Computer-Assisted, Quantitative Cytokine Enzyme-Linked Immunospot Analysis of Human Immune Effector Cell Function

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ABSTRACT

Originally developed for detecting antibody production from B lymphocytes, the enzyme-linked immunospot (ELISPOT) assay was later modified to assess cytokine production from various immune effector cells. Although the ELISPOT assay can detect antibody or cytokine production at the single-cell level, the visual counting of spots in a 96-well plate under a microscope makes this method unsuitable for handling large sample sizes. Here, we introduce a computer-assisted image analysis system to overcome this problem. This system makes the data analysis step of the ELISPOT assay convenient, objective, sensitive and suitable for handling large sample pools. Studies requiring lymphocyte proliferation assay, cytotoxic lymphocyte assay and precursor frequency assay can be conducted through the ELISPOT assay. This is demonstrated here using examples such as mixed lymphocyte allogeneic reactions and human immunodeficiency virus antigen-specific, cell-mediated immune responses.

INTRODUCTION

Enzyme-linked immunospot (ELISPOT) assay, first developed to quantitatively analyze specific antibody-secreting cells (7,10,13), was later modified for the detection of cytokine-secreting cells (5). Since cytokine secretion is important for the modulation of distinct host immune function [for example, secretion of interferon gamma (IFN- γ) and Interleukin-12 (IL-12) directs Th1 development, while secretion of Interleukin-4 (IL-4) and Interleukin-10 (IL-10) directs Th2 development (11)], assessing immune responses at the single-cell level is critical to the study of the development of immunity. The ELISPOT assay has been demonstrated to be useful in such applications (9,14). The commonly used methods for evaluating immune responses include cell proliferation and cytotoxic lymphocyte (CTL) assays. These methods, howev-

er, do not determine the frequency of effector responses to specific antigens unless a more tedious limiting-dilution step is included. In contrast, ELISPOT has been adapted in convenient protocols to determine precursor frequency and has been shown to work equally as well as the conventional limiting-dilution method (9). Despite the advantages of being easy, quantitative and nonradioactive, ELISPOT has been confined to small sample sizes, mainly because of the necessity for laborious enumeration of colored spots in 96-well plates under the microscope. Thus, further improvements to data acquisition are critical to the wide application of ELISPOT for immunological studies.

The purpose of this study is to establish a simple and reliable procedure for the enumeration of ELISPOT results. Using an IFN- γ ELISPOT assay, we examined two different immune responses, allogeneic mix lymphocyte reaction and human immunodeficiency virus type 1 (HIV-1) antigen-specific response. The specificity and sensitivity of the ELISPOT assay in these applications are demonstrated. By adapting a computer-assisted, video-imaging analysis system to the enumeration process, we present here an improved ELISPOT protocol that is very convenient, objective and reproducible.

MATERIALS AND METHODS

Cells

AA-2 is derived from the WIL-2 human splenic Epstein Barr Virus (EBV) + β -lymphoblastoid line, which expresses high levels of CD4 (2). AA-2 was obtained from the AIDS Research and Reference Reagent Program (National Institutes of Health [NIH], Bethesda, MD, USA) and maintained at 37°C, 5% CO₂ in 1640 RPMI medium supplemented with 10% fetal calf serum (FCS), 0.1 mg/mL penicillin-streptomycin, 2 mM L-glutamine (all from Life Technologies, Gaithersburg, MD, USA), 1 mM nonessential amino acids and sodium pyruvate (both from Sigma Chemical, St. Louis, MO, USA). Human peripheral blood lymphocytes (PBLs) were obtained from healthy or repeatedly HIV-1-exposed seronegative donors and separated by Ficoll[®] gradient as previously reported (3) and frozen in liquid nitrogen until use.

Separation of Subsets of Human Lymphocytes

PBLs were thawed at 37°C and used only if the viability was higher than 90%. They were incubated with mouse anti-human CD4-, CD8-, CD56- or mouse IgG₁-labeled BioMeg[®] magnetic beads (PerSeptive Biosystems, Framingham, MA, USA) at 50 beads per cell on ice for 30 min. Cell depletion was carried out by sorting the cells on a magnet for 5 min twice, using mouse IgG₁ sorting as a control. After sorting, cells were washed with medium and seeded with either stimulator or target cells in each experiment as described below.

Preparation of Stimulators and Target Cells

We used AA-2 as stimulator or target cells in the allogeneic reaction. For stimulation, AA-2 was treated with 5 µg/mL of mitomycin C (Bristol, Montreal, Canada) for 2.5 h, washed with phosphate-buffered saline (PBS) three times and seeded at 5×10^4 cells per well in a round-bottomed, 96-well plate together with various subsets of T cells. The T cells were prepared as described above from 2×10^2 to 5×10^3 per well in triplicates in RPMI 1640 containing 10% FCS and 20 U/mL of IL-2 (Boehringer Mannheim Canada, Laval, QC, Canada) in a total volume of 200 µL. The cells were incubated at 37°C, 5% CO₂ for 3 days before being used in the ELISPOT assay. To prepare target cells for the detection of HIV-1-specific effector function, PBLs from either healthy or HIV-1-exposed but uninfected donors were treated with phytohemagglutinin (PHA) (5 µg/mL; Sigma Chemical) for 2 days and infected with wild-type HIV-1 at multiplicity of infection doses of 0.2 for 3 h. After infection, PBLs were incubated in RPMI 1640 containing 10% FCS in the presence of IL-2 (20 U/mL) for 5 days. HIV-1 infection was confirmed by reverse transcriptase assay as reported previously (3). The infected PBLs were frozen in liquid nitrogen for later use. PHA-stimulated but uninfected PBLs of the same individual were used as a negative control.

ELISPOT Assay

The ELISPOT assay consists of five steps, which were carried out over four consecutive days similar to previously reported methods (5) with the following modifications: **Day 1:** 96-well nitrocellulose-bottomed plates (MultiScreen[®]-HA; Millipore, Mississauga, ONT, Canada) are coated with 70 µL/well mouse anti-human IFN-γ monoclonal antibody (MAb) (10 µg/mL; PharMingen, San Diego, CA, USA) and incubated at room temperature overnight. **Day 2:** The wells are washed 2 times with PBS and incubated at 37°C for 1 h with 100 µL/well of RPMI 1640 containing 10% FCS. The cells of interest are then added to each well to a total volume of 200 µL as follows. For determining the allogeneic response, 1×10^5 mitomycin C-treated AA-2 in 100 µL of RPMI 1640 containing 10% FCS and IL-2 (20 U/mL) were added to each well. Then, the PBLs, which had been incubated for 3 days with stimulators (AA-2), were directly transferred from the round-bottomed, 96-well plate to the nitrocellulose-well plate in 100 µL and incubated at 37°C, 5% CO₂ for 24 h. Control wells contained either AA-2 or PBLs alone in the presence of IL-2, which did not interfere with IFN-γ production. HIV-1-specific immune responses were studied

using unstimulated, frozen PBLs (4×10^4 to 1×10^6), which were obtained from seronegative HIV-1-exposed, high-risk or unexposed, low-risk donors. In each well, the thawed effector PBLs were incubated with 1×10^5 mitomycin C-treated autologous HIV-infected PBLs in a total volume of 200 µL for 24 h. The uninfected, autologous PBLs were used as controls.

Day 3: Each well is washed with PBS/T (0.05% Tween[®] 20) 4 times and blocked with 100 µL of 20% FCS in PBS/T for 15 min. Then, 75 µL of biotinylated anti-human IFN-γ MAb (2.5 µg/mL; PharMingen) are added to each well and incubated overnight at 4°C. **Day 4:** The wells are washed four times using PBS/T and incubated with 100 µL of peroxidase-labeled streptavidin (1:800 dilution; CALTAG Laboratories, South San Francisco, CA, USA) in PBS for 1 h. After the wells are washed twice with PBS/T and twice with PBS, 100 µL 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma Chemical) solution containing 0.3% NiCl₂ are added for another 15–20-min incubation, followed by four rinses using ddH₂O. After being air-dried, the number of spots in each well is counted by computerized video-imaging as described below.

Enumeration of ELISPOTs by Computerized Video-Imaging

The dried nitrocellulose 96-well plates were placed on top of an illumination box, and images were captured using a Cohu 4910 Series Monochrome CCD Camera, which was connected to an Appligene computer using the program "The Imager[™]" Version 2.03 (Appligene, Pleasanton, CA, USA). The image was optimized by adjusting contrast, grayscale and exposure time and saved in a picture file format [tag image file format (TIFF)] before it was analyzed using the NIH Image Version 1.55 shareware (<http://rsb.info.nih.gov/ni-image>). The readings of 6–24 wells can be captured and saved in one file and processed together. When retrieved, the image was sharpened and/or smoothed by adjusting the grayscale to obtain the best possible resolution. The image was then converted to a binary (black and white) format by setting up a proper threshold. The individual well was selected, and the spots were counted using the "analysis particles" option in the program. The size of particles can be predefined (e.g., 1–9 square pixels at magnification of 6) so that background spots of different size will be automatically excluded.

RESULTS AND DISCUSSION

In a normal immunocompetent individual, the allogeneic antigen stimulation induces a strong cell-mediated immune response, accompanied with marked IFN-γ production. This reaction serves as a good model for the ELISPOT assay. To induce an allogeneic response, unmatched donors' PBLs were mixed with irradiated AA-2 as target cells for three days, and the frequency of the IFN-γ-producing cells (IPCs) in the PBL population was determined by ELISPOT assay as described in the Materials and Methods. The spots formed by IFN-γ-secreting cells in the nitrocellulose-bottomed, 96-well plates when viewed under the stereomicroscope were dark purple in the center and a little lighter in the surrounding

region, similar to previous observation (5). These ELISPOTs were distinct and easily distinguished from the artifact spots, which were smaller, denser and mostly uniform. When the computer video-image system was used, the smaller artifacts did not appear from the background at the magnification used. Figure 1A shows a printout of a fine-tuned computer ELISPOT image processed by the free NIH Image shareware. The well on the left had the target AA-2 alone. The wells in the center and on the right contained 1000 and 200 PBLs, respectively, along with the target AA-2. Using a binary format (Figure 1B), the image of ELISPOTs is easily viewed, appearing as black spots, which can be counted automatically (Figure 1C). The number of IPCs counted using this system is similar to that determined with the aid of a stereomicroscope. This processing system avoids the tedious and unpleasant enumeration experience under the microscope. Most importantly, as both the darkness and minimal and maximal size of the ELISPOT can be predefined using the analysis program, the result uniformly eliminates unwanted artifacts. When compared with manual microscopic counting, the computerized analysis is accurate and objectively distinguishes the smaller artificial spots from the real ones. In addition to the NIH Image program, any picture-oriented program can be used to retrieve and print the captured image.

To determine the sensitivity of ELISPOT assay for immune effector function, a nonspecific allogeneic response was first studied. The PBLs of a healthy donor reacted to AA-2 showed over 1000 IPCs in 1×10^4 PBLs after a 3-day stimulation, and the number of IPCs doubled after a 6-day stimulation. In contrast, the allogeneic immune response of a cancer patient, who had been under chemotherapy two weeks before

the test, was 50 times less than that of the healthy donor. This result demonstrates that ELISPOT is a simple, fast and yet sensitive protocol for assessing the frequency of a strong, nonspecific immune response.

To demonstrate the specificity and sensitivity of the ELISPOT assay, we studied the frequency of HIV-1-specific IPCs in a repeatedly HIV-1-exposed (high-risk) but uninfected individual, compared to that in a control, low-risk individual. We used autologous HIV-1-antigen-presenting cells as the target cells. The secretion of IFN- γ upon encountering HIV-1-antigen-presenting cells would be an indication of prior exposure to HIV-1 (8). The target cells were prepared by infecting autologous PBLs from the corresponding individuals as described in the Materials and Methods. The results of this study indicate that the PBLs from the low-risk donor do not contain any HIV-1-specific responders in up to 1×10^6 T cells. In contrast, the PBLs from the high-risk donor have a precursor frequency of 28 HIV-1-specific responders per 1×10^6 lymphocytes without in vitro stimulation. The existence of HIV-1-specific IPCs in the high-risk donor is in concordance with previous reports that high-risk seronegative indi-

Figure 1. Computer-assisted, image-capturing and enumeration of ELISPOT assay. (A) A retrieved image of ELISPOT using NIH Image Version 1.55. The well on the left is a control, which contains only AA-2. The wells in the middle and on the right received 1000 and 200 PBLs together with AA-2, respectively. (B) A converted binary image by setting up a proper threshold. (C) Computer enumeration of ELISPOTs using the "Particle Analysis" option.

Figure 2. ELISPOT analyses of the contribution of different lymphocyte subsets to IFN- γ production upon nonspecific, allogeneic or HIV-1-specific antigen stimulation. (A) CD4-, CD8- and/or CD56-depleted, allogeneic PBL responses to AA-2 (standard errors for CD56 and CD4, 8, 56 depletion are 0.83 and 0.73, respectively, which do not register in the figure); (B) HIV-1-specific autologous PBL responses of an HIV-1-exposed, seronegative individual.

viduals have developed HIV-1-specific CTL responses (4,10,12,16). While HIV-1-specific CTLs can be detected in HIV-1-infected individuals without in vitro stimulation (15), HIV-1-specific CTLs and the precursor frequencies in high-risk individuals were demonstrated only after in vitro stimulation (1,4,12). This result thus demonstrates that the ELISPOT assay is sensitive enough to detect a weak antigen-specific immune response and to determine the precursor frequency without the additional step of in vitro stimulation.

To study the roles of individual T-cell subsets in the allogeneic and HIV-1-specific immune reactions, we depleted CD4, CD8, CD56 (NK) or all of the three subsets of lymphocytes in a separate experiment and performed the ELISPOT assay. As shown in Figure 2, A and B, all three cell types contributed, albeit to different extents, to IFN- γ production in both the allogeneic and the HIV-1-specific reactions. In the allogeneic reaction, NK cells accounted for about 30%, CD8 for 26% and CD4 for 16% of the IFN- γ production. However, in the HIV-1-exposed but uninfected individual, CD8 cells contributed the most (up to 71%) to the HIV-1-specific IFN- γ secretion; CD4 and NK cells only accounted for 14% and 10%, respectively, of total IFN- γ production. Together these studies demonstrated a quick analysis for the quantitation of IFN- γ -producing cells in antigen-specific and nonspecific immune reactions.

In conclusion, the modified ELISPOT assay with a computer-assisted data acquisition and processing system simplifies the experimental procedures and should be suitable for clinical or experimental assessment of general and specific immune responses, especially when dealing with a large sample size. With these improvements, ELISPOT can be used more conveniently in many immunological studies, including new applications such as establishing bioassays for miscellaneous interleukin functions (Y. Cui and L.-J. Chang, unpublished). Similar techniques can be used to improve a two-color ELISPOT method (7) using a color image-capture system or a pair of appropriate filters with the monochrome image system.

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