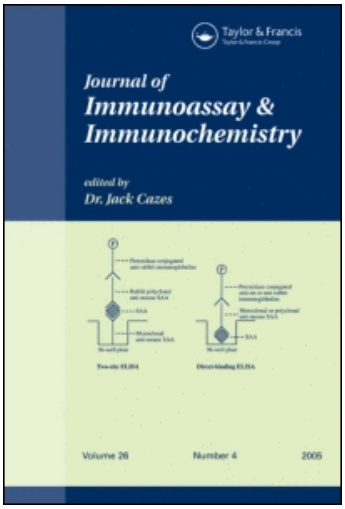

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A Time-Resolved Fluorescence Immunoassay (DELFIA) Increases the Sensitivity of Antigen-Driven Cytokine Detection

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ABSTRACT

In an effort to improve the quantification of the low levels of cytokines released in response to antigenic stimulation of T cells, a sandwich dissociation-enhanced lanthanide fluoroimmunoassay (DELFIA) was developed and compared to a standard sandwich ELISA. The DELFIA enhanced the sensitivity of a mouse IL-2 assay 8- to 27-fold, and a human GM-CSF assay 10-fold, as compared to colorimetric ELISA. The increase in sensitivity allows for the use of lower sample volumes per well, and the ability to run more assays per supernatant sample. This sensitive, nonisotopic alternative to other cytokine detection methods will be useful for

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those researchers wanting to quantitate low levels of antigen-driven cytokine production.

Key Words: DELFIA; ELISA; Time-resolved fluorescence; Cytokine; *T* cell.

INTRODUCTION

The need to detect and quantify cytokine production as a measure of antigen induced *T* cell response has spawned a number of different methods to date, including bioassay, radioimmunoassay, and ELISA. While all of these methods are currently being used to measure cytokines, they each have drawbacks that are well known. Bioassays are considered to be the most sensitive; however, the cells used may lose their dependence on the specific cytokine, or there may not be a bioassay available for a particular cytokine. Radioimmunoassays require the use of radioisotopes with the inherent problems associated with handling radioactive samples and waste disposal. For these reasons, as well as the general ease of use, ELISA has remained a popular method for cytokine detection despite the issue of the sensitivity of the assay. As evidenced by several studies, there has been much interest in increasing the sensitivity of this method, either by ELISA with repetitive rounds of amplification[1] or by the incorporation of cultured cells (celELISA) into the assay.[2,3]

The dissociation-enhanced lanthanide fluoroimmunoassay (DELFIA) presents an alternative to the more commonly used cytokine detection assays. In the last ten years, three articles describing cytokine detection by DELFIA have been published, indicating that this is an under-utilized technique for the measurement of cytokines. Culture supernatants of peripheral blood mononuclear cells have been assayed by DELFIA for IL-6 and TNFα.[4] DELFIA has also been used to measure the production of these same cytokines in a macrophage cell line,[5] and to detect the presence of serum interferon-alpha in clinical samples.[6] However, DELFIA is a widely used method in a number of areas of research such as lipoprotein metabolism studies, steroid and peptide hormone measurements, and enzyme activity assays.[7–11] The cytokine detection assays using DELFIA are sandwich assays that utilize one of four lanthanide chelates as the fluorophore, and are measured with time-resolved fluorometry. The assays use the same antibodies as a sandwich ELISA, and either the detection antibody or streptavidin can be labeled with the lanthanide chelate. These chelates have a longer

half-life and a wider Stoke's shift than conventional fluorophores, which allows the measurement of light at an emission wavelength and fixed time point after background fluorescence from plastic and serum components has receded. This produces significantly enhanced sensitivity with greatly reduced background interference. Typically, time-resolved fluorometry is 10 to 100 times more sensitive than a standard colorimetric ELISA.[12]

The issue of sensitivity is critical in our particular application, wherein mixture-based synthetic combinatorial libraries are used to study T cell recognition and to identify and optimize *T* cell clone epitopes. The successful use of these libraries has been demonstrated and has been recently reviewed.[13] In order to be able to detect the lowest possible levels of cytokine production, with the lowest number of cells, it is important to have optimized assays for the detection of *T* cell activation. To develop a more sensitive method of direct detection of cytokine production, we have compared the sensitivities of a colorimetric ELISA to a europium (Eu) DELFIA assay for the detection of murine interleukin 2 (mIL-2) and human granulocyte-macrophage colony-stimulating factor (GM-CSF). The use of the DELFIA method produced IL-2 detection limits that are 8 to 27 times lower than ELISA. For GM-CSF, there was a ten-fold improvement in the detection limit. This translates into an IL-2 detection limit of 5–16 pg/mL and 7 pg/mL for GM-CSF.

EXPERIMENTAL

Antibodies

Monoclonal antibodies were purchased from Pharmingen (San Diego, CA,). Anti-IL-2 capture antibody (catalog number 554424) and biotinylated anti-IL-2 detection antibody (catalog number 554426) were used for the murine IL-2 assays at concentrations of 1.0 and 0.125 μg/mL, respectively. Additionally, a nonbiotinylated version of the detection antibody (catalog number 554425) that had been directly labeled with europium was used for some assays at the same concentration as the biotinylated version. The europium-labeled anti-IL-2 antibody was kindly provided by Dr. Thomas Campbell, Perkin-Elmer Life Sciences, Norton, Ohio. Pharmingen recombinant murine IL-2 (catalog number 550069) was used as the standard for all IL-2 assays.

Anti-human GM-CSF capture antibody (catalog number 554502) and biotinylated anti-human GM-CSF detection antibody (catalog

number 554505) were also purchased from Pharmingen. They were used at concentrations of 2.0 and 0.5 μg/mL, respectively. Pharmingen recombinant human GM-CSF (catalog number 550068) was used as the standard for all GM-CSF assays.

Sandwich DELFIA Protocol

In the sandwich DELFIA for IL-2 or GM-CSF, high binding, 1/2 area ELISA plates (96-well; Corning Science Products, Rochester, NY) were coated with capture antibody diluted in carbonate buffer (0.05 M, pH 9.6). After overnight incubation at 4°C, plates were blocked with 3% bovine serum albumin in Dulbecco's phosphate buffered saline (BSA/DPBS) at pH 7.4 for 1 to 2 h at 37°C. Plates were then washed 6 times with 0.05% Tween in DPBS using a SkanWasher 300 plate washer (Skatron Instruments, Inc., Sterling, VA). IL-2 standards diluted in RPMI 1640 media and 8% fetal bovine serum (FBS), or GM-CSF standards diluted in IMDM media and 8% human serum were added to the plates along with the corresponding assay supernatants and incubated overnight at 4°C. Plates were washed as before and biotinylated or direct-labeled detection antibody in DELFIA assay buffer (catalog number 1244-111) from PerkinElmer LifeSciences, (Boston, MA) was added. Plates were incubated for 1 h at room temperature. Europium labeled streptavidin (catalog number 1244-360), also from PerkinElmer LifeSciences, was used at a 1:2000 dilution in DELFIA assay buffer for the IL-2 assay (1:1000 dilution for GM-CSF). The diluted streptavidin–europium (SA–Eu) was placed on the biotin-labeled antibody plates and incubated for 1 h at room temperature. Plates were washed 9 times and DELFIA enhancement solution (catalog number 1244-105) from PerkinElmer LifeSciences was added. Direct-labeled antibody plates were also washed 9 times and enhancement solution was added as before. In both cases, plates were allowed to sit at room temperature for 10 min prior to reading time resolved fluorescence measurements at 615 nm with a Wallac VICTOR^2 1420 multilabel HTS counter (Wallac Oy, Turku, Finland).

Colorimetric Sandwich ELISA Protocol

The sandwich DELFIA protocol was followed with the following modifications. Biotin-labeled detection antibody was diluted in the 3% BSA/DPBS buffer. Streptavidin peroxidase-conjugate (catalog

number 189733) from Calbiochem (La Jolla, CA) was used in place of streptavidin-europium at a 1:1000 dilution, also in the BSA buffer. Color was developed by the addition of *o*-phenylenediamine (catalog number P 8287) from Sigma (St. Louis, MO) at 1.7 mg/mL in distilled water and 3% H_2O_2. The reaction was allowed to proceed for 4–8 min and was stopped by the addition of 4N H_2SO_4. The optical density was measured at 492 nm in a UVMax plate reader (Molecular Devices, Sunnyvale, CA).

Culture Supernatants for IL-2 and GM-CSF Detection

For measurement of IL-2, LMR 17, a *T* cell hybridoma specific for the LACK peptide sequence of Leishmania was incubated with LB 27.4 antigen presenting cells in the presence or absence of dilutions of LACK peptide sequences. The LMR 17 hybridoma originated in the laboratory of Dr. Nicolas Glaichenhaus, Institut de Pharmacologie Moleculaire et Cellulaire, Valbonne, France, and was kindly provided to us by Dr. Claudia Raja Gabaglia, La Jolla Institute for Allergy and Immunology, San Diego, CA. After incubation for 24 h in RPMI 1640 media (Bio-Whittacker, Walkersville, MD) and 8% FBS (Hyclone, Logan, Utah), supernatants were collected and transferred to capture antibody-coated plates as described above. For the measurement of GM-CSF, TL3A6, a *T* cell clone specific for myelin basic protein (MBP) 83-99 was stimulated with agonist peptides and matched PBMC (peripheral blood mononuclear cells) as antigen presenting cells in IMDM media (Bio-Whittacker, Walkersville, MD) and 8% AB^+ human serum. After 72 h supernatants were collected and transferred to capture antibody-coated plates.

RESULTS

Cytokine Standards

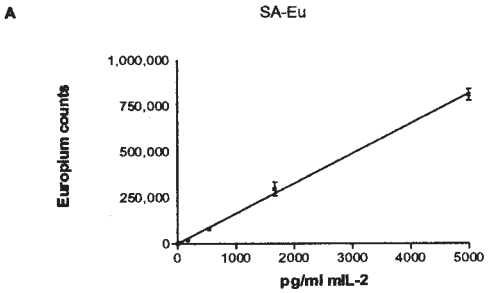
We first determined the minimum detection limit of the classical colorimetric ELISA and the europium time resolved fluorescence DELFIA with the cytokines IL-2 and GM-CSF. In the IL-2 assays, a standard sandwich ELISA was compared to a streptavidin-europium (SA–Eu) DELFIA and a direct europium-labeled antibody (Eu–Ab) DELFIA. In all cases plates were coated with capture antibody, blocked, and the standards were incubated at 4°C overnight. The ELISA and SA–Eu plates were bound with biotinylated secondary antibody,

while the direct-labeled assay received europium-secondary antibody. The SA–Eu and ELISA plates were further labeled with the appropriate streptavidin conjugate. Three-fold serial dilutions of mIL-2 were tested under all three protocols (Fig. 1). In the GM-CSF assays, two-fold serial dilutions of hGM-CSF were tested under the sandwich ELISA and SA–Eu DELFIA protocols as described above (Fig. 2). The linear regression data for 4 replicates in one assay was used and comparable results have been found in other assays. The table accompanying each graph shows the average and percent coefficient of variation (%CV) values of the data used to generate the curves.

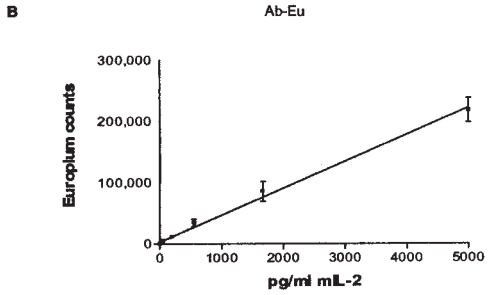
For both cytokines tested, the sandwich DELFIA method was more sensitive than the ELISA method. When assay sensitivity is defined as the average background +3 standard deviations (S.D.) of the mean, the detection limit for IL-2 was 8–27 times more sensitive when measured with the DELFIA methods. Using an alternative definition for assay sensitivity of twice the average background, the DELFIA methods were 9–32 times more sensitive for IL-2. For GM-CSF, a detection limit of background +3 S.D. was 10 times more sensitive with DELFIA, and at twice the background, it was 6 times more sensitive (Table 1). The DELFIA method at a given cytokine concentration has a greater signal to background ratio than the ELISA, e.g., 550 pg/mL IL-2 when measured with SA–Eu has a ratio of 127 as compared to a ratio of 6 for the ELISA method. At a lower concentration of IL-2, such as 21 pg/mL, the ratio is 4.4 vs. 1.2 for the ELISA. The signal to background ratios at 500 pg/mL for GM-CSF are 18 for DELFIA and 4 with ELISA. The comparable ratios at 31 pg/mL are 2.1 and 1.1, respectively.

Supernatants

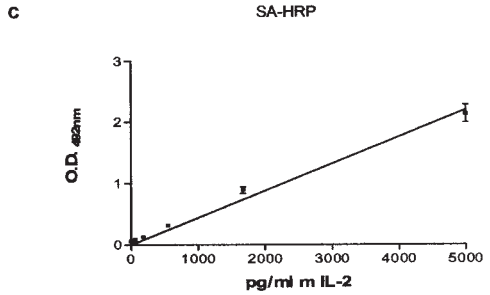
Once the detection methods had been optimized with cytokine standards, in vitro activation assays were run to assess the ability of the different methods to detect these same cytokines in culture supernatants. For the IL-2 studies, LMR 17, a *T* cell hybridoma that secretes IL-2 in the presence of soluble Leishmania antigens such as LACK (Leishmania homolog of receptors for activated C kinase) peptide was used.[14,15] The hybridoma was incubated with antigen presenting cells in the presence of dilutions of either the LACK peptide or a LACK analog #670. Supernatants were collected after 24 h and aliquots were analyzed by SA–Eu or Eu–Ab DELFIA as well as ELISA. In Figs. 3A and 3C, the two DELFIA methods are able to detect IL-2 produced in response to



pg/ml IL-2	Avg	SD	%CV
5,000	810,600	54,831	6.8
1,670	297,725	65,329	21.9
550	78,358	15,272	19.5
185	22,649	2,864	12.6
62	7,311	1,008	13.8
21	2,728	416	15.3
6.9	1,288	151	11.7
2.3	833	117	14.0
0.76	669	95	14.3
0.25	623	75	12.0
0.08	757	264	34.8
0	618	25	4.1

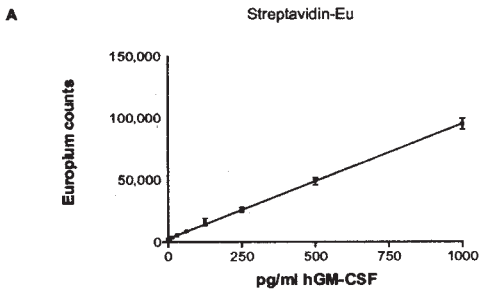


pg/ml IL-2	Avg	SD	%CV
5,000	218,068	19,574	9.0
1,670	85,473	16,106	18.8
550	34,269	6,181	18.0
185	12,228	1,925	15.7
62	4,809	751	15.6
21	2,028	319	15.7
6.9	1,081	102	9.5
2.3	770	128	16.7
0.76	631	70	11.1
0.25	598	79	13.1
0.08	572	82	14.4
0	642	19	2.9

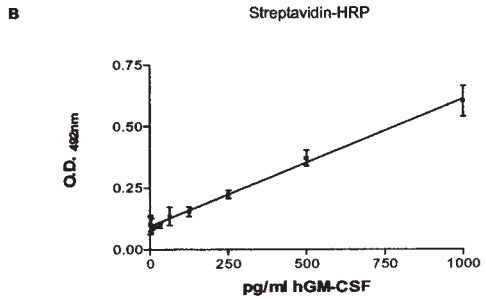


pg/ml IL-2	Avg	SD	%CV
5,000	2.137	0.144	6.8
1,670	0.887	0.053	6.0
550	0.314	0.014	4.5
185	0.134	0.007	5.3
62	0.077	0.005	6.6
21	0.060	0.006	10.4
6.9	0.051	0.004	7.1
2.3	0.050	0.003	5.1
0.76	0.052	0.003	5.9
0.25	0.058	0.007	11.5
0.08	0.049	0.002	3.1
0	0.049	0.001	2.3

Figure 1. Comparison of the IL-2 DELFIA with an ELISA. IL-2 was serially diluted and measured by the two DELFIA protocols (A, B) and the ELISA (C) to generate standard curves. The SA–Eu DELFIA and the ELISA used an identical biotinylated secondary antibody. The direct-labeled DELFIA used a europium-antibody conjugate in place of the biotinylated secondary antibody. For each concentration of cytokine an average of four replicates of either europium counts or optical density is shown. The standard curve was determined by linear regression analysis using a scientific graphics software program (GraphPad Prism; GraphPad Software, Inc., San Diego, CA).



pg/mL GM-CSF	Avg	%CV
1,000	94,982	4.7
500	48,776	5.8
250	26,124	7.0
125	16,200	18.1
62.5	8,822	12.9
31.3	5,497	9.6
15.6	4,154	13.8
7.81	3,210	10.0
3.91	2,817	11.8
1.95	2,629	10.3
0.98	2,530	8.0
0	2,667	9.7



pg/mL GM-CSF	Avg	%CV
1,000	0.603	10.3
500	0.372	8.5
250	0.224	7.0
125	0.154	12.1
62.5	0.135	27.1
31.3	0.096	9.2
15.6	0.091	3.9
7.81	0.089	8.8
3.91	0.097	31.1
1.95	0.107	30.7
0.98	0.098	36.7
0	0.089	15.1

Figure 2. Comparison of the GM-CSF DELFIA with an ELISA. GM-CSF was serially diluted and measured by the DELFIA protocol (A) and the ELISA (B) to generate standard curves. Both methods used an identical biotinylated secondary antibody. For each concentration of cytokine an average of four replicates of either europium counts or optical density is shown. The standard curve was determined by linear regression analysis using a scientific graphics software program (GraphPad Prism; GraphPad Software, Inc., San Diego, CA).

2.2 μg/mL of stimulating peptide (LACK or the #670 analog). At that same concentration, these peptides produce an IL-2 response that is below the detection limit of the ELISA (Fig. 3B), even though the ELISA used the same antibodies as in the SA–Eu assay. The only difference between the two is the use of europium vs. peroxidase.

For GM-CSF studies, an MBP superagonist derived from the screening of a decapeptide positional scanning library, plus the ten alanine substitution analogs of this superagonist compound were tested in a proliferation assay with TL3A6, an MBP-specific *T* cell clone established from the peripheral blood lymphocytes of a multiple sclerosis patient (data not shown). After 72 h, and prior to pulsing with ^{3}H-thymidine, supernatants were collected and tested in the SA–Eu DELFIA and

Table 1. Comparison of the DELFIA and ELISA methods for detection of two cytokines in *T* cell media.

Assay	ELISA		DELFIA (Ab–Eu3+)		DELFIA (streptavidin–Eu3+)	
Cytokine	Bkgd + 3S.D (pg/mL)	2 × Bkgd (pg/mL)	Bkgd + 3S.D (pg/mL)	2 × Bkgd (pg/mL)	Bkgd + 3S.D (pg/mL)	2 × Bkgd (pg/mL)
Murine IL-2[a]	136	227	16	26	5	7
Human GM-CSF[b]	69	163	NT	NT	7	27

[a]Capture antibody concentration of 1.0 μg/mL and detection antibody concentration of 0.125 μg/mL used for IL-2 assays.
[b]Capture antibody concentration of 2.0 μg/mL and detection antibody concentration of 0.5 μg/mL used for all GM-CSF assays.

ELISA assays (Fig. 4). GM-CSF produced by TL3A6 was detected in all of the supernatants by the DELFIA method (Fig. 4A), whereas the less sensitive ELISA method was only able to detect the cytokine in two of the ten supernatants tested (Fig. 4B). It is clear that for both IL-2 and GM-CSF, DELFIA is a more sensitive method for the measurement of the low levels of these cytokines produced in antigen-driven *T* cell activation assays.

DISCUSSION

In this study, we have demonstrated that the DELFIA method is more sensitive than the comparable ELISA method for both mIL-2 and hGM-CSF. Additionally, we have shown that a DELFIA protocol utilizing europium-labeled streptavidin is equivalent to both the double amplified ELISA[1] and the celELISA[3] methods for detecting mIL-2 at 5 pg/mL without the additional rounds of amplification or culturing of cells involved in these two methods. At mid-range concentrations for both cytokines, the europium based assays have greater signal to background ratios when compared to ELISA. In practical terms, this translates into the ability to analyze lower sample volumes per well, and to run more assays per supernatant sample. The DELFIA method also compares favorably to the published quantitation ranges of 1–700 pg/mL for IL-2 bioassay with CTLL-2 cells, and 5–1,000 pg/mL for GM-CSF bioassay with M-O7e cells.[16]

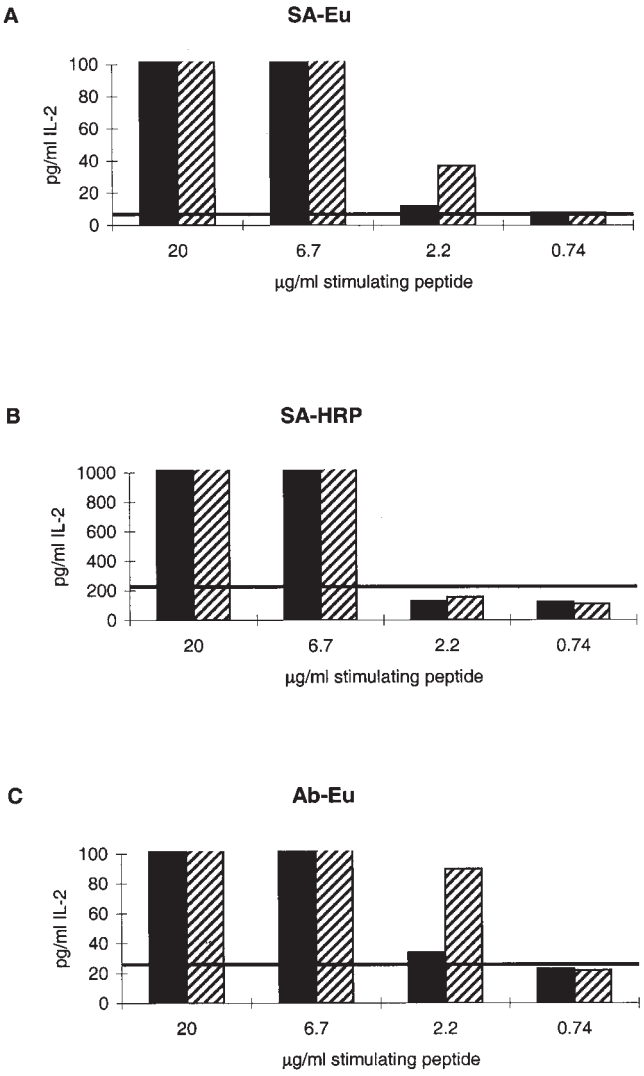


Figure 3. Murine IL-2 in culture supernatants analyzed by (A) streptavidin europium, (B) streptavidin peroxidase and (C) europium-labeled antibody. Cells were incubated with appropriate antigen presenting cells plus dilutions of LACK (■) or #670 (▨) peptide. Supernatants were transferred at 24 h to capture antibody coated plates and analyzed according to the protocols described. Horizontal line represents the detection limit, defined as twice the average background for each method. Note: concentration values below assay detection limits are not reliable.

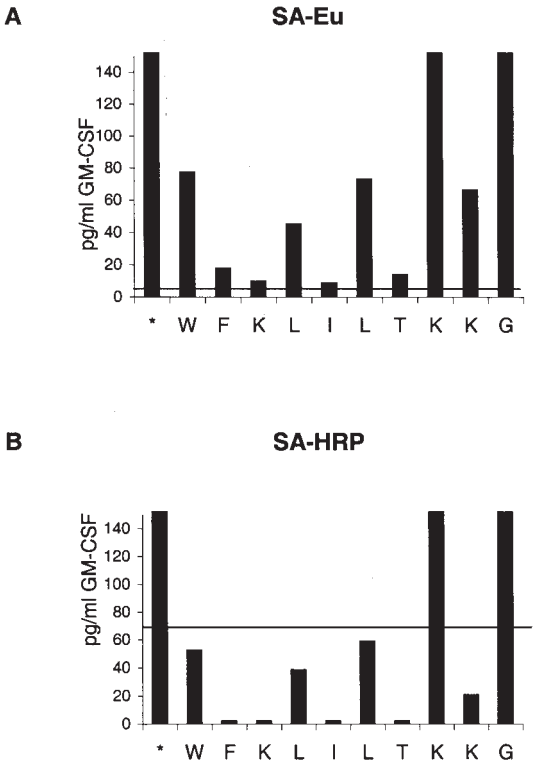


Figure 4. Human GM-CSF in culture supernatants analyzed by (A) streptavidin europium or (B) streptavidin peroxidase. Cells were incubated with appropriate antigen presenting cells plus agonist peptides at 2.5 μg/mL. Supernatants were transferred at 72 h to capture antibody coated plates and analyzed according to protocols described. Horizontal line represents the detection limit, defined as twice the average background for each method. (*) MBP superagonist. Each residue of this peptide was substituted with alanine, and the effect of this substitution on GM-CSF production is shown as bars above each residue of the parent peptide. Note: concentration values below assay detection limits are not reliable.

This study also compared two variations of the DELFIA method for the detection of mIL-2 in a standard curve, as well as in culture supernatants. The direct-labeled secondary antibody was somewhat better at detecting low levels of IL-2 production in culture supernatants (Fig. 3C). It was also a faster method as the assay was complete after the secondary antibody-binding wash step. However, the researcher must

either label their own secondary antibody or have it custom labeled. In comparison, streptavidin–europium is commercially available and can be used with any biotinylated secondary antibody to easily convert an existing sandwich ELISA protocol to a DELFIA protocol. Due to the greater sensitivity of europium chelates as compared to colorimetric methods, it is necessary to use a DELFIA assay buffer for the preparation of secondary antibody and streptavidin–europium solutions to obtain optimal detection limits. Also, the use of a plate washer with buffer rather than hand washing the plates with water is recommended to achieve the best results. These two criteria are necessary in order to obtain the low background counts required to achieve maximum assay sensitivity.

Our findings support the use of DELFIA as a sensitive, reproducible, nonisotopic alternative to other cytokine detection methods. Using DELFIA, researchers can make use of the multifunction counters with photometry, luminometry, fluorometry and time-resolved fluorescence capabilities that are now commonly found in many laboratories. These assays should prove useful in applications requiring the detection of low levels of antigen-driven cytokine production.

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