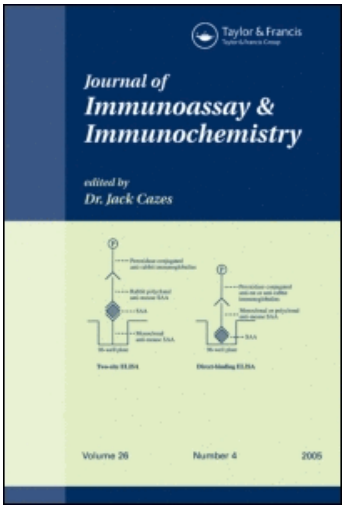

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Yoshihiro Okamoto[a]; Yoshimitsu Gotoh[b]; Yoshiharu Kobayashi[c]; Akira Mizuno[a]; Hirotaka Tokui[a]; Mikio Nishida[a]

[a] Department of Clinical Pharmacy, Faculty of Pharmacy, Meijo University, Tempaku-ku, Nagoya, Japan [b] Department of Pediatrics, Nagoya Daini Red Cross Hospital, Showa-ku, Nagoya, Japan [c] Clinical Chemistry Laboratory, Kobe Pharmaceutical University, Higashinada-ku, Kobe, Japan

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DETECTION OF INTERLEUKIN-12 (IL-12)-SECRETING CELLS IN NORMAL MICE WITH ENZYME-LINKED IMMUNOSPOT (ELISPOT) ASSAY

Yoshihiro Okamoto[1], Yoshimitsu Gotoh[2], Yoshiharu Kobayashi[3], Akira Mizuno[1], Hirotaka Tokui[1], and Mikio Nishida[1].

[1] Department of Clinical Pharmacy, Faculty of Pharmacy, Meijo University, 150 Yagotoyama, Tempaku-ku, Nagoya 468-8503, Japan.
[2] Department of Pediatrics, Nagoya Daini Red Cross Hospital, 2-9 Myoken-cho, Showa-ku, Nagoya 466-8650, Japan.
[3] Clinical Chemistry Laboratory, Kobe Pharmaceutical University, 4-19-1, Motoyamakita-machi, Higashinada-ku, Kobe 658-8558, Japan.
e-mail: yokamoto@meijo-u.ac.jp

ABSTRACT

To assess mice interleukin-12 (IL-12)-secreting cells at a single cell level, we have developed a murine IL-12 specific enzyme-linked immunospot (ELISPOT) assay. The application of the newly developed method clearly showed the frequency of IL-12-secreting cells in the resident peritoneal exuded cells was higher than other organs of normal DBA/1J mouse. Moreover, we determined the frequency of IL-12-secreting cells in the spleens of five strains of inbred mice, and found the incidence of IL-12 secretors in the strain C57BL/6 to be greatest, and significantly greater than four of the others. These results are compatible with the predicted evidence, supporting this ELISPOT assay for IL-12-secreting cells is accurate. The procedure provides a useful tool for investigating complicated immune responses at a single cell level. (KEY WORDS: interleukin-12, ELISPOT assay, Th1, Th2, mice)

INTRODUCTION

Interleukin-12 (IL-12) is a pleiotropic cytokine that is produced by antigen presenting cells (APCs), macrophages, and B cells (1-4). IL-12 promotes the growth of activated T cells and natural killer (NK) cells (1-4). IL-12 selectively induces the development of naive T helper (Th) cells into Th1 cells and stimulates Th1 type cytokine production (5-7). The significance for IL-12 in the pathogenesis of several diseases has been reported. For example, experimental allergic encephalomyelitis (EAE) (8) and acute graft versus host diseases (9) are prevented by administration of anti-IL-12 antibody.

The enzyme-linked immunospot (ELISPOT) assay is an efficiently sensitive technique for the enumeration of cytokine- or immunoglobulin-secreting cells at the single cell level (10). This technique has been modified by the author's group (11, 12) and other investigators to detect various cytokines (13-16). However, the IL-12-secreting cell has never been analyzed with ELISPOT assay.

In the present study, we have developed a highly specific ELISPOT assay for IL-12. We applied the improved assay system to detect IL-12-secreting cells in various organs of normal DBA/1J mice, and to investigate the differences in the frequencies among several strains of inbred mice.

MATERIALS AND METHODS

Animals

Specific-pathogen-free DBA/1J, DBA/2, BALB/c, C3H/He, and

C57BL/6 male mice were obtained from Charles River Japan Co. (Tokyo, Japan). The mice were maintained in laminar flow cabinets. Sterile food and water were provided ad libitum. All animals were 8 - 9 weeks of age when used.

Cell preparation

Spleen cells were prepared as described previously (11). The spleen was aseptically removed and mechanically disrupted. A dispersed-cell suspension was filtered through a mesh (150 mesh), and red blood cells (RBC) were lysed with RBC lysis solution (0.83% NH_4Cl, 0.017mol/l Tris-hydroxymethyl-aminomethane, pH 7.65). After washing three times with RPMI 1640 medium (Nissui Co. Ltd., Tokyo, Japan), spleen cells were resuspended in the medium supplemented with 10% heat-inactivated fetal bovine serum (FBS, GIBCO BRL Co. Ltd., Grand Island, NY, USA). The lymph node cells were prepared by essentially the same method as that for spleen cells. The peritoneal exuded cells were harvested by peritoneal lavage with 8 ml of RPMI 1640 medium. After washing three times with the same medium, the peritoneal exuded cells were resuspended with 10% FBS.

IL-12 specific ELISPOT assay

Sterile 96-well nitrocellulose-based microtiter plates (Multi Screen®-HA plate, Millipore Co., Bedford, MA) were coated with an anti-mouse IL-12 p35 monoclonal antibody (mAb) (5 μg/ml, Genzyme, Cambridge, MA, USA) in 0.1 mol/l bicarbonate buffer (pH 8.2), washed

with phosphate buffer saline (PBS) containing 0.05% Tween 20 (PBS-T), and then blocked with 5% bovine serum albumin (BSA, Sigma Chemical Co. St. Louis, MO, USA) in PBS for 2 hours at room temperature. Pooled cells resuspended in RPMI 1640 supplemented with 10% FBS were inoculated into individual wells ($1x10^3$ to $1x10^6$ cells/100μl/well) and, were incubated for 18 hours at 37°C in a humidified atmosphere of 5% CO_2 in air. The plate was thoroughly washed with PBS-T and incubated overnight at 4°C with 100 μl of biotinylated anti-mouse IL-12 p70/p40 (2 μg/ml, Genzyme, Cambridge, MA, USA) in PBS-T containing 0.5% BSA. The plate was thoroughly washed and further incubated with avidin conjugated horseradish peroxidase (diluted 1:2,000, GIBCO BRL., Grand Island, NY, USA) in PBS-T containing 0.5% BSA for 2 hours at room temperature. Plates were rinsed as above. After rinsing, the wells were exposed to 100 μl of aminoethylcarbazole (AEC) / H_2O_2 substrate solution (Vector Laboratories, Inc., Burlingame, CA, USA) and examined for red spots to identify IL-12. Stained spots of IL-12 secreted by individual cells were enumerated under low magnification (X 40) with a microscope. The cell specimen was sequentially diluted to detect appropriate numbers of the spots in a well, and the conditions to produce 10 - 50 spots/well were used to count the total number of cytokine-secreting cells per sample, except for the IL-12-secreting cell-rich organs, where the condition up to 100 spots/well was applied. Stained artifacts were easily distinguished from the spots of secreted cytokine due to the size, stainability and density of contaminated objects.

RESULTS

Determination of IL-12-secreting cells

To identify individual IL-12-secreting cells, the optimal conditions for IL-12 specific ELISPOT assay were examined by some preliminary experiments. Spleen cells from normal DBA/1J mice, which produce IL-12 constitutively (17), were subjected to the assay system. When the number of inoculating cells in a well were increased, the developed spots were also increased (Figure 1). When the population of $1x10^5$ cells was inoculated in a well, the most adequate number of spots to count was obtained. Thus, the following experiments were carried out using $1x10^5$ cells inoculation.

Specificity

Whether or not the newly developed ELISPOT assay is specific for an IL-12-secreting cell, was tested. First, the specificity of both the coating mAb and the detection mAb to mouse IL-12 was examined by enzyme-linked immunosorbent assay (ELISA) with recombinant mouse IL-12 as a positive control and other types of recombinant cytokine (mouse IL-1β, IL-2, IL-4 and IL-6) as negative controls. The ELISA assay using a combination of these antibodies was only positive with mouse IL-12 in a dose dependent manner, and was negative when tested with other cytokines (data not shown). Second, anti-mouse IL-12 mAb was replaced with anti-mouse IL-4 mAb (clone BVD6-24G2, Pharmingen, CA, USA) as an unrelated antibody. No IL-12 specific spots were formed (data not shown). Third, as stated before, when the

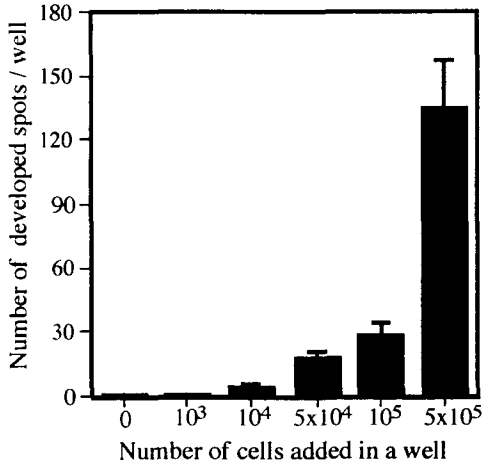

FIGURE 1. Evaluation of IL-12-secreting cells. Various numbers of spleen cells were inoculated into anti-IL-12 antibody-coated well, and subsequently spots developed as described in Materials and Methods. Each bar represents the mean values ± SD in 6 experiments.

number of inoculating cells in a well were increased, the spot formation also increased in parallel fashion (Figure 1). Fourth, after the spleen cells were pretreated with 0.5 mM cycloheximide for 2 hours, the treated cells were tested. The treatment with cycloheximide reduced the number of IL-12-secreting cells by 80 percent from control. The concentration of cycloheximide did not affect cell viability which was assessed by trypan blue dye exclusion test.

To demonstrate the accuracy of the detection procedure, we applied the new method to the two following investigations.

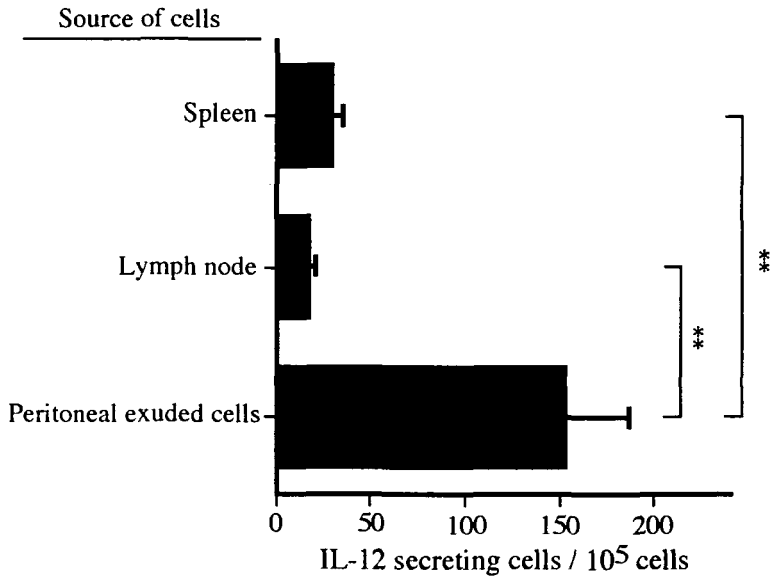

FIGURE 2. Detection of IL-12-secreting cells in DBA/1J mice. The number of cells secreting IL-12 per 10^5 spleen, lymph node and peritoneal exuded cells was measured in normal DBA/1J mice by the IL-12 specific ELISPOT assay. The results represent the mean ± SD in 8 experiments, consisting of the pooled specimens from 3 to 4 mice in each experiment. Significant difference was determined by Kruskal-Wallis non-parametric one-way analysis of variance and Scheffé's F test. **$p<0.01$.

Detection of a major site of IL-12 production

Single cell suspensions were prepared from the spleen, lymph node, and resident peritoneal exuded cells of normal DBA/1J mice. The results in Figure 2 show the population of detected IL-12-secreting

cells in each tested cell suspension. The estimated frequency of IL-12-secreting cells under physiological conditions was 153 ± 43 cells / 10^5 resident peritoneal exuded cells, 29 ± 5 cells / 10^5 spleen cells, and 17 ± 4 cells / 10^5 lymph node cells. The frequency of IL-12-secreting cells in peritoneal exuded cells was significantly greater than the other cells we tested.

IL-12-secreting cells of several mouse strains

The frequency of IL-12-secreting cells in various strains of mice were determined. Single cell suspensions were prepared from the spleen of normal DBA/1J, DBA/2, BALB/c, C3H/He and C57BL/6 male mice. The results in Figure 3 show the population of IL-12-secreting cells in the spleen of each strain. The estimated frequency of IL-12-secreting cells under physiological conditions was 29 ± 5 cells / 10^5 cells in DBA/1J, 40 ± 5 cells / 10^5 cells in DBA/2, 34 ± 5 cells / 10^5 cells in BALB/c, 29 ± 5 cells / 10^5 cells in C3H/He, and 48 ± 8 cells / 10^5 cells in C57BL/6 mice. The frequency of IL-12 secreting cells in C57BL/6 mice was relatively higher than in the other strains of mice.

DISCUSSION

The purpose of the present study was to identify and enumerate cells producing IL-12 in vivo. To this end, we developed an IL-12 specific ELISPOT assay system. Czerkinsky et al. were the first to

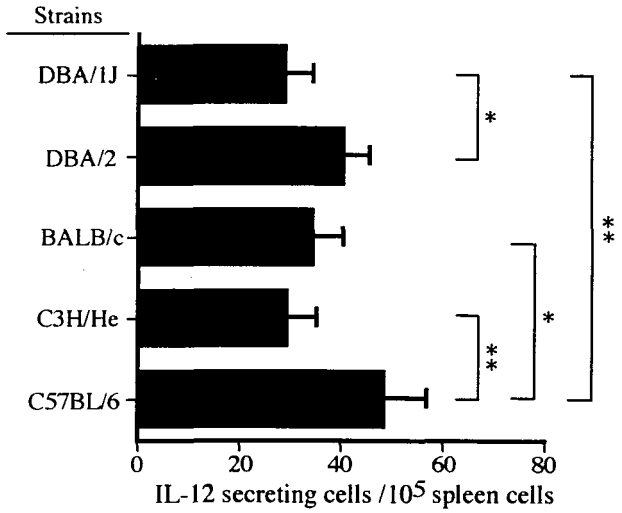

FIGURE 3. The frequency of IL-12-secreting cells from several mouse strains. Spleen cells obtained from five different strains of mice were subjected to the IL-12 specific ELISPOT assay. The results represent the mean ± SD in 6 - 8 experiments, consisting of the pooled specimens from 3 to 4 mice in each experiment. Significant differences were determined by Kruskal-Wallis non-parametric one-way analysis of variance and Scheffé's F test. **$p<0.01$, *$p<0.05$.

describe a new assay system for the detection and enumeration of specific antibody-producing cells at the single cell level (18). This technique is a modification of an ELISA, and is commonly known as an ELISPOT assay. The ELISPOT assay has been modified by other investigators including ourselves to detect several cytokines (11-16). The specificity of our developed ELISPOT assay was characterized by

various results that were described in the text: the stained spot formation required the right combination of antibodies. And, treatment with cycloheximide reduced the IL-12 specific spot formation by spleen cells due to the inhibition of the de novo synthesis of protein.

Single cell suspension prepared from several organs in normal male mice under physiological conditions was applied to enumerate the frequency of IL-12-secreting cells (Figure 2). We revealed that resident peritoneal exuded cells were the major source of IL-12 secretion in all preparations we tested. The IL-12- secreting cells were also detected in spleen and lymph node. This result was the first evidence of comparing frequencies of IL-12-secreting cells in various organs. The results obtained were consistent with evidence reported by other methods : 1) macrophages/monocytes were major sources of IL-12 production (1-4); and, 2) macrophages/monocytes and lymphoid organs constitutively expressed mRNA encoding IL-12 p70 (17).

It was reported that mouse genotype affects cytokine production (19-21). For example, C57BL and BALB/c mice were considered to be Th1- and Th2- dominant strains, respectively (19). Spleen cells obtained from mice of five different inbred strains were subjected to the IL-12 specific ELISPOT assay (Figure 3). The result clearly demonstrated the constant frequency of IL-12-secreting cells in the spleen. The frequency of IL-12-secreting cells in C57BL/6 mice was significantly higher than that of BALB/c mice. IL-12 was a cytokine that induced Th1-dominant immune responses. The differences in

frequency of IL-12-secreting cells could be one of the causes for the dominance of Th-cell in C57BL/6 versus BALB/c mice.

IL-12 has attracted the attention of many researchers since it inhibits the growth of a variety of experimental tumors in vivo and has anti-angiogenic effects in vivo (22). IL-12 seems to be a potential candidate for the treatment of some types of tumors. Thus further research for IL-12 is needed. The assay system we have developed in this laboratory should be one of the useful tools for progress on this research.

In summary, we have developed a valuable method to differentiate cells secreting IL-12 at a single cell level. The application of the method clearly showed the frequency of IL-12-secreting cells in several organs of normal mouse. Moreover, we determined the frequency of IL-12-secreting cells in the spleen of several inbred mice. All the results obtained confirmed its consistency with the previous evidence from other procedures. This procedure provides a useful tool for investigating complicated immune responses at a single cell level.

Correspondence to : Yoshihiro Okamoto, Department of Clinical Pharmacy, Faculty of Pharmacy, Meijo University, 150 Yagotoyama, Tempaku-ku, Nagoya 468-8503, Japan.
FAX: +81 52 834 8780, e-mail: yokamoto@meijo-u.ac.jp

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