Development of Fluorescence-linked Immunosorbent Assay for High Throughput Screening of Interferon-γ

Eiji Matsukuma1, Zenichiro Kato1, Kentaro Omoya1, Kazuyuki Hashimoto1, Ailian Li1, Yutaka Yamamoto1, Hidenori Ohnishi1, Hidenori Hiranuma2, Hisakazu Komine2 and Naomi Kondo1

ABSTRACT
Background: Human interferon-gamma (hIFN-γ) is produced by lymphocytes and has a variety of biological properties. Measurement of hIFN-γ is widely used for various immunological responses for allergic or autoimmune diseases. Enzyme-linked immunosorbent assay (ELISA) is an established immunoassay used to quantify cellular metabolites or cytokines. ELISA requires many incubation and wash steps and is not practically suitable for screening large numbers of samples.

Methods: We have developed a fluorescence-linked immunosorbent assay (FLISA) method for the detection of hIFN-γ. We measured the 50% inhibitory concentration (IC50) value of the hIFN-γ production by interleukin (IL)-18 binding protein and anti-IL-18 monoclonal antibody. The IC50 described by FLISA was compared with that by ELISA.

Results: We developed a new system for measuring hIFN-γ using Allophycocyanine (APC) fluorescent protein and compared it with the previous method using Cy5.5. The proposed FLISA had a smaller coefficient of variation than ELISA, and the means of coefficient of variation using the same samples measured by ELISA and FLISA were, respectively, 11.1% and 3.8%, suggesting that the edge effect giving non-specific results may be smaller in FLISA than in ELISA.

Conclusions: The improved FLISA system proposed is ideally suited for efficient measurements of hIFN-γ. This homogeneous and multiplex method will be a powerful tool for high throughput screening for drug discovery research.

KEY WORDS
enzyme-linked immunosorbent assay, fluorescence-linked immunosorbent assay, fluorometric microvolume assay technology, high-throughput screening, interferon-gamma

INTRODUCTION
Several studies have shown that cytokines participate in the induction and effector phases of inflammatory responses in allergies. Studies of the cytokine network are important for identifying candidates for drugs or drug targets. In addition, measurement of cytokines is required for screening of drugs.1 The regulation of human interferon-gamma (hIFN-γ) is particularly important for protective immunity in Th1 cells.2 hIFN-γ is implicated with interleukin (IL)-12 in the adoption of a Th1 phenotype. On the other hand, Th2 cytokines such as IL-4, IL-5, IL-10, and IL-13 affect allergic responses.3,4 Many studies have shown that hIFN-γ secretion associated with IL-12 or 18 plays an important role in the Th1 and Th2 balance.5,6 Hence, measurement of hIFN-γ has been widely used for monitoring immune responses in allergic diseases such as atopic dermatitis.7,8

Enzyme-linked immunosorbent assay (ELISA) is an established immunoassay used to quantify various cytokines. Conventional ELISA, which is not readily amenable for screening large numbers of samples in drug screenings, requires many processes and large quantities of antibody. For drug discovery and development, high throughput screening (HTS) would...
benefit from an immunoassay that requires minimal
manipulation and uses just a small amount of the
compounds required. Fluorescence-linked immuno-
sorbent assay (FLISA) is particularly well suited
for HTS.\(^9\)

We have developed a FLISA method for the mea-
surement of hIFN-\(\gamma\). We constructed the assembly of
capture antibody and fluorescent conjugated detec-
tion antibody for our FLISA system. This system en-
ables high throughput measurement of hIFN-\(\gamma\) and
could be applied to the assessment of other cytokines
or chemical mediators in allergic responses.

METHODS

INSTRUMENTATION

A fluorescent microvolume assay technology
(FMAT\(^\text{\textregistered}\)) instrument consists of a detector (Applied
Biosystems, Tokyo, Japan) integrated with a bar code
reader and robotic plate handler (Zymark Corp, MA,
USA) that can accommodate 60 plates. An FMAT\(^\text{\textregistered}\)
8100 HTS has a macro confocal imaging system with a helium/neon laser (633 nm) which auto-
matically focuses on and scans fluorescent-bound beads resting on the bottoms of multiwell trays. The
laser performs 256 scans across an area 1 mm \(\times\) 1
mm\(\times\)100 \(\mu\)m deep (Fig. 1). Because the depth of fo-
cus, 100 \(\mu\)m, is small in comparison with the remain-
ing volume, background fluorescence is minimal in relation to bead-bound fluorescence; thus, there are no washing steps to remove unbound fluorophores. The emitted fluorescence passes through the same optical path as the excitation beam and through a di-
choic beam splitter for detection by photomultiplier
tubes through the filters (680–730 nm).

PREPARATION OF ANTIBODY COATED BEADS

200 \(\mu\)l of streptavidin beads (0.5% w/v; 6 \(\mu\)m bead di-
ameter; SVP-60-55, Spherotech, were diluted in 800 \(\mu\)l
of phosphate-buffered saline (PBS) +0.01% sodium az-
ide (NaN3). After centrifugation at 10,000 \(g\) for 2min-
utes, the supernatant was discarded and resuspended
in 1 ml of PBS+0.01% NaN3. Next, a 4 \(\mu\)g portion of
biotinylated mouse anti-hIFN-\(\gamma\) monoclonal antibody
(mAb) (AHC4539, Biosource, California, USA) was
added to the streptavidin beads solution and incubated
with gentle mixing for 18 hours at room tem-
perature. Washing was repeated 2 times and the cap-
ture antibody coated beads were stocked in PBS +
0.01% NaN3 (8.4 \(\times\) 10\(^6\) beads/ml) at 4\(\Celsius\).

PREPARATION OF RECOMBINANT hIL-18 PRO-
TEIN

We used recombinant wild-type hIL-18 for the inhibi-
tion assay. Expression and purification were carried out
as described previously with minor modifica-
tions.\(^{10-12}\) The concentration of purified hIL-18 protein
was estimated using the absorbance constant (6160)
for hIL-18.

PREPARATION OF hIFN-\(\gamma\) BIOLOGICAL SAMPLE

hIL-18 inhibition assay based on hIFN-\(\gamma\) induction
was carried out as previously described.\(^{13,14}\) Briefly,
human myelomonocytic KG-1 cells (ATCC CCL246)
were grown in a culture medium consisting of RPMI
1640 supplemented with 10% heat-inactivated fetal calf
serum, L-glutamine (2 mmol/L), penicillin (100 U/mL)
and streptomycin (100 \(\mu\)g/mL). hIL-18
preparations prepared at our laboratories (in a final concentra-
tion of 2 ng/ml) was preincubated with anti-hIL-18 mAb
(Medical & Biological Laboratories, Nagoya, Japan)
or hIL-18 binding protein Fc chimera (IL-18BP) (Re-
search and Development Systems Inc, Minneapolis,
USA) (in final concentrations between 0 and 500 ng/
ml) at 37\(\Celsius\) for 1 hour and then 100 \(\mu\)l of the mixture
added to 100 \(\mu\)l of KG-1 cells (3 \(\times\) 10\(^6\) cells/ml) per
well in a 96-well plate (Nunc, Roskilde, Denmark), fol-
lowed by incubation at 37\(\Celsius\) for 24 hours in 5% CO\(_2\).
The culture supernatants were collected, and the
hIFN-\(\gamma\) production in each of the samples was deter-
mined by ELISA (Japan Immunoresearch Laborato-
ries, Co. Ltd., Takasaki, Japan) and FLISA. Data are
presented as the percent inhibition of total hIFN-\(\gamma\)
production in the absence of hIL-18 antagonists. IC\(50\),
the concentration of antagonists required to inhibit
50% of hIFN-\(\gamma\) production by KG-1 cells stimulated
with hIL-18, was then calculated.

FLUOROMETRIC MICROVOLUME IMMUNOAS-
SAY FOR hIFN-\(\gamma\)

A 50\(\mu\)l aliquot of sample or hIFN-\(\gamma\) standard (Re-
search and Development Systems Inc) was placed
into a 96-well plate. 50 μl of beads mixture (antibody bead: allophyocyanin (APC) conjugated mouse anti-hIFN-γ mAb (554702, Pharmingen, California, USA): FLISA buffer (PBS, 1% BSA, 0.35 M NaCl, 0.1% Tween-20, 0.01% NaN3) = 100:6.4:6500) was then mixed with the sample or standard in each well and incubated for 4 hours overnight at room temperature in the dark. After incubation, the 96-well plate was scanned using the FMAT® scanner. The average fluorescence per bead was recorded.

RESULTS

FMAT® 8100 HTS includes a bead-based assay system and is designed to negate the unbound fluorescent dye-labeled antibody. At measurement, FMAT® scans for fluorescent-bound beads resting on the bottom of multiwell trays (Fig. 1). No beads are found in the upper portions of the wells and non-specific fluorescence is not involved in background fluorescence intensity. In the lower 100 μm of the wells, the signals of unbound fluorescent dye-labeled antibody are deleted due to the limited size and form of the beads. Actually, the fluorescence intensity of negative control wells is 31.6 + / – 2.1 and that of the wells, which have 3.9 pg/ml of the minimum IFN-γ is 77.2 + / – 4.9. This is an obvious difference between negative control wells and minimum IFN-γ wells.

A standard solution of hIFN-γ was two-fold serially diluted in RPMI-1640 + 10% FCS. The intensity of the bead-bound fluorescence increased according to hIFN-γ concentration (Fig. 2). A standard curve for hIFN-γ was generated using the FMAT® scanner (Fig. 3). The linear dynamic range of hIFN-γ by FLISA was 3.9–1500 pg/ml. These results indicate that our system can recognize minute changes of hIFN-γ values. With previously established methods for IL-6 and IL-8, the linear dynamic ranges were 15.6–1000 pg/ml and 15.6–2000 pg/ml, respectively. Our FLISA system has an equivalent dynamic range comparable to these results, a range that is sufficient to be applied in clinical use.

To test whether FLISA could accurately quantify hIFN-γ in a biological sample, inhibition assays of hIL-18 were performed. We measured about 150 samples using FLISA and about 90 samples using ELISA. Sample preparation time of FLISA was equal to that of ELISA. hIFN-γ produced by KG-1 cells was measured using ELISA and FLISA (Figs. 4A, 4B). hIL-18 was neutralized by anti-hIL-18mAb or IL-18BP before stimulation. The IC50s for anti-hIL-18mAb and hIL-18BP using FLISA were 19.7 ng/ml and 5.2 ng/ml, respectively (Fig. 4B). Similarly, those using ELISA were estimated as 27 ng/ml and 5.4 ng/ml, respectively (Fig. 4A). However, as shown in Figure 4, the results of ELISA did not show a regression curve suitable for estimating precise IC50 values. The means of the coefficient of variation using the same samples measured by ELISA and FLISA were respectively 11.1% and 3.8%, suggesting that the edge effect often giving non-specific results may be smaller in FLISA than in ELISA.

Our proposed FLISA method was modified to use a combination of capture antibody and fluorescent dye-labeled detection antibody. As shown in Figure 5,
streptavidin beads and a biotinylated mouse anti-hIFN-γ mAb were used as IFN-γ capture particles and APC conjugated mouse anti-hIFN-γ mAb was used as the IFN-γ detection reagent. Previously, goat anti-mouse IgG (Fc) beads and mouse anti-hIFN-γ mAb have been used as the IFN-γ capture particles and Cy 5.5 conjugated mouse anti-hIFN-γ mAb as the IFN-γ detection reagent. Figure 6 shows our standard curve for IFN-γ estimated IFN-γ values in detail compared with that from the previously proposed FLISA method.

**DISCUSSION**

When measuring various cytokines, the effect of the environmental temperature is one of the factors influencing inter-experimental accuracy. ELISA requires washing and application of a detection antibody following the first incubation, and washing and application of a detecting reagent following the second incubation. On the other hand, with FLISA, measurements can be performed following the first incubation. In all ELISA steps it is therefore very easy to expose the samples to environmental temperatures. The edge effect, which is the difference in temperature between the outer and inner wells, is therefore one of the factors resulting in inter-experimental errors. Accurate screenings are very much required in working toward drug discoveries.

We used two kinds of mouse hIFN-γ mAb in our FLISA method: one a capture antibody, the other an APC conjugated antibody as a detection antibody. Measurements of hIFN-γ in a previous report used a...
goat anti-mouse IgG antibody as the bead-coating antibody and two kinds of mouse anti-hIFN-γ mAb, a capture antibody and a Cy5.5 conjugated antibody (Fig. 5). The sensitivity of our proposed FLISA method was about four-fold higher than that of previous methods (Fig. 6). That is, the minimum unit for measurement in our method is four times smaller: 0.5 pg/mL/fluorescence (FL) in the previous method by Komatsu et al., 0.125 pg/mL/FL in our method. Therefore, we were able to obtain detailed IFN-γ values. The sensitivity of the FLISA method proposed in this paper allowed the discrimination between differences.

The sensitivity of each method may depend on the structural difference between APC and Cy5.5. One APC molecule binds to one antibody. The APC is a trimer that consists of α and β subunits (αβ). Each subunit has one fluorophore; so as a result an APC conjugated antibody contains six fluorophores (Fig. 5A). However, one Cy5.5 binds to one antibody, and the Cy5.5 is a single fluorophore itself. Thus an antibody conjugated Cy5.5 has one fluorophore (Fig. 5B). Although the fluorescent dye/antibody ratio of APC is equal to that of Cy5.5, an APC conjugated antibody has six times as many fluorophores as a Cy5.5 conjugated antibody. The difference in fluorescent intensity may also be a factor increasing sensitivity.

To quantify hIFN-γ in large amounts of sample, we developed a more efficient assay method than conventional ELISA, which can involve many incubation and washing steps, and requires large amounts of antibodies. In contrast, our FLISA method is a homogeneous bead-based immunoaassay that requires no wash steps. Over 100-fold less capture antibody is needed in FLISA than in conventional ELISA. However, the most striking difference is the time required for the assay. In our study, the occupation time of the FLISA method was about 1 hour, demonstrating that FLISA can be performed in a much shorter time than conventional ELISA, which requires about 5 hours.

A minimal time requirement is a crucial factor for applications such as HTS in drug discovery research. FLISA is thus an attractive method as it involves less hands-on time and lower running costs.

In immunological studies using FLISA, measurements of IL-6 and IL-8 and CD3+/CD4+ lymphocyte counts in whole blood have been reported. The bead-based system of the FLISA is readily applicable to any other plate-based assay, such as nonradioactive kinase, phosphatase, and protease assays. A multiplexed bead-based receptor-ligand binding assay has already been demonstrated using FLISA. In addition, FLISA is capable of detecting and quantifying fluorescence on live cells, allowing for such diverse assays as apoptosis and cytotoxicity, and cellular immunoaassays, and receptor ligand binding assays. The FLISA system can be adapted for HTS of large libraries of chemical and natural products, thus having a place in any laboratory that routinely performs multiple, repetitive assays.

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