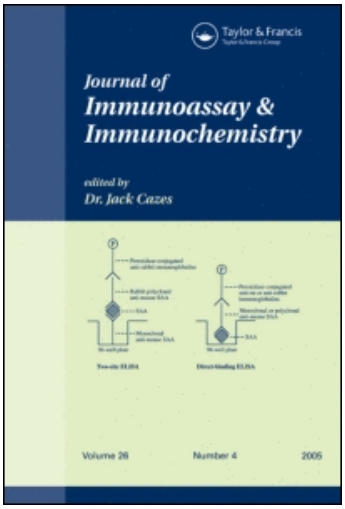

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Enhanced Cytokine Detection by a Novel Cell Culture-Based Elisa

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ENHANCED CYTOKINE DETECTION BY A NOVEL CELL CULTURE-BASED ELISA

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ABSTRACT

Production of some cytokines, such as IL-4 and IL-10, often occurs at low levels and is difficult to detect by standard ELISA techniques. In many cases the level of detection is at or near to the limits of sensitivity of the assay due either to minimal synthesis and/or cytokine consumption. In an effort to enhance the quantitation of weakly detected cytokines we have developed a unique cell culture-capture ELISA. Lymphocytes are incubated in an anti-cytokine antibody coated ELISA plate for the last 6 hours of a 24 hour *in vitro* activation period. Use of this cell culture capture method consistently enhanced detection of several T cell cytokines compared to conventional ELISA techniques. Moreover, this technique was found to enhance detection without altering the rate of cytokine secretion which occurred prior to the cell culture capture period. Thus, the cell culture capture ELISA may be useful for detection of a variety of cytokines which are produced at low levels and have traditionally been difficult to quantify.

(KEY WORDS: enzyme-linked immunosorbent assay; ELISA; cytokines; enhanced detection; capture; culture technique)

INTRODUCTION

Mitogen- or antigen-activated lymphocytes produce a variety of soluble factors which may have pleiotropic effects on other cells in their environment.

Cytokines secreted by activated lymphocytes have been shown to have both autocrine and paracrine activities. Due to their importance in immune regulation, cytokine levels are regularly investigated and reported in the literature. Although sensitive bioassays have been described for many cytokines, the reliability of bioassays is often compromised by presence of inhibitory substances or the ability of other cytokines to display similar bioactivities. Most of these cytokines can also be detected rapidly and more specifically by the enzyme-linked immunosorbant assay (ELISA). However, the sensitivity of an ELISA is limited by the affinity of the antibodies used in the assay. The need for greater sensitivity of detection often arises with cytokines which are either secreted in very low amounts or are utilized by cells in an autocrine fashion, resulting in levels of the cytokine in *in vitro* culture supernatants that are often too low for detection. Cytokines such as IL-2 and IL-4 are growth-promoting factors that are used by lymphocytes soon after production. Unlike IL-2, which is produced in high amounts, IL-4 is produced in lower quantities but can also be consumed by the producer cells or other cells that express IL-4 receptors. Consequently, we and others (1,2,3) have observed that IL-4 is often difficult to detect in lymphocyte cultures.

In order to enhance the detection of low level cytokines, such as IL-4 and IL-10, we developed a cell culture capture-ELISA procedure that was designed to capture secreted cytokines before they could be bound to surface receptors and consumed by the producer cell or other cells during the *in vitro* activation period. While antibody specific for cytokine receptors has been used to inhibit cytokine

uptake to enhance detection in bioassays and ELISAs, it is clear that some anti-receptor antibodies transduce signals (positive or negative) to the cytokine-producing cells which could alter cytokine synthesis and confuse interpretations. To avoid this possible complication, we reasoned that incubation of cytokine-producing cells directly on an anti-cytokine antibody coated ELISA plate would result in an enhanced signal compared to a conventional ELISA in which cells are first incubated separately and culture supernatants are then assayed. Moreover, the rate of cytokine synthesis would not likely be altered, since anti-cytokine receptor antibodies would not be utilized. To test the validity of this idea, we compared the quantitation of IL-2, IL-4, IL-10 and IFN-γ secreted by activated spleen cells as measured by conventional ELISA and the cell culture capture-ELISA. Results demonstrate that the cell culture capture-ELISA is superior to the conventional ELISA in detecting cytokine production by lymphocytes.

MATERIALS AND METHODS

Animals

Female C57BL/6 mice were obtained from the National Cancer Institute (Frederick, MD) and maintained by the Division of Laboratory Animal Resources at the University of Kentucky. Mice were 8-12 weeks old at the time of use in all experiments.

Isolation of Splenocytes

Mice were killed by CO_2 asphyxiation and spleens were removed. Single cell suspensions were obtained by processing each spleen separately in RPMI-1640

medium (supplemented with 10% FCS, 2mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.05 mM 2-mercaptoethanol) using a Stomacher tissue disrupter (Seward Medical, London, England). Lysis of erythrocytes was achieved by incubation with Tris-NH_4Cl (pH 7.2) for 5 minutes. Following two washes with culture medium (supplemented RPMI-1640), the cells were counted using a hemacytometer and resuspended appropriately. Viability, assessed via trypan blue exclusion, was routinely greater than 95% for all experiments.

Reagents

Concanavalin A (Sigma Chemical, Co., St. Louis, MO), affinity-purified anti-CD3 mAb (145-2C11), Phorbol myristate acetate (Sigma) and ionomycin (Calbiochem, La Jolla, CA) were used to stimulate cells for cytokine secretion.

For ELISA, the following pairs of monoclonal antibodies (mAbs) were purchased from Pharmingen Corp. (San Diego, CA) and used: anti-IL-2, purified JES6-1A12 (lot # 8507-04) and biotinylated JES6-5H4 (lot # F511273); anti-IL-4, purified 11B11 (lot # M013791) and biotinylated BVD6-24G2 (lot # 8578-04); anti-IL-10, purified JES5-2A5 (lot # 8897-04) and biotinylated SXC-1 (lot # H511520); and anti-IFN-γ, purified R4-6A2 (lot # 511538) and biotinylated XMG1.2 (lot # B510298). Recombinant IL-2 (BioSource International, Camarillo, CA), IL-4 (Pharmingen), IL-10 (Sigma), and IFN-γ (Sigma) were used to prepare cytokine standards used in the ELISAs. Horseradish peroxidase (HRP) conjugated streptavidin (Zymed Laboratories, Inc., San Francisco, CA) and 3,3'5,5'-

tetramethylbenzidine base substrate (TMB-ELISA) (Gibco BRL, Gaithersburg, MD) were other components of the ELISAs.

Cell Culture

Splenocytes were dispensed into 96-well flat bottom tissue culture plates (Becton-Dickinson, Lincoln Park, NJ) at 5 X 10^5 cells per well in 100 µl volume of culture medium. Con A (10 µg/ml or 5 µg/ml), anti-CD3 mAb (8 µg/ml or 4 µg/ml), or PMA (5 ng/ml) plus ionomycin (0.75 µM) were then added in 100 µl volumes to appropriate wells. The plate was incubated for 18 h at 37°C/5% CO_2. Following this incubation, non-adherent cells were resuspended by pipetting and 100 µl of the suspension was transferred to a mAb-coated and blocked ELISA plate (Nunc-Immunoplate MaxiSorp C96) (Nunc, Roskilde, Denmark) as describe below for the cell culture capture-ELISAs or in a fresh tissue culture plate to obtain supernatants for the conventional ELISAs.

Cell Culture Capture-ELISA

The ELISA plates into which the non-adherent cell suspension was transferred were previously coated overnight at 4°C with 100 µl of primary antibody at 2.5 µg/ml in coating buffer (68 mM $NaHCO_3$, 32 mM Na_2CO_3, pH 8.9). The plates were washed 3X with wash buffer (0.9% NaCl, 0.05% Tween-20, pH 7.3) using an automatic plate washer (Dynatech Ultrawash Plus)(Dynatech Laboratories, Chantilly, VA) and blocked for 1h at 37°C with 200 µl of 20% fetal bovine serum in PBS (pH 7.2). Subsequently, the plates were washed 3X and 100 µl of serially-

diluted recombinant cytokine standards was added to each well or 100 µl of the cell suspensions was dispensed into each well in triplicate. After a 6h incubation at 37°C/5% CO_2, the plates were washed 5X and 100 µl biotinylated mAb in dilution buffer (0.9% NaCl, 1% BSA, 20 mM Trizma base, 0.04% Tween-20, pH 7.3) was added to each well at a concentration of 1µg/ml. Following a 1h incubation at 37°C, the plates were washed 5X and 100 µl streptavidin-HRP was added to each well at a dilution of 1:2000 in dilution buffer. The concentrations of coating mAb, biotinylated mAb, and streptavidin-HRP used were previously determined by standardization on a conventional ELISA for maximal detection of each cytokine. After a 45 min incubation at 37°C, the plates were washed 8X and 100 µl of TMB substrate was added per well. Following a 10 min - 20 min incubation at room temperature, the reaction was stopped with 100 µl of 0.18 M H_2SO_4 per well. Optical density (O.D.) of the product was measured using a VMAX microplate reader (Molecular Devices, Menlo Park, CA) with a test wavelength of 490 nm and a reference of 570 nm. Cytokine concentration in each sample was calculated from absorbance data interpolated from the fitted standard curve by using the SOFTmax analytical program (Molecular Devices). All samples were run against a media control (blank) which was subtracted from their O.D. values.

Conventional ELISA

Following the entire 24h incubation, the plate was spun at 200g and then 75 µl of supernatant fluid from each well was collected and frozen at -70°C until used. For cytokine quantitation, 100 µl of serially diluted recombinant cytokine standards

and supernatant samples (diluted 1:2 in culture media) were incubated for 6h at at 37°C/5% CO_2. Following the sample incubation, all the steps were identical to the cell culture capture-ELISA described above.

Sensitivity of the Cell Culture Capture-ELISA

To determine whether the capture-ELISA was more sensitive than the conventional assay over a range of cell concentrations, the two assays were set up as described above for the detection of IL-2 and IL-4. Between 1 X 10^6 and 3.13 X 10^4 cells were cultured for 24h in a final volume of 200 µl in the wells of a 96-well plate and activated with either 5 µg/ml of ConA (for IL-2) or PMA (5 ng/ml) plus ionomycin (0.75 µM) (for IL-4).

Control Experiment

The following experiment was set up to test the possibility that incubation of cells for 6h on primary antibody coated plates might alter the rate of secretion of cytokine by previously activated cells. Splenocytes were activated with PMA plus ionomycin on regular tissue culture plates for 18h at 37°C/5% CO_2. The preactivated cells were then transferred into mAb coated or uncoated wells of an ELISA plate (all wells were blocked with 20% FBS prior to the addition of cells). Cells were again stimulated for 6h with 100 µl of PMA plus ionomycin, after which the plate was spun at 200g and the supernatant was discarded. Cytokine synthesis was then measured after 6h of additional incubation in the presence of fresh culture medium containing PMA plus ionomycin. Culture supernatants were collected and frozen at -70°C until

assayed by conventional ELISA to compare cytokine secretion between cells that were exposed to coating antibody for 6h compared to those that were not exposed to the coating antibody.

Statistics

Statistical differences between cytokine levels detected by conventional ELISA versus cell culture capture-ELISA was determined by Students' paired t-test using SigmaStat software (Jandel Scientific Co., San Rafael, CA). A value of $p<0.05$ was considered significant.

RESULTS

Comparison of IL-4 and IL-10 Detected by Cell Culture Capture-ELISA versus a Conventional ELISA

The experiment depicted schematically in Figure 1 was utilized to compare the level of detection of IL-4 and IL-10 by the cell culture capture-ELISA versus the conventional ELISA. Data in Figure 2 show that in every instance tested, IL-4 detection was significantly enhanced ($p<0.05$) whereas IL-10 detection was enhanced only upon stimulation with anti-CD3 mAb ($p<0.05$). However, following stimulation with ConA or PMA plus ionomycin IL-10 signal was enhanced in two of three groups (Figure 1- D, F). Therefore, regardless of the technique used to activate the cells, the levels of IL-4 and IL-10 detected from stimulated splenocytes of three individual mice were substantially higher when the cell culture capture-ELISA was used.

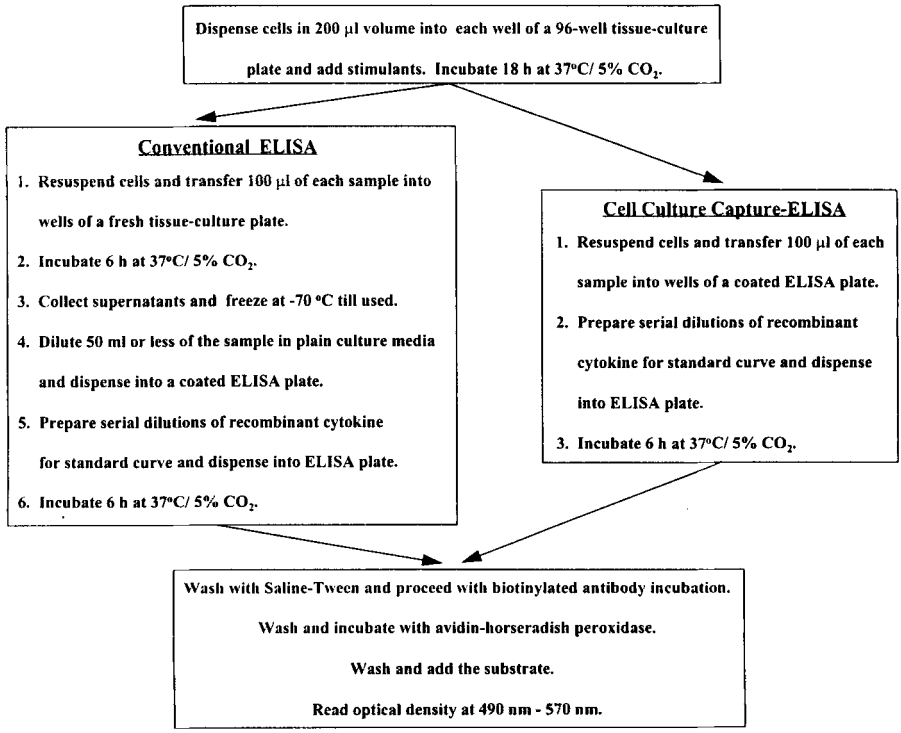

FIGURE 1. Schematic representing the experimental set up used to compare cytokine concentration detected by the conventional ELISA versus the cell culture-based ELISA.

Comparison of the Culture-based Technique versus Conventional ELISA for other Cytokines

In order to determine whether the cell culture capture technique could also be used to detect additional cytokines, we also evaluated the levels of IL-2 and IFN-γ in identical experiments. Figure 3 shows that higher levels of IL-2 released by ConA or anti-CD-3 mAb activated cells were also detected with the cell culture capture-

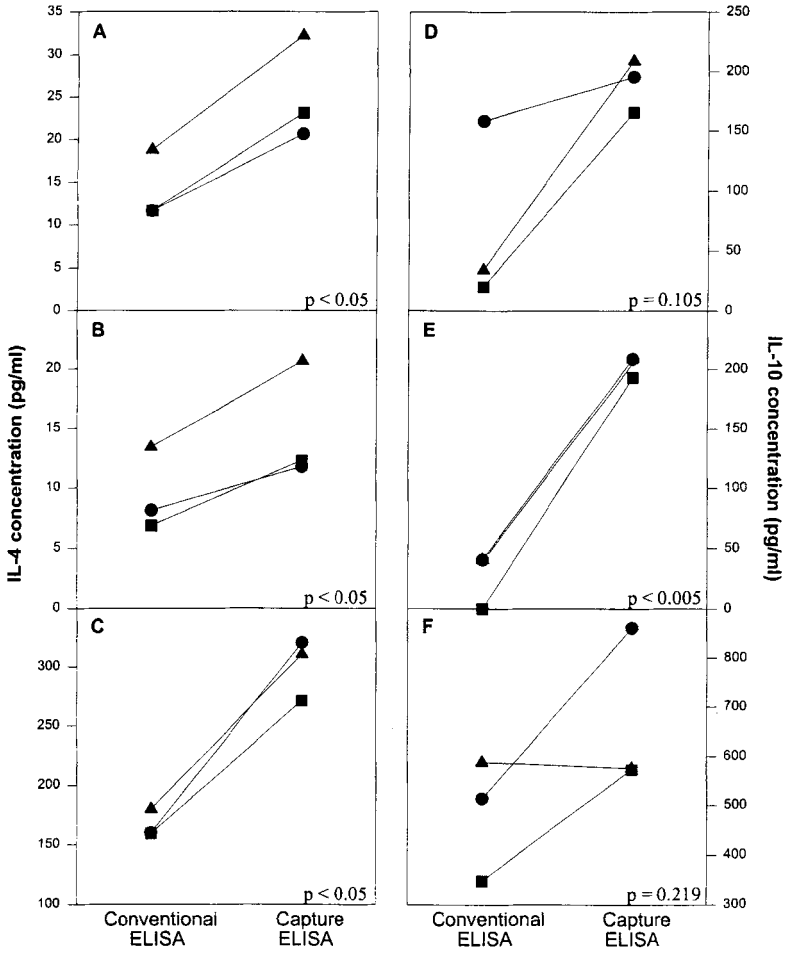

FIGURE 2. Comparison of IL-4 (A,B,C) and IL-10 (D,E,F) concentration detected from stimulated *in vitro* cultures of spleen cells from 3 individual mice by conventional ELISA and the cell culture based ELISA. Cells were stimulated with Con A (A- 10 μg/ml; D- 5 μg/ml), anti-CD3 mAb (B- 8 μg/ml; E- 4 μg/ml), or PMA (5 ng/ml) plus ionomycin (0.75 μM) (C,F) and cytokine levels were analyzed after 24 h. Each of the 3 pairs of points represents cell cultures from an individual mouse. Data shown are from a typical experiment.

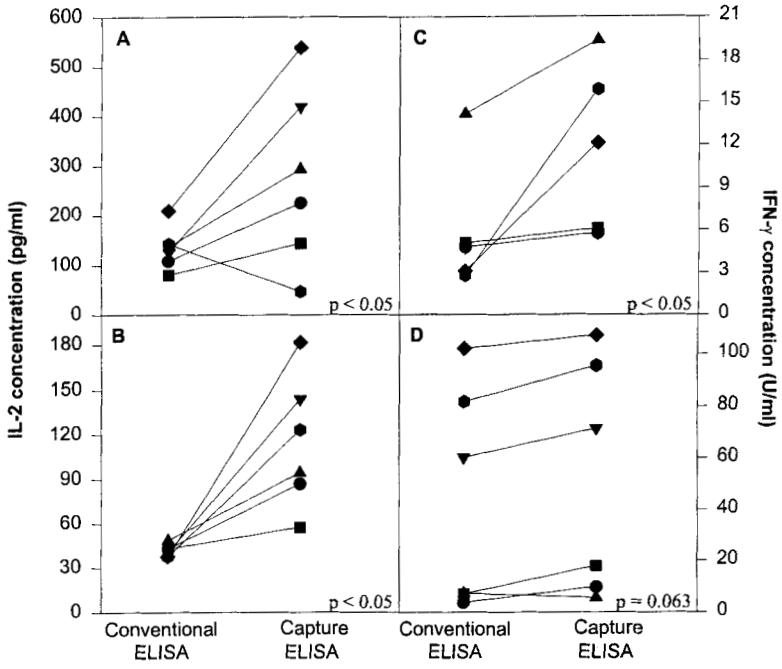

FIGURE 3. Comparison of IL-2 (A,B) and IFN-γ (C,D) concentration detected from stimulated *in vitro* cultures of spleen cells from 6 individual mice by conventional ELISA and the cell culture-based ELISA. Cells were stimulated with Con A (A- 5 μg/ml; C- 10 μg/ml), anti-CD3 mAb (8 μg/ml) or PMA plus ionomycin and cytokine levels were analyzed after 24 h. Each of the 6 pairs of points represents cell cultures from an individual mouse. Results shown represent combined data from two separate experiments.

ELISA ($p<0.05$). The enhancement of IFN-γ detection by the capture technique following ConA stimulation were statistically significant ($p<0.05$) while it approached significance ($p=0.063$) upon stimulation with anti-CD3 mAb. While the concentration of IFN-γ was also shown to be detected at higher levels by this technique, generally the enhancement was less than observed with IL-2, IL-4 and IL-10.

Evaluation of the Sensitivity of the Cell Culture Capture-ELISA

A comparison of the cell culture capture technique to conventional ELISA was performed with lower numbers of cells in culture as a means to assess the sensitivity of the capture technique. Tables 1 and 2 depict IL-2 and IL-4 concentrations, respectively, detected by the capture technique or in culture supernatants by a conventional ELISA from cultures of six serial concentrations of cells. The cell culture capture technique was more sensitive than the conventional ELISA, even at concentrations of cells as low as 6.25 X 10^4 cells per well.

Exposure of Lymphoid Cells to Coating Antibody does Induce a Higher Rate of Cytokine Secretion

Since the cell culture capture-ELISA was found to detect higher amounts of cytokines, the possibility was evaluated that the exposure of cells for 6 h to the coating antibody could trigger cells to secrete greater amounts of cytokines rather than just capturing more during synthesis. Hence the control experiment described in Materials and Methods was conducted for IL-4 and IL-10. Thus, we determined the amount of cytokine released by spleen cells previously activated and exposed (or not exposed) to plate-bound anti-cytokine antibody. An increased level of cytokine in the supernatants of cells exposed to coating antibody for 6 h was predicted if indeed such an exposure stimulated enhanced secretion. However, as indicated in Table 3, cells exposed to plate-bound anti-cytokine antibody did not secrete higher levels of any of the cytokines tested compared to cells which were not exposed to the plate-bound antibody.

TABLE 1

Sensitivity of Enhanced IL-2 Detection by the Capture-ELISA Technique is Consistent in Cultures Containing Low Numbers of Cells

# Cells per well	1×10^6	5×10^5	2.5×10^5	1.25×10^5	6.25×10^4	3.13×10^4
Conventional ELISA*	528.03	445.46	285.36	130.29	44.58	14.27
Cell Culture Capture-ELISA*	860.94	721.32	452.00	191.43	67.00	19.14
Fold Enhancement in detection	0.6	0.6	0.6	0.5	0.5	0.3

* Values shown represent cytokine concentration in pg/ml.

TABLE 2

Sensitivity of Enhanced IL-4 Detection by the Capture-ELISA Technique is Consistent in Cultures Containing Low Numbers of Cells

# Cells per well	1×10^6	5×10^5	2.5×10^5	1.25×10^5	6.25×10^4	3.13×10^4
Conventional ELISA*	45.61	34.94	21.07	15.76	7.65	5.05
Cell Culture Capture-ELISA*	127.61	82.78	55.45	31.42	20.48	6.06
Fold Enhancement in detection	1.8	1.4	1.6	1.0	1.7	0.2

* Values shown represent cytokine concentration in pg/ml.

TABLE 3

Plate-bound Anti-cytokine Antibody does not Enhance Cytokine Synthesis by Activated Spleen Cells

Cytokine	Spleen No.	24h cytokine concentration (pg/ml)	
		No Antibody	Plate-bound Antibody
IL-4	1	8.61	8.27
	2	10.52	7.13
	3	8.76	9.60
		(9.30 +/- 0.60)*	(8.33 +/- 0.71)*
IL-10	1	223.90	162.48
	2	200.35	200.35
	3	219.14	162.44
		(214.50 +/- 7.20)*	(175.10 +/- 12.63)*

* Shown within parentheses are the mean +/- SEM of the data from the three individual samples shown in the table.

DISCUSSION

IL-4 and IL-10 are two cytokines that have often been reported as being difficult to detect. Both these cytokines are recognized to play very important roles in immunomodulation and thus many immunologic studies remain incomplete without an accurate evaluation of levels of secretion *in vivo* as well as *in vitro*. We have observed that the concentrations of IL-4 and IL-10 produced by Con A- or anti-CD3-activated murine splenocytes as detected by conventional ELISA were sometimes at or below the level of sensitivity. Marginal detection of these cytokines

occurred in spite of the high sensitivity of conventional ELISAs in our laboratory (IL-4 and IL-10 ELISAs were generally around 10 pg/ml and 40 pg/ml, respectively), which were equally as sensitive as previously reported with these combination of mAbs (4,5). In addition to low level synthesis, utilization of secreted cytokines could also contribute to the marginal levels of detection. Use of anti-receptor blocking antibody to inhibit cytokine uptake may reduce this phenomenon. However, the available anti-IL-4R mAbs (4G3, 3E12, and TUGm2) are known react with the common γ subunit of IL-2R, IL-7R, IL-9R, and IL-15R as well. Hence, use of such antibodies may alter the capacity of cytokine-secreting cells in culture and as such may not reflect the actual cytokine concentration due to the *in vitro* stimulation. In this context we hypothesized that incubation of the cytokine-producing cells on an anti-cytokine coated ELISA plate might result in enhanced signal, due to the fact that coating mAb on the plate would bind cytokine soon after secretion and prevent its uptake by the cells. As predicted, this culture-based technique detected higher levels of IL-4 and IL-10 in every instance tested. We next tested the same for IL-2 and IFN-γ. The culture-based ELISA detected enhanced levels of IL-2 as in the case of IL-4 and IL-10; however, marginal enhancement was seen in the case of IFN-γ. This method may be considered as a combination of ELISPOT and conventional ELISA assays. While ELISPOT assays usually involve a 6h - 12h incubation of cells immediately upon *in vitro* activation and ELISA assays involve detection of cytokine from 24h - 72h culture supernatants, we cultured pre-activated cells on mAb-coated ELISA plates for the last 6h of a 24h total culture period. Although a 24h culture

period on the anti-cytokine mAb coated ELISA plates conceivably could capture more cytokine, we intended to investigate whether enhanced signal (i.e., higher sensitivity) would result if pre-activation was done prior to the transfer of cells to the mAb coated plates. While we find that this protocol sufficiently enhances cytokine detection, we suggest that investigators who intend to use this method must determine their own optimal pre-activation and capture periods.

Stimulation of cells with PMA and ionomycin resulted in higher cytokine production. Such stimulation could thus be used to enhance cytokine detection by the conventional ELISA. However, PMA plus ionomycin treatment does not accurately reflect physiological stimuli, such as induced by anti-CD3 mAb binding the T cell receptor. Hence in many investigations it may be more relevant to examine lower levels of cytokine release stimulated by more physiologic stimuli. In such cases we propose that the cell culture capture-ELISA would prove to be a better method of choice.

An important control study was to determine whether the exposure of stimulated cells to plate-bound coating mAb for 6h could trigger the cells to produce greater amounts of cytokine rather than just increase the level of detection. It is possible that the anti-cytokine mAb could bind some cell surface protein such as a membrane-bound form of the cytokine, and induce greater secretion of the cytokine. On the other hand, it is also possible that the cytokine in the medium, once bound to the plate-bound mAb, could bind cell surface cytokine receptors and cross-link them, resulting in enhanced secretion of the cytokine. However, studies to test this

possibility found no difference in the ability of cells exposed to mAb to secrete cytokine compared to cells that were not exposed to mAb.

While Con A, anti-CD3, and PMA plus ionomycin stimulated cytokine secretion to varying degrees, the fold-enhancement of cytokine detection with the capture-ELISA over the conventional method was consistent for each cytokine, regardless of the method used to activate the cells. Based on these findings we conclude that the cell culture capture-ELISA produces enhanced detection compared to the conventional ELISA and should therefore be useful in detection of cytokines that are traditionally difficult to detect.

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