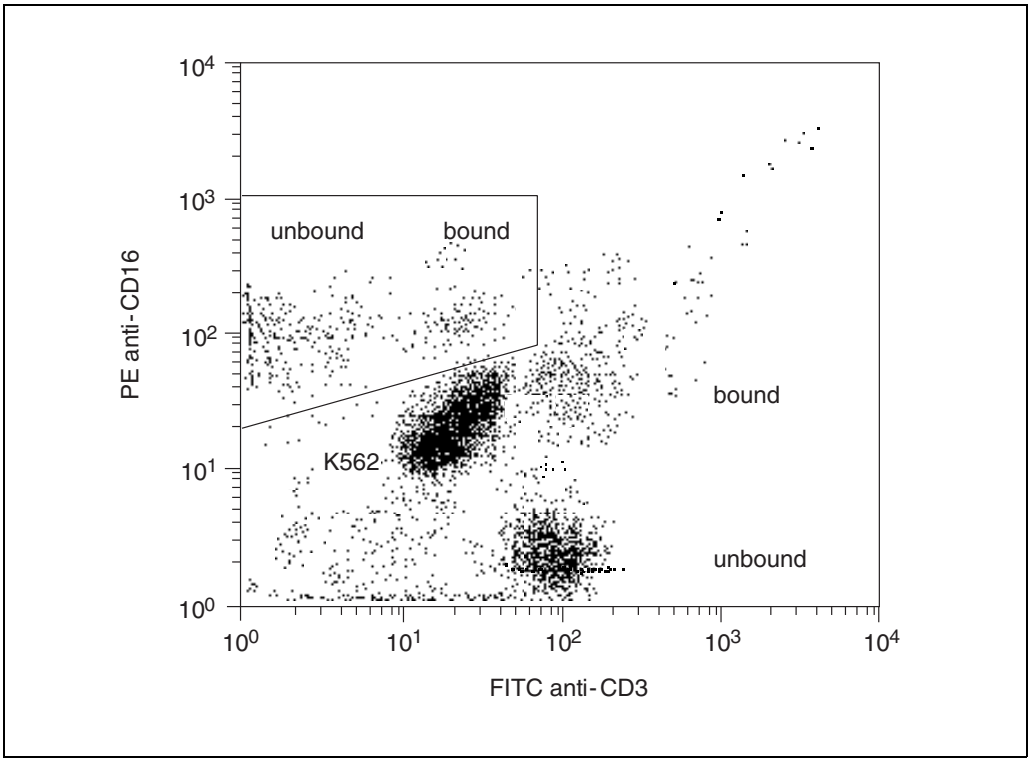

Figure 9.10.2 Fluorescence dot-plot of lymphocytes identified with two different monoclonal antibodies, FITC-conjugated anti-CD3 and PE-conjugated anti-CD16, and autofluorescent K562 cells. It is possible to identify $CD3^+$ (T-lymphocytes) and $CD16^+$ (NK) cells conjugated with target cells.

Perform flow cytometry

14. Analyze the target cell population alone. In the scatter plot (see Fig. 9.10.1), position live K562 cells in the upper right quadrant of the plot to allow good resolution of the lymphocytes when the two populations are analyzed together.

    If the K562 population is in another position, correct the amplification of the scatter signals. The position of K562 cells in the fluorescence cytogram is essential to allow discrimination between bound and unbound effector cells.

    K562 tumor cells are larger than lymphocytes, and monocytes are between the two in size.

    In a fluorescence cytogram, K562 cells should have autofluorescence values similar to the ones shown in Figures 9.10.2 and 9.10.3. Dead cells also show higher autofluorescence values and can compromise the results if the starting percentage of dead cells is >5% to 10%.

15. Analyze effector cells alone, using a scatter plot to check their position and to check for the presence of residual monocytes.

16. Using a fluorescence cytogram, compensate the fluorescence of labeled effectors.

    Simultaneous analysis of two subpopulations can be performed using lymphocytes labeled with two MAbs that recognize antigens not coexpressed by the same subpopulations. The MAbs must be conjugated with different fluorochromes (e.g., FITC-conjugated anti-CD4 versus PE-conjugated anti-CD8 or FITC-conjugated anti-CD3 versus PE-conjugated anti-CD16).

17. Analyze a 1:1 mixture of target and effector cells (see Fig. 9.10.2).

18. Draw a gate (R1) around all the population positive for the antigen(s) of interest.

    In Figure 9.10.2, only the CD16-positive cells are gated.

19. Draw another gate (R2) around the bound cells inside the previous gate.

    If the fluorescence compensation is carried out correctly and the target cell viability is 90% to 95%, bound cells are recognized as those acquiring bound target cell fluorescence (see Fig. 9.10.2).

20. Acquire ≥50,000 total events. Divide the number of cells in R2 (bound) by the number of cells in R1 (bound + unbound) to obtain the percentage of bound cells belonging to the effector subpopulations being examined.

    If the effector subpopulations are poorly represented, acquire only that population using gate R1 as a "live" gate so only cells positive for that antigen will be acquired.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

Propidium iodide solution

Dissolve propidium iodide to a final concentration of 10 µg/ml in RPMI 1640. Store up to 6 months at 4°C.

CAUTION: *Propidium iodide is a potentially hazardous agent; therefore always use all necessary precautions when handling it, especially while weighing the powder.*

RPMI-10

RPMI 1640 medium containing:
10% (v/v) fetal bovine serum (FBS; heat inactivated 30 min at 56°C; *APPENDIX 2A*)
100 U/ml penicillin
0.1 mg/ml streptomycin sulfate
Store at 4°C

COMMENTARY

Background Information

During the last decade a number of optical microscopy– or flow cytometry–based methods have been developed for quantitative determination of the lytic activity of NK/CTL cells against specific targets (Kimberley et al., 1986; Papa et al., 1988). Other methods, meanwhile, were developed for recognition and quantitative/qualitative evaluation of cells forming conjugates with tumor targets (Segal and Stephany, 1984; Storkus et al., 1986; Vitale et al., 1992). These techniques are characterized by being single-cell assays; they are more reliable and controllable than total cell assays such as the ^{51}Cr release assay (Albright and Albright, 1983) and the FDA assay (Blomberg et al., 1996). Moreover, they are based on the ability to distinguish effector cells, living and dead target cells, and conjugates. This ability to distinguish the different cells is based on differences between scattering signals produced by effector and target cells (Fig. 9.10.1), autofluorescence displayed by different cell types in flow analysis as seen for K562 cells (Fig. 9.10.2), and fluorescence acquired after binding of specific fluorochrome-conjugated monoclonal antibodies to different populations of effector cells (Fig. 9.10.2 and 9.10.3).

Measurement of light scatter is advantageous because it avoids any possible interference with lytic activity due to previous sample preparation. The relationship that links forward scatter to cellular dimension through the diffraction pattern is well known. However, the forward-scatter signal is also affected by the relative refractive index (Mullaney and Dean, 1970), which undergoes large variation during cell death, thus inducing a constant lowering of the forward-scatter signal and allowing detection of cell death on the cytogram. On the other hand, side scatter has been shown to depend mainly on cytoplasmic granularity and consequently on cytoplasmic vacuolization during cell death (Vitale et al., 1989). Therefore, the morphological changes that take place after killing produce significant modifications of cellular light-scattering properties.

The main requirement of these techniques is the ability to clearly discriminate effector cells from target cells and conjugates. While

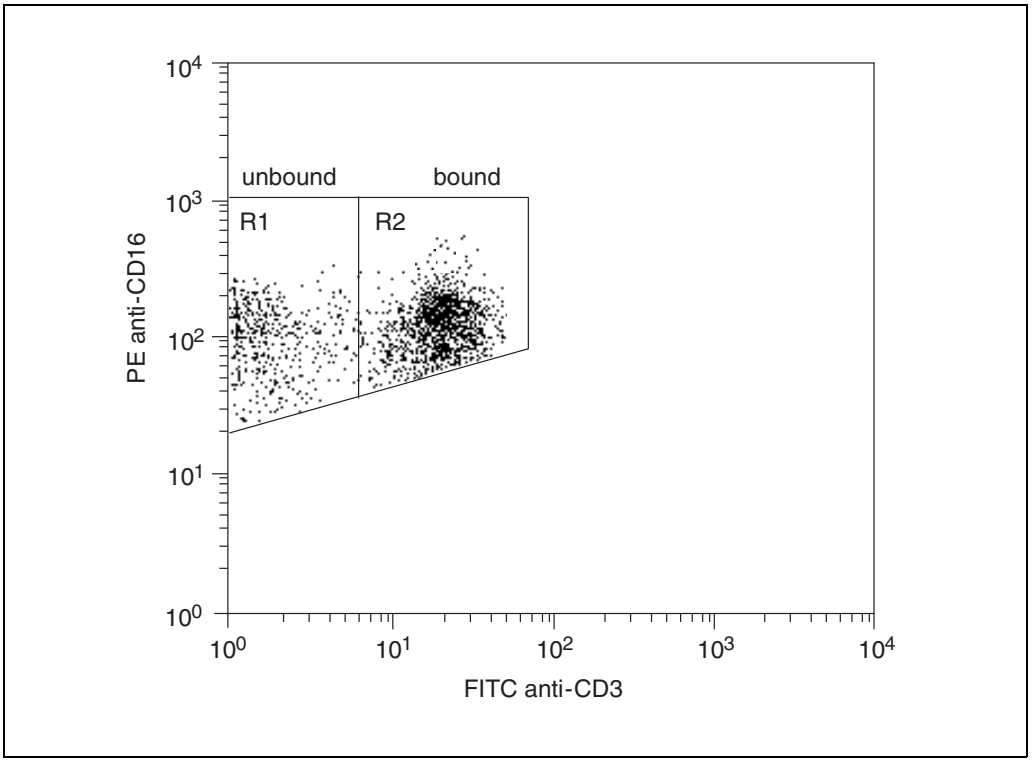

Figure 9.10.3 Fluorescence dot-plot showing gating methods for calculating the proportion of NK cells bound to the target cells. A gate is drawn for $CD16^+$ cells (bound and unbound, R1 + R2). Another gate is drawn only around the bound portion (R2). The first gated region contains 1905 of 50,000 cells (3.81%); there are 1003 bound cells (2.81% of the total and 52.65% of the gated cells). The abscissa mean value for unbound cells is 23.2 and for bound cells it is 35.4

this can be obtained on a scattering matrix for evaluation of cytotoxicity, for evaluation of conjugates this discrimination must be made on the fluorescence matrix, making this method more flexible for the use of different target cells. Furthermore, this binding assay permits identification of different effector cells attached to the targets (the conjugates) based on effector cell labeling with different directly conjugated monoclonal antibodies. Different monoclonal antibodies are identified based on the conjugated fluorochrome, so this technique resembles a typical dual- or triple-fluorescence analysis as in a normal flow phenotype analysis.

These methods have been set up primarily in order to investigate non-MHC-restricted cytotoxicity (Herberman et al., 1986). They are not restricted to this application but can be extended to antibody-dependent cellular cytotoxicity (ADCC) or other adhesion-based mechanisms. The authors' experience demonstrates the usefulness of these methods with different target cells (data not shown). The only limitations are that the methods can be used to monitor only reactions that occur in suspension, and the effector compartment (lymphocyte in this case) must be clearly distinguishable on the basis of scatter signals.

Critical Parameters and Troubleshooting

This method requires an accurate and complete depletion of monocytes. Monocytes can alter the assay results because their autofluorescence can overlap the K562 tumor cell autofluorescence and their size is similar to that of small target cells, so they may be counted as lymphocytes. However, monocyte contamination is easily monitored: monocytes are present in a specific area between lymphocytes and K562 cells in a scatter plot of a mixed sample (see Fig. 9.10.1), and they do not possess any of the antigens used to recognize lymphocyte subpopulations. Monocyte contamination can be relatively low in a 1:1 mixture of effector and target cells, but it becomes larger when the effector-to-target ratio is increased to 2:1 or 4:1 for better resolution of bound cells. This protocol is also preparatory for the single-cell cytotoxicity assay where effector to target cell ratios are 6.25:1 to 25:1 and the monocyte contamination can be relatively high.

Regarding targets, when cell lines are employed, it is necessary to work with cells in the logarithmic phase of growth to reduce the number of spontaneous dead cells present in the samples. Nonviable cells affect both assays: the SCCA, for the high level of dead cells present in controls, and the binding assay, for the increased autofluorescence of dead cells. This last problem can be avoided by gating on the scattering cytogram (Fig. 9.10.1 and 9.10.2).

As for other flow techniques, these methods need an accurate setting of the cytometer because of the use of all parameters, including scatter, for analysis. Moreover, when these samples are run on analyzers (such as FACScan), amplification of scatter signals must be corrected. For setting the proper signal amplification at E:T = 1:1, the mixed sample must be run in order to optimize the cytogram position of effector cells and target cells (Fig. 9.10.1).

Another critical parameter is fluorescence compensation. It is preferable to proceed with a dual-parameter compensation setting done using directly labelled cells (for example, lymphocytes labelled with PE-conjugated anti-CD8 and FITC-conjugated anti-CD4) from the mononuclear samples under investigation. In this way, the sample can be used both for setting the proper signal amplification for the scatter and for amplification and compensation of the fluorescence channels (PE for red and FITC for green). Compensation should be employed in order to perform accurate calculations.

Many problems can be due to the type of cytometer used and its optic and fluidic systems. For this assay, the set of filters employed is composed of a 488-nm bandpass filter on the orthogonal scatter, a 530 ± 15-nm bandpass filter on the green channel, and a 585 ± 30-nm bandpass filter on the red channel. The optics and hydrodynamics of analyzers such as FACScan, Ortho Absolute, Coulter Profile, and Coulter XL are perfectly optimized to perform these assays.

If a sorter is used to perform this assay, it must be equipped with a nozzle tip of diameter ≥100 μm to avoid disruption of conjugates when they pass through the orifice (Vitale et al., 1989).

Sample analysis must be performed at a slow flow rate with a sheath pressure ~10 lb/in.^{2} in order to obtain a good orientation of conjugates within the jet.

In the case of triple staining for effector cells, the setting of the third fluorescence channel should be obtained according to the manufacturer's instructions.

Whereas analyzers are fixed machines, sorters can be adjusted with proper filters for different fluorescent probes; for example, a dual-laser instrument will need a 660 ± 20-nm bandpass filter for APC and Tricolor and a 630 ± 20-nm bandpass filter for RED 613.

MAbs for these experiments must be carefully selected. Antigens recognized by the MAbs cannot be expressed in the target cell membrane or on the monocytes. If the antigen is expressed by monocytes, the monocyte population must be completely depleted. The antigens recognized by the MAbs cannot be coexpressed on the same effector subpopulations. If the antigens are coexpressed, triple staining should be done using two other MAbs—one FITC-conjugated and the other PE-conjugated—to distinguish the two subpopulations and their binding to the target cells. The presence of the shared antigen in both populations is then detected using an argon laser and a third MAb labeled with a fluorochrome, such as PerCP and Red 670, that has an emission >600 nm. Different MAbs belonging to the same cluster should be pretested to avoid using antibodies that increase or inhibit effector-to-target cell binding. For example, when using cells that express the Fc receptor, $F(ab')_2$ MAbs must be used to avoid antibody-mediated binding. Finally, the brighter the fluorescence of the MAb, the better the measurement of binding.

Anticipated Results

The behavior of effector and target cells can be detected in flow cytometry on the basis of scatter signals. This method has an advantage because of the relative difference in signal intensity between lymphocytes and target cells (K562). Lymphocytes are morphologically one of the simplest cells in the organism, consisting of a nucleus and a little cytoplasm, and their signal is restricted to a specific area in both forward and side scatter. In contrast, all cells derived from peripheral tumors and cell lines are much larger and present a large cytoplasmic compartment enriched with granules; this enhances their side scatter and autofluorescence signals (see Fig. 9.10.1). In the binding assay, autofluorescence of target cells plays a major role in detecting conjugates and assessing the type of effector cell present. This assay was designed to identify the lymphoid subpopulation able to bind to targets in a multiparametric analysis, including small subsets of lymphocytes which can only be distinguished by dou-

ble staining with directly labeled monoclonal antibodies.

The discovery of new 488 nm–excitable fluorochromes, such as RED 613, that emit in the far-red region of the spectrum has given a further boost to the application of this technique by allowing the use of MAbs in triple-staining experiments to discriminate between three different effector populations in a single experiment.

Because the targets are clearly distinguishable on the fluorescence cytogram (Fig. 9.10.2), a two- or three-color analysis is best when effector analysis is based on two antigens that do not overlap (Fig. 9.10.2). A third antigen could be one that is partially represented in the subset defined by the first two monoclonal antibodies. The third antigen could also be one that displays a dim fluorescence only just overlapping the autofluorescence region of the target cells.

Evaluation of binding values can be obtained directly from the fluorescence cytogram for the first two subsets by gating the whole subset population (bound and unbound lymphocytes; Fig. 9.10.2) then extracting the value of the bound ones (Fig. 9.10.3).

Time Considerations

Lymphocyte sample preparation takes ~2 hr. Target cell preparation takes only a few minutes. The whole binding process takes just over an hour. Immunophenotyping takes ~1 hr. Flow cytometry analysis takes ~5 min per sample.

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