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Fluorospot Assay

Methodological Analysis

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Summary

The conventional enzyme-linked immunospot (ELISPOT) technique detects only one secreted cytokine, which constitutes a major pitfall for the accurate characterization of the various T-cell subpopulations. We have therefore developed a Fluorospot assay, which is a modification of the ELISPOT and is based on the use of multiple fluorescent-labeled anticytokines detection antibodies. A special automated ELISPOT reader consisting of a light microscope with incident fluorescence illumination and an integrating digital color camera has been adapted for this technique. This technique has been applied for the analysis of subpopulations of T-cells and polarized antigen-specific T-cells.

Key Words : ELISPOT; cytokines; T-lymphocytes; fluorospot.

1. Introduction

Upon activation, T-lymphocytes up-regulate expression of and secrete a number of cytokines ***(1)***. The enzyme-linked immunospot (ELISPOT) assay is based on solid-phase immunoenzyme technology ***(2)***. This test allows the detection of functionally specific T-cells secreting cytokines at a single cell level. When T-cells are incubated in the plates, the cytokines released are directly bound by capture antibodies; therefore, cytokines are not diluted in the culture supernatant or bound by cytokine receptors present in the supernatant or adjacent cells; this may explain the higher sensitivity of this technique compared with ELISA ***(3,4)***. However, in most cases the ELISPOT procedure detects only one secreted cytokine; this constitutes a major drawback for the characterization of the various T-cell populations, which are identified by their profile or co-expression of cytokines ***(5,6)***. Recent studies indicate that the

From: *Methods in Molecular Biology, vol. 302: Handbook of ELISPOT: Methods and Protocols*
Edited by: A. E. Kalyuzhny © Humana Press Inc., Totowa, NJ

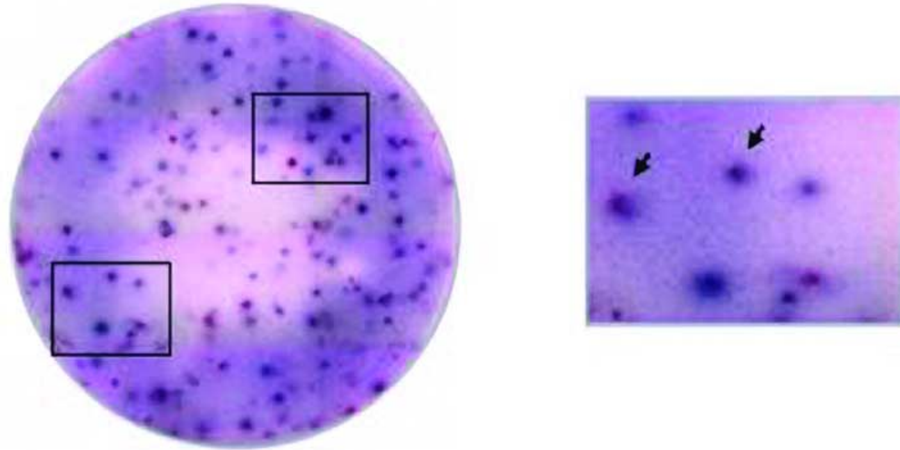

Fig. 1. Dual-color immunoenzymatic ELISPOT for the detection of IFN-γ and IL-4 producing cells. Left, B.EBV cells and a TH2 T-cell clone were mixed and activated with PMA–ionomycin. IFN-γ and IL-4 derived from secreting cells were detected using substrates specific for horseradish peroxidase (3-amino-9-ethylcarbazole, $C_{14}H_{14}N_2$) or alkaline phosphatase (5-bromo-4-chloro-3-indolylphosphate/Nitroblue tetrazolium chloride), respectively. Enzymes were linked to detection antibodies for IFNγ and IL-4. Red spots corresponded to IFN-γ secreting cells, whereas blue spots belong to IL-4 producing cells. Right: Greater enlargement of a quadrant from the left. The arrows showed the difficulties in the interpretation of mixed color spots. *See* **Color Plate X**, following page XXX.

detection of double interferon (IFN)-γ-interleukin (IL)-2-producing T-cells provides additional clinical information regarding the prognosis of patients with human immunodeficiency virus than enumeration of IFN-γ– or IL-2-secreting T-cells alone ***(7–10)***.

As others and we have experienced, attempts to develop an immunoenzymatic dual-color ELISPOT failed because of difficulties in the interpretation of mixed color spots (**Fig. 1**; **refs. *11,12***). Therefore we have developed a fluorospot assay, which is based on a modification of the ELISPOT. The fluorospot assay is based on the use of multiple fluorescent-labeled anticytokine detection antibodies. This assay clearly provides better discrimination and characterization of double cytokine-producing cells than does an enzymatic reaction. This technique allows for the detection of regulatory T-cells and polarized type 1 and type 2 specific tetanus toxoid T-cells ***(13)***. The availability of a large range of fluorophores should permit the extension of this technique to multiparameter analysis.

2. Materials

1. Ethanol (Merck-Eurolab-Polylabo, Denmark).
2. 96-Well polyvinylidene diflouride flat bottom plates (Millipore, Molsheim, France).
3. Cells were maintained in AIM V medium (Gibco-Life Technologies, Paisley, Scotland).
4. Tween 20 (Merck, Schardt, Germany).
5. Phosphate-buffered saline (PBS), pH 7.2–7.4 (Gibco-Life Technologies).
6. Anticytokine antibodies (*see* **Note 1**): Mouse IgG1 anti-IFN-γ (clone B-B1), mouse IgG1 anti-IL-2 (clone B-G5), fluorescein-labeled mouse anti-IFN-γ (clone BG-1), and biotinylated rabbit polyclonal anti-IL-2. All the antibodies were obtained from Diaclone (Besançon, France).
7. Secondary reagents for amplification step: anti-fluorescein rabbit IgG labeled with Alexa Fluor 488 (Molecular Probes, Eugene, OR), biotinylated goat anti-rabbit IgG (Southern Biotechnology), and phycoerythrin-conjugated streptavidin (Dako, Trappes, France). Fluorophores labeled conjugates are stored in aliquots undiluted at 4°C and protected from light.
8. Equipment required to read the fluorescence spots. For evaluation, we used an automated ELISPOT reader (KS ELISPOT, Carl Zeiss Jena) equipped with a light microscope (Axioplan 2 imaging mot, Carl Zeiss Jena) with incident fluorescence illumination (double band path fluorescence filter for FITC and Rhodamine), motor stage and automatic focusing unit.

3. Methods

To illustrate this technique, we describe a double-color fluorospot to detect IFN-γ and IL-2, but this technique also can be applied for monocolor fluorospot or for the detection of other cytokines.

1. Ninety-six well polyvinylidene diflouride (PVDF) flat-bottom plates (Millipore, Molsheim, France) were first treated with ethanol 70% for 10 min at room temperature (*see* **Notes 2** and **3**).
2. The plates then were washed three times with PBS; this step increases the binding efficiency of the plates.
3. The plates were then coated overnight at 4°C with 100 µL of mouse monoclonal anti-IL-2 and anti-IFN-γ antibodies in PBS at 10 µg/mL.
4. The plates were then blocked with 2% milk in PBS for 2 h at room temperature and washed twice with PBS containing 0.05% Tween 20. Less background was obtained when milk was used compared with medium that contained serum.
5. Cells diluted in AIMV medium in a volume of 100 µL were then added in serial dilutions in triplicate and were incubated for various times ranging from 18 h to 48 h at 37°C in a humidified atmosphere of 5% CO_2 in air (*see* **Notes 4** and **5**). We demonstrated that for human peripheral blood mononuclear cells, the plates were saturated at a concentration 2×10^5 cells and, therefore, it is not recommended to exceed this number of cells in the plates.

6. Then cells were removed and the plates were incubated with PBS containing 0.05% Tween 20 for 10 min to lyze all the remaining cells. This step was followed by three washes with PBS containing 0.05% Tween 20.
7. For the detection of IFN-γ and IL-2, 100 μL of a fluorescein labeled mouse monoclonal anti-IFN-γ antibody (2 μg/mL) and 100 μL of biotinylated rabbit polyclonal anti-IL-2 (1.5 μg/mL) were added for 1.5 h at 37°C in a place protected from light (*see* **Note 6**). In general, the concentrations of the detection antibodies are always lower than that used for the capture antibodies.
8. For IFN-γ the signal was amplified with 15 μg/mL of an anti-fluorescein rabbit IgG conjugated with Alexa Fluor 488 and, for IL-2, the reaction was detected with 15 μg/mL of phycoerythrin-conjugated streptavidin. All the incubations were performed in a place protected from the light. It is necessary to avoid using anti-fluorescein rabbit IgG conjugated to fluorescein to prevent the formation of auto-aggregates of this antibody.
9. Images were taken with an integrating digital color camera (Axiocam MRc, Carl Zeiss Jena; *see* **Note 7**), which allows one to record high-resolution images. This camera, an alternative to an analog color camera, can be adjusted to an optimum fluorescence exposure time directly from the evaluation software. Various settings for different experiments may be saved in their own configuration files and easily recalled when necessary. **Figure 2** illustrates a dual-color fluorospot for IL-2 and IFN-γ recorded using this equipment.
10. After the digitization, the KS ELISPOT software proceeded with automatic data processing. In the user interface, the user elements are reduced to a minimum. Only four buttons are needed for routine evaluation. Finally, all data were transferred directly into a spreadsheet program for further evaluation and graphical display. The system settings were tested before the evaluation of a complete plate with the "check well" function. In this mode, the spot recognition can be adapted with the "teach" function, or a new configuration setting where multiple evaluation patterns can be defined.
11. Finally, the evaluation of the entire plate was started. The stage was moved to the center of the first well position and the auto-focus initiated. Then, the well position was scanned in a meander mode. From all fields, a complete well image was generated and displayed in a reduced mode in the image field for user control. The result was displayed in the overlay (spot indication and spot diameter). The stage moved to the next well position and the sequence repeated until the last well was evaluated. All rejected positions were skipped. After the evaluation of the last well position, the stage will automatically return to the start position. A detailed description of the evaluation software is given in Chapter 8 in this volume.

4. Notes

1. The use of antibodies without azide and with low endotoxin levels may help to reduce background levels when plate is developed.
2. The PVDF flat-bottom plates gave the best results when compared to plastic plates. This technique did not require the use of black plates.

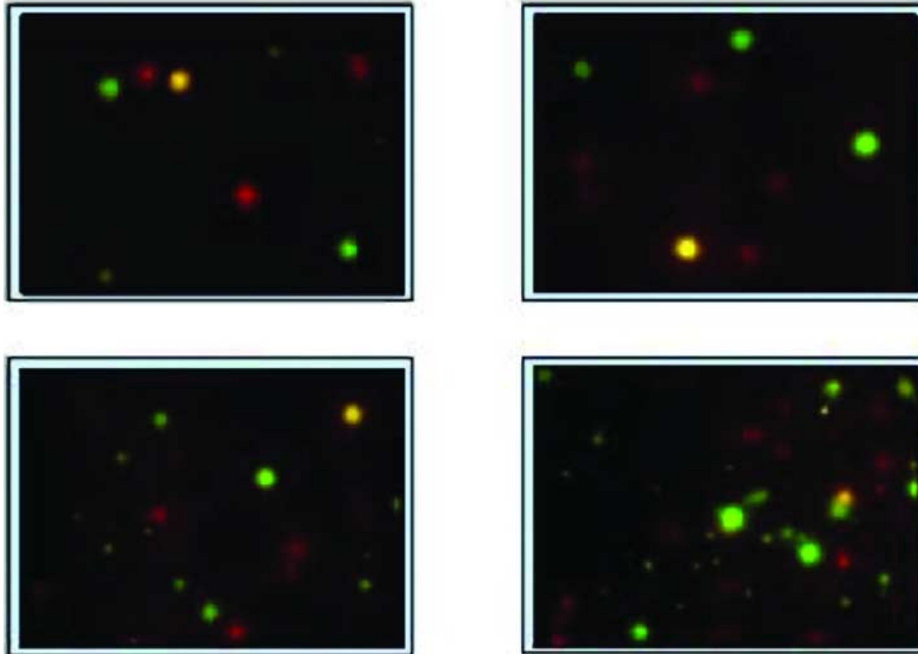

Fig. 2. Dual-color fluorospot for the detection of IL-2 and/or IFN-γ– producing cells. Peripheral blood mononuclear cells were stimulated with PMA and ionomycin in PVDF plates. IFN-γ- and/or IL-2-producing cells were characterized by a dual-color fluorospot assay. Green spots corresponded to IFN-γ secreting cells, whereas red spots belong to IL-2-producing cells. Yellow spots corresponded to cells coexpressing IFN-γ and IL-2. No spots were observed when non-stimulating cells were used for the dual-color fluorospot. *See* **Color Plate X**, following page XXX.

3. When dual-color fluorospot is performed, competition between capture antibodies may occur and this will introduce bias in the detection of spots. This difference may be related to the variable efficiency of antibodies binding to PVDF membrane. When antibodies are used for the first time, to ensure that the same concentrations of antibodies directed against IL-2 or IFN-γ were bound, we simultaneously tested the frequency of IL-2 and IFN-γ-producing cells with single color or double color fluorospot procedure. The number of cells producing IFN-γ and IL-2 in the single-color fluorospot assay has to match the sum measured with the double-color fluorospot.
4. Medium with fetal calf serum or human serum may non specifically activate the cells; therefore, we recommend medium without serum (either AIMV [Gibco] or X-Vivo [Cambrex]).
5. Cell incubation times vary depending on the cytokine to be detected. For IL-2 or IFN-γ, times ranges from 10 to 18 h. On the contrary, for the detection of IL-10 or IL-12, cells may need to be incubated up to 48 h.

6. Amplification system: when directly labeled anti-cytokine detection antibodies were used without amplification, fluorescence was not detectable. During dual-color fluorospot, one must be aware of crossreactivity between secondary and/or primary antibodies. Therefore, we recommend that one use species depleted antibodies and Fab'2 antibodies. For the detection of some cytokines a supplementary amplification step further increases the signal. For example, when detecting of IL-2, an amplification step with 100 μL of biotinylated goat anti-rabbit IgG for 1.5 h at 37°C could be added. During the double IFN-γ/IL-2 fluorospot assay, we first incubated the cells with the biotinylated goat anti-rabbit IgG and phycoerythrin-conjugated streptavidin, and the anti-fluorescein rabbit IgG conjugated with Alexa Fluor was added in a second step, after three washes, to avoid cross reactivity between the anti-rabbit IgG and the anti-fluorescein rabbit IgG. For the detection of IL-10, we use mouse IgG1 anti-IL-10 antibodies for the capture, biotinylated mouse IgG 2b anti-IL-10 and, in that case, the amplification step may include a biotinylated rabbit anti-mouse IgG 2b (Rockland, Gilbertsville)
7. Because the fluorescence illumination does not cross the PVDF membranes, the light from illuminator has to come from the top of the plates.

Acknowledgments

This work was supported by grants from Fondation de France, Agence Nationale de Recherche sur le SIDA (RIVAC program), Ligue Nationale Contre le Cancer, Association pour la Recherche sur le Cancer, INSERM (ATC Biotherapy), and the Canceropole.

Benoit Vingert is a fellow of the Fondation de France.

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