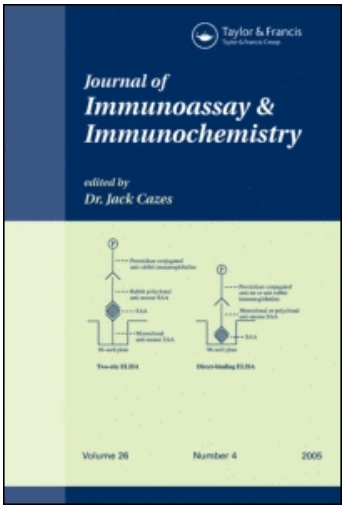

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A SENSITIVE IN SITU ELISA FOR QUANTITATIVE MEASUREMENTS OF CYTOKINES AND ANTIBODIES SECRETED BY CULTURE LYMPHOCYTES

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A SENSITIVE IN SITU ELISA FOR QUANTITATIVE MEASUREMENTS OF CYTOKINES AND ANTIBODIES SECRETED BY CULTURE LYMPHOCYTES

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ABSTRACT

Cytokines are usually measured using ELISA and ELISPOT. We have developed an in situ ELISA by combining the advantages of the highly sensitive ELISPOT and the quantitative conventional ELISA techniques to measure cytokines and antibodies secreted by culture lymphocytes. In this assay, lymphocytes were cultured on ELISA plates pre-coated with either a special antigen or mAbs against IL-4, IFN-γ. The caprtured lymphokines or immunoglobulins were subsequently measured by conventional ELISA procedures. As a comparison, a conventional ELISA was carried out in parallel. The in situ ELISA measured a 5-fold increase in

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antigen-stimulated IL-4 and IFN-γ, compared to the conventional ELISA. It also rquired 10,000 times fewer lymphocytes to produce a detectable level of antigen-specific antibodies than the conventional ELISA. We conclude that the in situ ELISA is much more sensitive than a conventional ELISA and less costly and easier to perform than ELISPOT, providing a useful tool to quantitatively measure cell-secreted molecules.

INTRODUCTION

Analysis of cytokine production by human peripheral blood mononuclear cells (PBMCs), or animal lymphocytes, has been hampered by technical difficulties because antigen-driven cytokine production in primary cultures were frequently below the limit of sensitivity of the assay.(1–4) This problem is more pronounced for cytokines such as IL-4 and IL-5, which have been difficult to detect using a conventional ELISA. As well, in order to meet the sensitivity of the assay for antigen-specific antibody secretion by culture lymphocytes, a long-term culture, usually 8–14 days, is often required.(5–7) These constraints have led investigators to develop alternative assays, such as cytokine mRNA analysis, or the derivation of long term T cell clones.

The ELISPOT assay was originally described by Czerkinsky et al.(8) and Sedgwick and Holt(9) as a method to enumerate antibody producing cells and was later adapted to quantitatively measure the cytokine-secreting cells.(10,11) It was suggested that ELISPOT was a more sensitive method than conventional ELISA for characterization of cytokine secretion patterns of different cell populations in vitro.(12) To date, the ELISPOT assay is one of the most sensitive methods to detect cytokine production for a particular single cell. One significant improvement of the ELISPOT has been the introduction of a nitrocellulose membrane as a solid support for the coating agent.(13) An ELISPOT assay in which plastic ELISA plates, instead of the nitrocellulose bottomed 96-well plates, used for the detection of cytokines, has been reported.(12)

A major limitation of the ELISPOT assay is that it requires serial dilution of cells to obtain the appropriate number of cells in each well. In addition, since the spots are enumerated by the examiner, it is possible that faint and overlapping spots are counted incorrectly.(14,15) Although computer-assisted video images may improve the objectivity and speed of analysis,(16–19) the expense of such a device would limit its broad application. Moreover, a large number of background spots are often present in

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unstimulated nitrocellulose-bottomed wells, introducing other problems for the analyst.

In this report, we decribe an in situ ELISA which we developed to quantitatively measure cytokine and antibody production by cultured lymphocytes. It is more convenient and accurate than the ELISPOT and more sensitive than conventional ELISA. We also investigated the sensitivity and reproducibility of this new assay by comparing our in situ ELISA with the conventional ELISA for the detection of antigen-specific IL-4, IFN-γ, and/or IgG and IgG1 production by lymphocytes in both animals and humans.

EXPERIMENTAL

Mice and Immunization

BALB/c mice (female, 8–10 weeks old) were obtained from the Central Animal Care Services, University of Manitoba. All animals were maintained under identical conditions at the service facility. The experiments were approved by the Animal Care and Use Committee, University of Manitoba and the investigators adhered to Canada Council Animal Care (CCAC) guidelines for humane treatment of animals.

The mice were immunized twice with purified rAed a 2, a 37 kDa recombinant salivary protein of the yellow fever mosquito *Aedes aegypti*.(20) Briefly, each mouse was injected i.p. with 50 μg of rAed a 2 mixed with complete Freund's adjuvant (Sigma, St. Louis, MO). After two weeks, the mice were boosted with the same amount of rAed a 2 in incomplete Freund's adjuvant (Sigma). Mice injected with saline served as controls.

Human Subjects

This project was approved by the University Faculty Committee for the Use of Human Subjects in Research, and participants gave written, informed consent before entering the study. The human peripheral blood mononuclear cells (PBMCs) used in the study were obtained from 6 mosquito-allergic subjects. These subjects, who had strong skin reactions in mosquito bite tests (the diameters of immediate wheal between 10 to 18 mm) and very high levels of serum mosquito saliva-specific IgE and IgG antibodies, were selected from our previous study of mosquito allergy.(21)

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Mouse Lymphocyte and Human PBMCs Preparation and Cell Culture

Mouse lymphocytes were isolated on day 7 following the last immunization. Briefly, single cell suspension was prepared by gently teasing spleens through sterile stainless steel meshes in incomplete RPMI 1640 medium supplemented with 2 mM L-Glutamine, 100 μg/mL of penicillin and 100 μg/mL of streptomycin. The cells were washed with the incomplete medium and exposed for 3 min to 1 mL of a solution containing 10 mM $KHCO_3$, 0.1 mM EDTA, and 0.16 M NH_4Cl, in order to lyse erythrocytes. The lysis reaction was stopped by the addition of 1 mL of fetal calf serum (FCS).

The cells were then washed and subsequently centrifuged through a Ficoll-Hypaque gradient (Sigma, St. Louis, MO). The lymphocytes collected from the interface of the gradient were washed and resuspended at 2×10^6 cells/mL in complete RPMI 1640 consisting of 10% FCS, 10 mM L-glutamin, 2×10^{-5} M β-mercaptoethanol, and antibiotic-antimycotics (Gibco BRL, Burlington, Ontario). The lymphocytes (200 μL/well) were cultured in 96-well flat-bottom plates (Corning, Rochester, NY) at 10-fold serial concentrations between 4 and 4×10^5 cells/well in either 5 μg/mL of rAed a 2 in complet RPMI 1640 medium or 5 μg/mL of Con A in medium or the medium alone. The plates were incubated at 37°C in a humidified atmosphere with 5% CO_2. The incubation was 24 h or 48 h in Con A induced IL-4 and IFN-γ production, 48 h for rAed a 2-induced IL-4 and IFN-γ production, and 4, 8, 12, 24, 36, and 48 h for Con A induced IL-4 and IFN-γ kinetic production.

To prepare human PBMCs, heparinized blood from donors was centrifuged on a Histopaque-1077 gradient (Sigma). After 2 washes in PBS, the PBMCs were resuspended in complete RPMI 1640 medium and cultured in 96-well flat-bottom plates (Corning) to a final concentration of 2×10^6 cells/mL at 200 μL per well. For each human subject, cultures were set up as below:

(i) in the presence of mosquito *Aedes vexans* head and thorax extract which was prepared by the method previously described,(22) at a concentration of 100 μg/mL.
(ii) in the presence of 5 μg/mL of Con A.
(iii) in the absence of stimuli. PBMC culture supernatants were collected at 48 h for detection of IL-4 and IFN-γ.

In Situ ELISA Measurement of Mouse and Human IL-4 and IFN-γ

To detect mouse IL-4, plastic ELISA plates (high binding with flat bottom, Costar corporation, Cambridge, MA) were coated with monoclonal

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rat anti-mouse IL-4 antibody (11B11, PharMingen, San Diego, CA) diluted to 1 μg/mL in sterile PBS, 100 μL/well, at 4°C overnight. After washing 4 times with sterile PBS, 100 μL/well of culture medium was added for plate blocking at 37°C for 4 h. 200 μL of the cell suspension were added in duplicate into each well of the antibody-coated plates after washing twice. The cells were incubated at 37°C with 5% CO_2 for 24 h or 48 h. Twenty four wells filled with 200 μL/well of culture medium were used as standard setting wells, while the wells filled with 200 μL/well of culture medium plus cells but without Con-A or rAed a 2 served as negative controls. After cell culture, cell suspensions were removed from the 96-well plates by flicking. The wells were then washed three times with sterile PBS, supplemented with 0.05% Tween 20 (PBST). 11 serial 2-fold dilutions of recombinant mouse IL-4 ranging in concentration from 0.5 pg/mL to 500 pg/mL (19231V, PharMingen) were added in duplicate to the 24 standard setting wells to obtain a standard curve. PBS was added to the remaining 2 wells to serve as blank controls.

The plates were then incubated at 37°C for 3 h and washed four times with PBST. 100 μL of 1 μg/mL of biotinylated rat anti-mouse IL-4 antibody (BVD6-24G2, PharMingen) were added to each well and the plates were incubated at room temperature for 1 h. Following extensive washing, 100 μL of avidin-alkaline phosphatase (AP) (13043E, PharMingen) diluted 1 : 1,000 was added to the wells and the plates were incubated at room temperature for 30 min. After 8 washes, 100 μL of 1 mg/mL n-nitrophenylphosphate solution (Sigma) in carbonate buffer (pH 9.6) was added and the colour reactions allowed to develop at room temperature. The optical density was measured with a Microplate Reader ($Thermo_{max}$, Molecular Devices, USA) at 405 nm. The sensitivity for mouse IL-4 measurement was 2 pg/mL.

To measure mouse IFN-γ, purified rat anti-mouse IFN-γ mAb (R4-6A2, PharMingen) at 1 μg/mL was used as a capture antibody, biotinylated rat anti-mouse IFN-γ mAb (XMG1.2, PharMingen) at 1 μg/mL as a detecting antibody, and recombinant mouse IFN-γ (19301 T, PharMingen) ranging from 4 pg/mL to 1,000 pg/mL as an IFN-γ standard. The sensitivity of the assay was 15.6 pg/mL for mouse IFN-γ.

To detect human IL-4 and IFγ-γ, purified mouse anti-human IL-4 (8D4-8, PharMingen) or IFN-γ (NIB42, PharMingen) mAb was used at 1 μg/mL as the capture antibody. Biotinylated rat anti-human IL-4 (MP4-25D2, PharMingen) or IFN-γ (4S.B3, PharMingen) mAb was used at 1 μg/mL as the detecting antibody. Recombinant human IL-4 (19641V, PharMingen) ranging from 1 pg/mL to 1,000 pg/mL, and IFN-γ (19751N, PharMingen) ranging from 1 pg/mL to 1,000 pg/mL served as human IL-4 or IFN-γ standards, respectively. The detection limits of the assay were 2 pg/mL for human IL-4 and 15.6 pg/mL for human IFN-γ.

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In Situ ELISA Measurement of Mouse Antigen-Specific IgG and IgG1 Antibodies

To detect mouse rAed a 2-specific IgG and IgG1 antibody production by in vitro cultured lymphocytes, ELISA plates were coated with rAed a 2 at 2 μg/mL in sterile PBS followed by blocking with a 10% FCS solution. Lymphocytes at 10-fold serial concentrations between 4 and 4×10^5 cells/well with or without 5 μg/mL of rAed a 2 were added to each well in triplicate, while two rows of wells were filled with medium to be standard setting wells. The plates were incubated at 37°C with 5% CO_2 for 96 h followed by four washes. A reference serum containing rAed a 2-specific IgG and IgG1 was added in triplicate in serial dilutions to rAed a 2-precoated standard setting wells. The plates were left at room temperature for 3 h. After washing, 100 μL of AP-conjugated goat anti-mouse IgG (Cat. 293355, Jackson ImmunoResearch Laboratories Inc., West Grove PA) or IgG1 (Cat. L356-N907, Southern Biotechnology Associates Inc., Birmingham, AL) antibodies were added to each well and incubated at room temperature for 1 h. Following washing and incubation with AP-substrate, the colour reaction was measured at 405 nm as described above.

Standardization of ELISA results between assays and estimation of the relative amount of rAed a 2-specific IgG and IgG1 in each sample were accomplished using a reference serum. The serum was pooled from mice with high titres of rAed a 2-specific IgG and IgG1 and defined as 1,000 U/mL for both rAed a 2-specific IgG and IgG1 antibodies. The sensitivity of the assay was 0.2 U/mL for the two rAed a 2-specific antibodies.

Conventional ELISA Detection of IL-4, IFN-γ, and rAed a 2-Specific IgG and IgG1

Mouse lymphocytes and human PBMCs were cultured in parallel, as described above, on the plates, which were not precoated with mAb or rAed a 2. The levels of mouse and human IL-4 and IFN-γ and mouse rAed a 2-specific IgG and IgG1 in the supernatants were determined by a conventional ELISA. Briefly, the ELISA plates were coated with mAb anti-IL-4 or anti-IFN-γ as a capture agent followed by sequential incubations with culture supernatants and then with biotinylated mAb anti-IL-4 or anti-IFN-γ. To detect rAed a 2-specific IgG and IgG1, the plates were coated with rAed a 2 followed by sequential incubations with culture supernatant and then AP-conjugated anti-mouse IgG or -IgG1 antibodies. The optimal concentrations of the ELISA reagents above had been previously determined(23) and were identical to those of the in situ ELISA.

Statistical Analysis

The concentrations of immunoglobulins, IL-4 and IFN-γ were expressed as mean SD. A two-tailed Student's t-test was used to compare the significance of groups.

RESULTS

Sensitivity of In Situ ELISA and Conventional ELISA in Detecting Con A-Stimulated IL-4 and IFN-γ Production

Lymphocytes from normal BALB/c mice were cultured with 5 μg/mL of Con A in 96-well plates pre-coated with anti-mouse IL-4 or IFN-γ mAbs. The secreted IL-4 and IFN-γ were captured by coating with mAbs and subsequently detected using in situ ELISA. For comparison, IL-4 and IFN-γ in the culture supernatants obtained from non-mAb coated plates were measured in parallel using a conventional ELISA. As shown in Figure 1a, after being cultured for 24 h, the in situ ELISA detected a significant increase in IL-4 production with a lymphocyte concentration as low as 4×10^3 cells/well, whereas the conventional ELISA failed to detect IL-4 production even in cultures with 4×10^5 cells/well. After 48 h of culturing with 4×10^4 cells/well, the conventional ELISA detected lower levels of IL-4 production at 48.7 pg/mL compared with the in situ ELISA at 209.9 pg/mL ($p < 0.05$) (Figure 1a). Interestingly, there was no difference in the detection of IFN-γ levels in Con A-stimulated lymphocytes between the two ELISAs (Figure 1b).

Sensitivity of In Situ ELISA and Conventional ELISA in Detecting Antigen-Stimulated IL-4 and IFN-γ Production

Since in vitro Con A is a more powerful stimulator of cytokine production than antigens, the high levels of cytokines in Con A-stimulated cultures may mask the sensitivity of the in situ ELISA, producing similar results in both ELISAs as found in Figure 1b. Hence, we re-evaluated the sensitivity of both ELISAs in the measurement of rAed a 2-stimulated IL-4 and IFN-γ production by lymphocytes cells in which IL-4 and IFN-γ were much lower than those induced by Con A. The in situ ELISA detected a significantly higher level of IL-4 production than the conventional ELISA ($p < 0.01$) (Figure 2a). Intriguingly, IFN-γ levels in rAed

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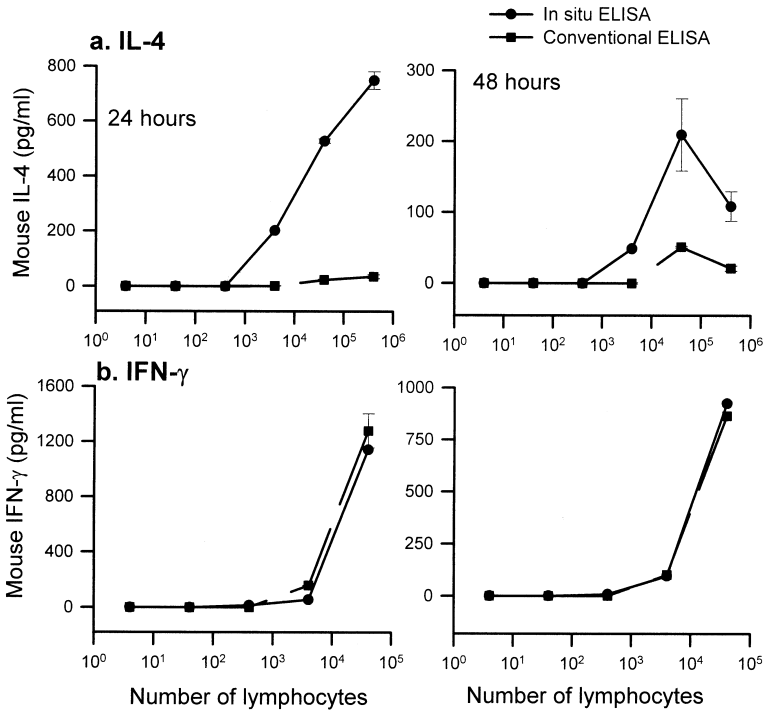

Figure 1. A comparison of an in situ ELISA and a conventional ELISA in the detection of Con A-induced IL-4 and IFN-γ production by mouse lymphocytes. BALB/c lymphocytes between 4 and 4×10^5 cells/well were stimulated with Con A (5 μg/mL) for 24 h (left) or 48 h (right). The levels of IL-4 (a) and IFN-γ (b) from culture lymphocytes were measured by the in situ ELISA (solid circles) or the conventional ELISA (solid squares) as described in Experimental.

a 2-stimulated cultures detected by the in situ ELISA were about 5-fold higher than those measured by the conventional ELISA ($p < 0.01$) (Figure 2b).

Comparison Between In Situ ELISA and Conventional ELISA in Detecting Kinetic Production of IL-4 and IFN-γ Induced by Con A

Con A induced a time- (Figure 3a) and dose-dependent (data not shown) production of IL-4 from the culture lymphocytes of normal mice.

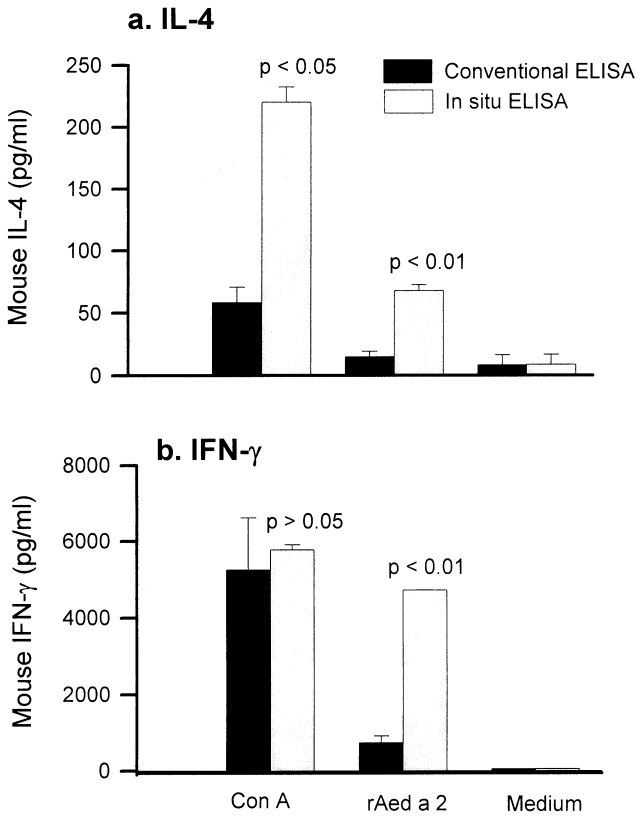

Figure 2. A comparison of the sensitivities of an in situ ELISA and a conventional ELISA in detecting Con A- or rAed a 2-stimulated IL-4 and IFN-γ production by mouse lymphocytes. 2×10^5 lymphocytes/mL in 200 μL per well in triplicate were stimulated with rAed a 2 (5 μg/mL), Con A (5 μg/mL) or culture medium alone for 48 h. The levels of mouse IL-4 (a) or IFN-γ (b) were determined by the in situ ELISA and the conventional ELISA.

Compared to the conventional ELISA, the in situ ELISA revealed an earlier and faster increase in IL-4 production from Con A-stimulated cultures (Figure 3a). In addition, Con A also induced a time- (Figure 3b) and dose-dependent (data not shown) increase in IFN-γ production. Interestingly, both the in situ ELISA and the conventional ELISA detected a similar curve of IFN-γ production (Figure 3b), suggesting that both assays have a similar sensitivity for the detection of kinetic production of Con A-induced IFN-γ.

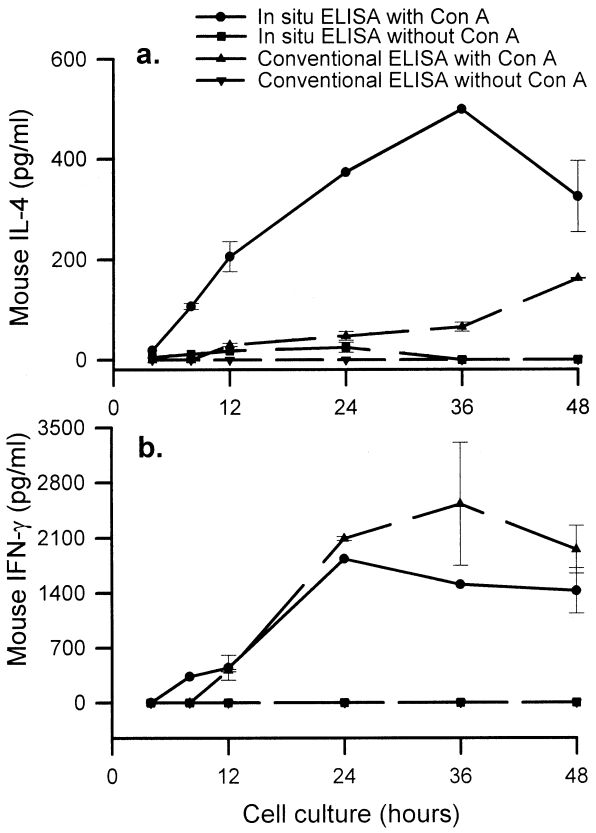

Figure 3. A comparison of an in situ ELISA and a conventional ELISA in the detection of Con A-stimulated IL-4 (a) and IFN-γ (b) kinetic production. 2×10^5 lymphocytes in 200 μL per well were stimulated with 5 μg/mL of Con A for 4, 8, 12, 24, 36 and 48 h (solid circles and solid stand triangles). Lymphocytes grown without Con A served as controls (solid squares and solid reverse triangles). The levels of IL-4 and IFN-γ were measured by the in situ ELISA (solid circles and solid squares) and the conventional ELISA (solid stand triangles and inverted triangles).

Sensitivity of In Situ ELISA and Conventional ELISA in the Detection of Mouse Antigen-Specific IgG and IgG1

In order to confirm the high sensitivity of the in situ ELISA, antigen-specific antibody production from in vitro culture lymphocytes was

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examined using both ELISAs. Lymphocytes from rAed a 2-sensitized BALB/c mice were cultured with rAed a 2 for 96 h in 96-well plates precoated with rAed a 2. The antibodies secreted by B cells were captured by coated antigens and subsequently detected using the techniques described in method. In parallel, the rAed a 2-specific IgG and IgG1 in the culture supernatants from plates not coated with rAed a 2 were measured by a conventional ELISA. As shown in Figure 4, the in situ ELISA detected rAed a 2-specific IgG and IgG1 secreted by lymphocytes with concentrations as low as 40 lymphocytes/well. In contrast, 10,000 times as many lymphocytes were required to produce detectable IgG and IgG1 levels in the conventional ELISA.

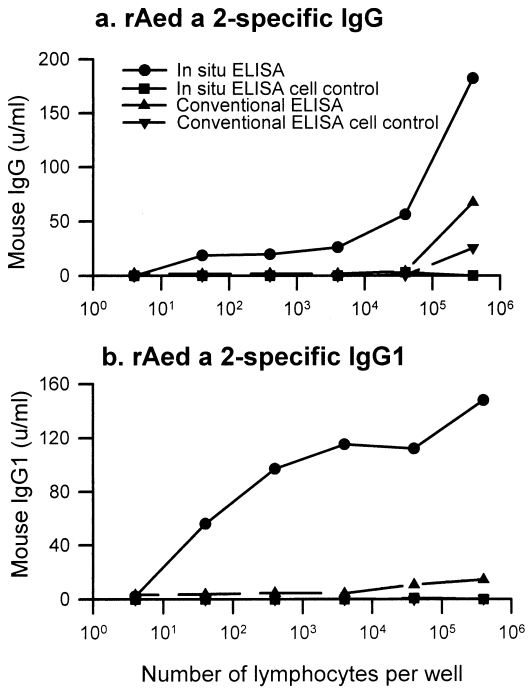

Figure 4. A comparison of an in situ ELISA and a conventional ELISA in the detection of anti-rAed a 2 IgG and IgG1 in the culture supernatants from rAed a 2-stimulated lymphocytes. Lymphocytes at different concentrations were cultured with and without rAed a 2 (5 μg/mL) for 96 h. The anti-rAed a 2 IgG (a) and IgG1 (b) antibodies were determined by the in situ ELISA (solid circles and squares) and the conventional ELISA (solid triangles and inverted triangles).

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Sensitivity of In Situ ELISA and Conventional ELISA in Detecting Antigen-Driven Human IL-4 and IFN-γ Production by PBMCs

The PBMCs from subjects allergic to mosquito bites were cultured with the mosquito head and thorax extract for 48 h on the plates precoated with mAb anti-IL-4 or -IFN-γ and the plates not coated with mAbs. The IL-4 and IFN-γ levels detected by the in situ ELISA and the conventional ELISA were compared. As shown in Figure 5, the in situ ELISA detected significantly higher levels of IL-4 and IFN-γ production in the six mosquito-allergic subjects than the conventional ELISA did ($p < 0.05$).

Sensitivity of In Situ ELISA Correlated with High Efficiency Binding of the Coating Antibody

In order to determine whether the coating antibodies efficiently capture secreted cytokines, we analysed the cytokine levels in the supernatants from plates precoated with anti-mouse IL-4 or -mouse IFN-γ. As shown in Figure 6, the IL-4 and IFN-γ levels in the supernatants from the plates precoated with mAbs were very low compared with those from the plates not coated with mAbs. This result suggests that the high sensitivity of the in situ ELISA is due to efficient binding of the in situ coating antibodies to cytokine molecules released from lymphocytes.

DISCUSSION

The in situ ELISA assay described here employs a direct capture step by precoating cytokine-specific mAbs or antibody-specific antigens of interest before setting up cell cultures on ordinary ELISA plates. The secreted cytokines and immunoglobulins bind specifically to the corresponding immobilized antibody or antigen on the plate. Compared to conventional ELISA techniques, in situ ELISA reactions yielded an earlier increase and greater maximum secretion of IL-4, IFN-γ (Figure 3), and antigen-specific IgG and IgG1 (Figure 4) by lymphocytes stimulated with antigens. In Con A-stimulated cultures, it appears that the in situ ELISA is comparable to the conventional ELISA in the detection of Con A-stimulated IFN-γ (Figure 1b and Figure 2b, Figure 3b, $p > 0.05$). This result may be due to the high levels of IFN-γ in the culture supernatants induced by Con A, a powerful mitogenic reagent. However, in antigen-stimulated IFN-γ production in which IFN-γ levels in the cell cultures are much lower than those induced by Con A, the in situ ELISA was much more sensitive

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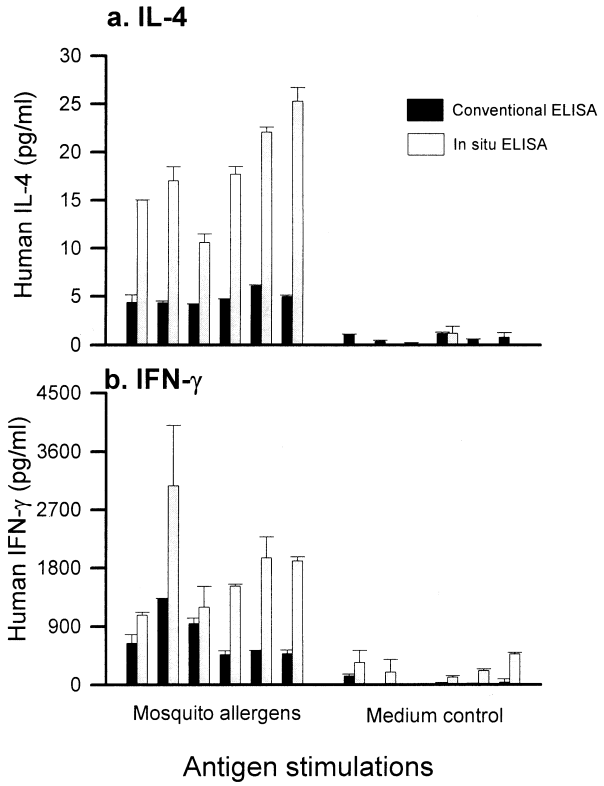

Figure 5. A comparison of an in situ ELISA and a conventional ELISA in the detection of antigen-driven IL-4 and IFN-γ production from human PBMCs of mosquito allergic subjects. PBMCs from six individuals at 2×10^6 cells/mL in 200 μL per well in triplicate were incubated with mosquito head and thorax antigens (100 μg/mL) for 48 h. IL-4 (a) and IFN-γ (b) levels in cell supernatants were measured by the in situ ELISA and the conventional ELISA.

than the conventional ELISA, detecting about a 5-fold higher level of IFN-γ than that measured by conventional ELISA (Figure 2b, $p < 0.01$). The data demonstrate the advantage of in situ ELISA in detecting the antigen-driven IFN-γ production in vitro. Moreover, IL-4, the most difficult cytokine to detect using conventional ELISA techniques,(1,2) was easily detected with the newly developed in situ ELISA.

The low background production of cytokines and antibodies in unstimulated cells in the in situ ELISA can be read by the ELISA reader and simply deducted from the test samples. This makes the in situ ELISA more

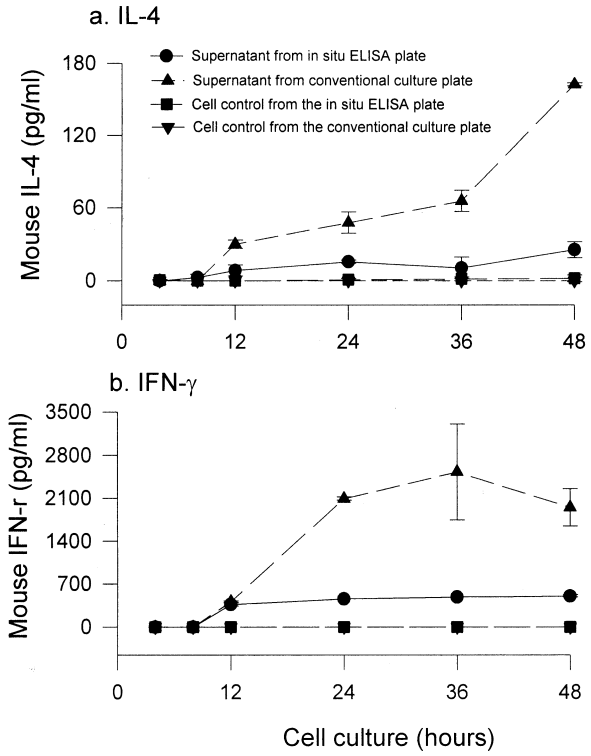

Figure 6. Comparison levels of Con A-induced IL-4 and IFN-γ in the supernatants from in situ ELISA plates and conventional ELISA plates. 2×10^5 lymphocytes in 200 μL per well were stimulated with Con A (5 μg/mL) for 4, 8, 12, 24 and 48 h as indicated. IL-4 and IFN-γ levels in the supernatants from mAb-coated ELISA plates (solid circles and solid squares) or antibody non-coated plates (solid and inverted triangles) were measured by the conventional ELISA.

accurate and reliable compared to the ELISPOT in which artificial spots may be introduced especially when the number of the spots is low or spots are faint. Unlike ELISPOT, the in situ ELISA method is performed very easily and efficiently, saving time and the lower cost of ordinary ELISA plates compared to nitrocellulose plates is also worthy of consideration.

In our kinetic studies, similar to a previous report,(15) we observed that the cytokine levels measured by in situ ELISA slightly declined over time after an initial maximum at 24–36 h of culture (Figure 3). This was interpreted to be due to the consumption of cytokines loosely bound to the

capture antibody.(15) Therefore, the choice of an optimal time to show a maximum T cell response is critical for this assay.

The reason for the higher sensitivity of the in situ ELISA is clearly the same as that for ELISPOT. Our data directly demonstrate that the precoated antibodies efficiently and specifically capture most target molecules released from culture cells in situ. In turn, there were a few target molecules remaining in the supernatant as shown in Figure 6.

The levels of standard cytokines and antibodies in the standard setting wells were not consistent during the incubation at 37°C for 24 to 96 h (data not shown). Therefore, in the present study, ELISA standards were added to the standard setting wells after the 24–96 h cell culturing and the plates were then incubated at 37°C with 5% CO_2 for 3 hours as described previously.(24) The standard setting we developed here is suitable not only for early expressed cell molecules but also late expressed molecules, such as antigen-specific antibodies that are secreted up to 96 h during the culturing.

In conclusion, the newly developed in situ ELISA is a simple and sensitive assay for the qualitative detection of low levels of cytokines, antibodies or any other components (if their antibodies are available) produced by culture cells.

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