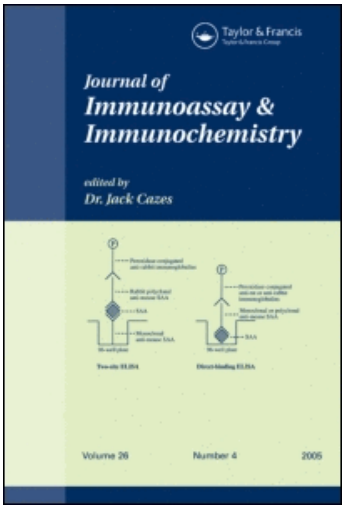

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Rapid Detection of Antigen-Specific Mouse IgE by Enzyme-Linked Immunosorbent Assay (Elisa)

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RAPID DETECTION OF ANTIGEN-SPECIFIC MOUSE IgE BY ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

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ABSTRACT

A rapid, sensitive, antigen-specific mouse IgE capture ELISA is described. A monoclonal rat anti-mouse IgE was used as the capture antibody, and a DNP-coupled BSA-biotinylated conjugate along with a peroxidase-avidin-biotin complex was utilized as the detection system. The lower detection limit of this assay is 8.5 ng/ml of antigen-specific IgE. With some modifications, this assay can be employed to screen for antigen specific antibodies of other isotypes and subtypes.

Keywords (murine IgE/ELISA/immunoassay/antigen-specific IgE)

INTRODUCTION

The standard assay to quantitate antigen-specific mouse IgE classically has been the passive cutaneous anaphylaxis (PCA) reaction in rats [1]. However, this assay requires a great deal of technical expertise, takes several days to complete, requires large numbers of animals, and has a small sample throughput. ELISA technology allows for high sample throughput, rapid assay times, and the ability to quantitate analyte. In recent times several ELISA based systems have been described to detect antigen-specific IgE [2-4]. These assays have limits of detection ranging from 200 pg/ml to 20 ng/ml. In comparison, the PCA test has been reported to have detection limits ranging from 2 ng/ml

to 100 ng/ml of specific IgE, depending upon the antibody sample source (polyclonal vs. monoclonal) [5,6]. We now report a sensitive, rapid IgE-capture ELISA for antigen-specific IgE which has an assay time of less than 3 hours when using pre-coated and blocked plates, but still retains sensitivity to less than 10 ng/ml of antigen-specific IgE.

Materials and Methods

Anti-mouse IgE

Monoclonal rat anti-mouse IgE was obtained commercially (U.S. Biochemical, Cleveland, Ohio). This preparation is a rat monoclonal of subclass IgG_{2a} with kappa light chains (clone #6HD5).

Biotinylation of DNP-BSA

DNP-BSA was labeled with N-hydroxysuccinimidobiotin (Sigma,St. Louis,MO) [7].

Dinitrophenol Conjugations

2,4-Dinitrobenzenesulfonic acid, sodium salt (Kodak, Rochester,NY) was coupled to bovine serum albumin (Miles,Elkhart,IN) or ovalbumin (ICN,Lisle,IL) as described previously [8].

Mouse IgE Against Dinitrophenol

Monoclonal mouse IgE to dinitrophenol (DNP) was obtained commercially (ICN ImmunoBiologicals, Lisle, IL).

Preparation of Mouse IgG Against DNP Standard

Six week-old female CBA/J mice (Jackson Laboratories, Bar Harbor, ME) were immunized intraperitoneally (i.p) on day 0 with a 0.1 ml injection volume containing 200 µg of DNP-bovine serum albumin (DNP-BSA) emulsified in complete Freund's adjuvant (Difco, Detroit, MI). A booster injection of 0.1 ml containing 200 µg of DNP-BSA emulsified in Freund's incomplete adjuvant (Difco, Detroit, MI) was given i.p. on day 14. Animals were sacrificed by CO_2 asphyxiation on day 21 and bled by cardiac puncture. The blood was allowed to clot overnight at 4°C, and the serum was removed and stored at -20°C until use. DNP specific IgG was harvested by passing the sample through Protein G Sepharose 4 Fast

Flow, and then by affinity purification of the bound fraction through a DNP-coupled CNBr activated Sepharose 4B column. Specificity to DNP was tested by ELISA (data not shown). Protein quantitation was performed using Bio-Rad Protein Dye Concentrate (Bio-Rad, New York, NY) using supplied BSA as the standard.

Mouse IgE Samples

Six week-old female CAF_1 mice (Jackson Laboratories,Bar Harbor,ME) were immunized i.p. on day 0 with a 0.1 ml injection volume containing 1 µg DNP-OA in 1 mg of alum (Accurate Chemical & Scientific Corp., Westbury, NY). Animals were sacrificed on various days and cardiac puncture performed for serum samples. Remaining animals were boosted with the same antigen preparation on day 35, and then sacrificed and bled as previously described at various times after the booster. All samples were stored at -20°C until analysis could be performed.

Mouse Immunoglobulin Samples

IgG_1,kappa (MOPC21); IgG_{2a},kappa (UPC21); IgG_{2b},kappa (MOPC141); IgG_3,kappa (FLOPC21); and IgM,lambda (MOPC104) were obtained commercially (Sigma,St. Louis,MO).

DNP Specific IgE Capture ELISA

Immulon 4 microtiter plates (Dynatech,Alexandria,VA) were coated for 16 hours at 4°C with 100 µl/well of a 5 µg/ml concentration of rat anti-mouse IgE (U.S. Biochemical,Cleveland,OH) diluted in 50 mM carbonate buffer, pH 9.6. The plates were emptied and 200 µl/well of a 1% (w/v) of bovine serum albumin (BSA) in PBS (Sigma, St. Louis,MO) was added to block unreacted protein binding sites. The plates were incubated for 2 hours at 37°C then washed using an Ultrawash plate washer (Dynatech,Alexandria,VA) set for 3 wash cycles of 300 µl/well with PBS containing 0.05% Tween 20. IgE standards and test sera were diluted in 20 mM potassium phosphate, pH 8.0, containing 2% (w/v) BSA and 0.05% Tween 20 with 100 ul/well of sample added to each well. The plates were covered and incubated at 37°C for 1 hour and then washed.

DNP-BSA-biotin conjugate was added (100 µl/well), diluted in sample diluent and the plates were incubated for 1 hour at 37°C. After washing, 100 µl of peroxidase-ABC (Vector Labs,Burlingame,CA) prepared according to manufacturer's directions in sample diluent was added to each well and the plates were incubated at ambient temperature for 20 minutes. Following a final wash, 100 µl/well of (2.2'-azino-di-[3-ethyl-benzthiazoline sulfonate]) (ABTS) substrate (Kirkegaard & Perry Laboratories, Gaithersburg,MD) warmed to 37°C was added and the plates were covered and incubated at 37°C for 15 minutes. The optical densities were recorded at 405/630 nm using a Lambda Reader and data processed using the ELISAsoft software package (Perkin-Elmer,Norwalk,CT). Antibody positive cutoff values were chosen as 2 standard deviations above background. The specificity of the IgE capture was determined by comparing the optical densities of antigen specific IgE against antigen specific IgG as described below.

ELISA to Determine Assay Specificity

Immulon 4 plates were coated as previously described with 5 µg/ml (100 µl/well) of rat anti-mouse IgE and blocked as previously described. Equal concentrations of purified IgG anti-DNP and purified IgE anti-DNP were added (200 ng/ml serially diluted through 3.1 ng/ml) in 20 mM potassium phosphate, pH 8.0, containing 2% (w/v) BSA and 0.05% Tween 20. The assay proceeded as described previously. Three separate assays with each point in triplicate were run.

ELISA to Determine Interference of Normal Mouse Serum

Purified IgE against DNP was prepared at a concentration of 1000 ng/ml in either ELISA diluent (20 mM potassium phosphate, pH 8.0, containing 0.05% Tween 20 and 2% (w/v) BSA) or in normal mouse serum. These samples were diluted serially from 1:5 through 1:320 (200 ng/ml through 3.1 ng/ml) in ELISA diluent and the assay was performed as previously described.

RESULTS

Figure 1 shows the standard curve generated by this ELISA assay when run on three separate plates with each sample point performed in

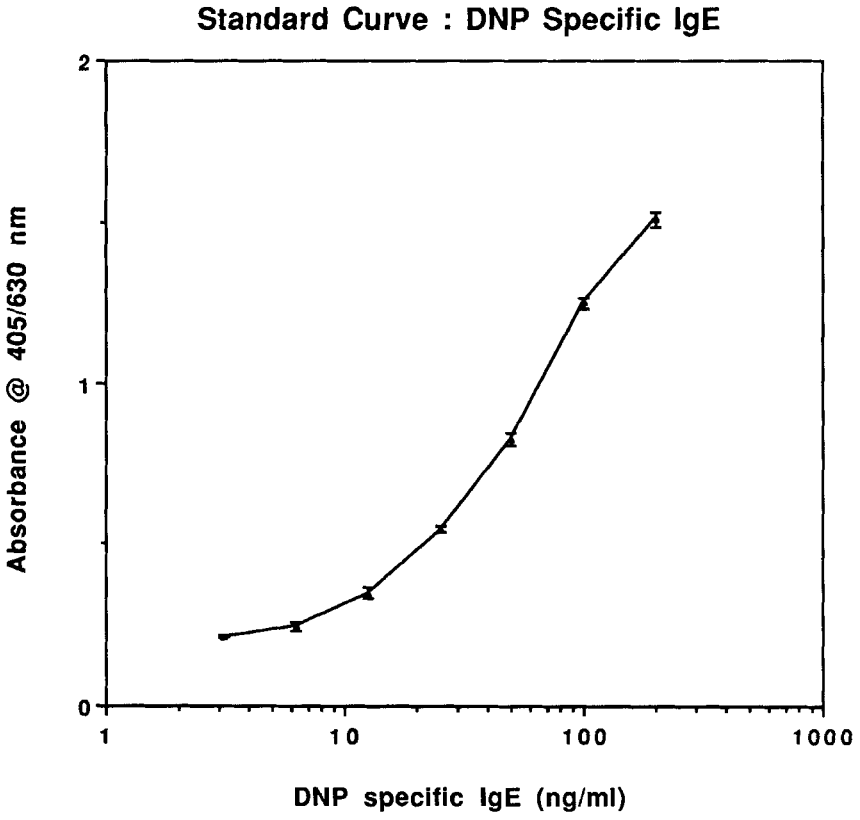

FIGURE 1. IgE_{DNP} Standard Curve Generated by ELISA (n=9 for Each Point).

triplicate. Purified DNP-specific IgE was run diluted serially from 200 ng/ml through 3.1 ng/ml resulting in a minimum antigen-specific IgE detection of 8.5 ng/ml.

A variety of blocking proteins were evaluated in this system, including BSA, milk protein (Kirkegaard & Perry Labs, Gaithersburg,MD), and fetal bovine serum (FBS). BSA was found to be superior and was incorporated into the ELISA reagent diluent at a concentration of 2% (w/v) (data not shown).

TABLE 1

Capture Antibody Specificity for IgE_{DNP} Over IgG_{DNP}

[Antibody] (ng/ml)	$Abs_{405/630\ nm}$ (IgE_{DNP})	$Abs_{405/630\ nm}$ (IgG_{DNP})
200.0	1.508	0.125
100.0	1.251	0.145
50.0	0.827	0.119
25.0	0.545	0.128
12.5	0.347	0.193
6.3	0.248	0.137
3.1	0.215	0.147
0.0	0.143	0.143

Various DNP and biotin loads were evaluated on the BSA carrier protein. A load of 7 DNP's per BSA in conjunction with a 50:1 molar excess of N-hydroxysuccinimidobiotin to carrier BSA was found to be optimal for this assay with respect to sensitivity and minimum background levels.

Rigorous controls were used to assure that the antibody capture was specific for IgE. Assays were run on three separate occassions with each point in triplicate using highly purified DNP-specific IgG diluted serially from 200 ng/ml through 3.1 ng/ml. The results were tabulated for the three assays (n=9 for each point) and are presented in Table 1. None of the absorbances were above the antibody positive cutoff value (OD=0.256). Additionally, to ascertain the interference of normal mouse serum (NMS) at high concentrations in the assay, CAF_1 pre-immune NMS was run serially diluted from 1:10 through 1:640 (n=9 for each point). Again, none of the absorbance values were above the 0.256 antibody positive cutoff value. These data are presented in Table 2.

To further show the specificity of the capture reaction the assay was run with the IgE standard being applied after blocking the wells with

TABLE 2

Background Effects of CAF_1 Normal Mouse Serum

Dilution of normal mouse serum	$Absorbance_{405/630\ nm}$
1:10	0.150
1:20	0.162
1:40	0.138
1:80	0.144
1:160	0.158
1:320	0.149
1:640	0.166
buffer only	0.143[a]

[a] antibody positive cutoff value (2 standard deviations above background) = 0.256 absorbance units

TABLE 3

Competition of Other Ig Isotypes and Subtypes with IgE_{DNP} for Capture Antibody Binding

	Absorbance at 405/630 nm					
$[IgE_{DNP}]$ (ng/ml)	IgE_{DNP} alone	IgG_1	IgG_{2a}	IgG_{2b}	IgG_3	IgM
200.0	1.460	1.443	1.425	1.405	1.402	1.462
100.0	1.297	1.309	1.268	1.272	1.253	1.353
50.0	0.966	0.932	0.908	0.923	0.939	1.025
25.0	0.631	0.560	0.545	0.572	0.626	0.676
12.5	0.378	0.398	0.389	0.426	0.399	0.412
6.3	0.250	0.263	0.313	0.299	0.279	0.319
3.1	0.199	0.200	0.254	0.244	0.268	0.248

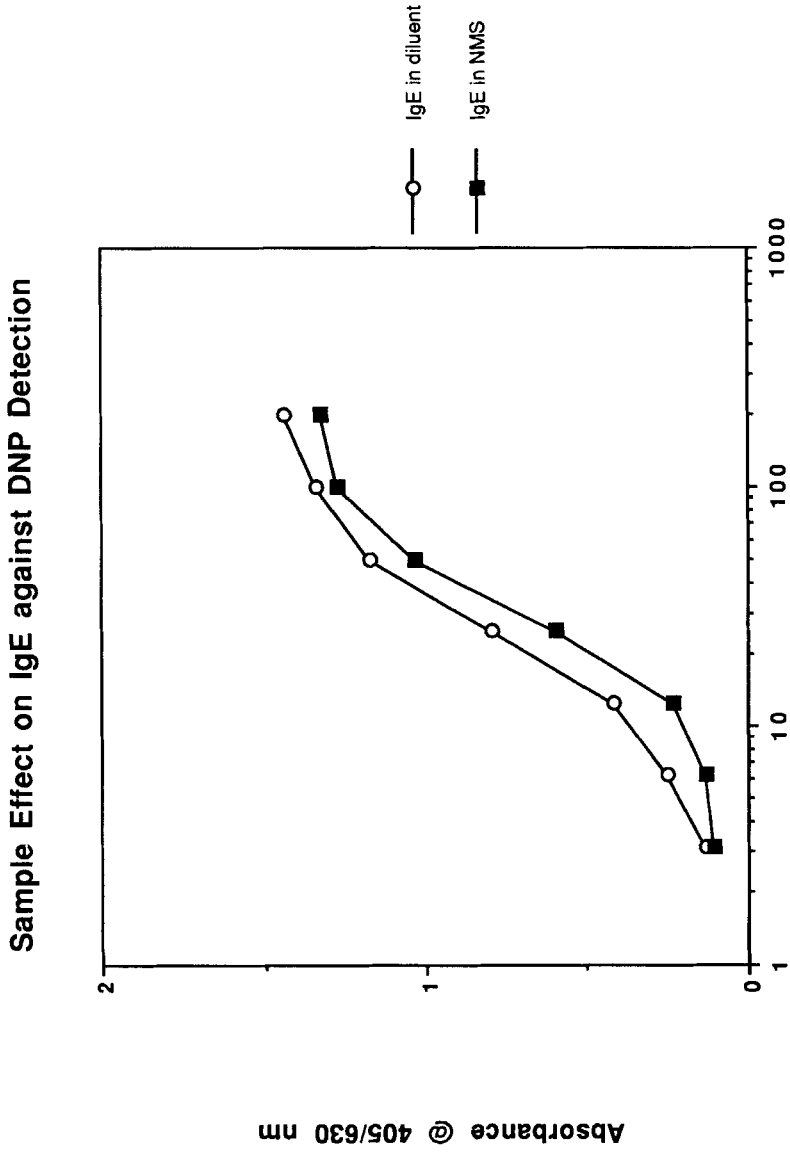

FIGURE 2. The Effect of Normal Mouse Serum on the Detection of DNP-Specific Mouse IgE.

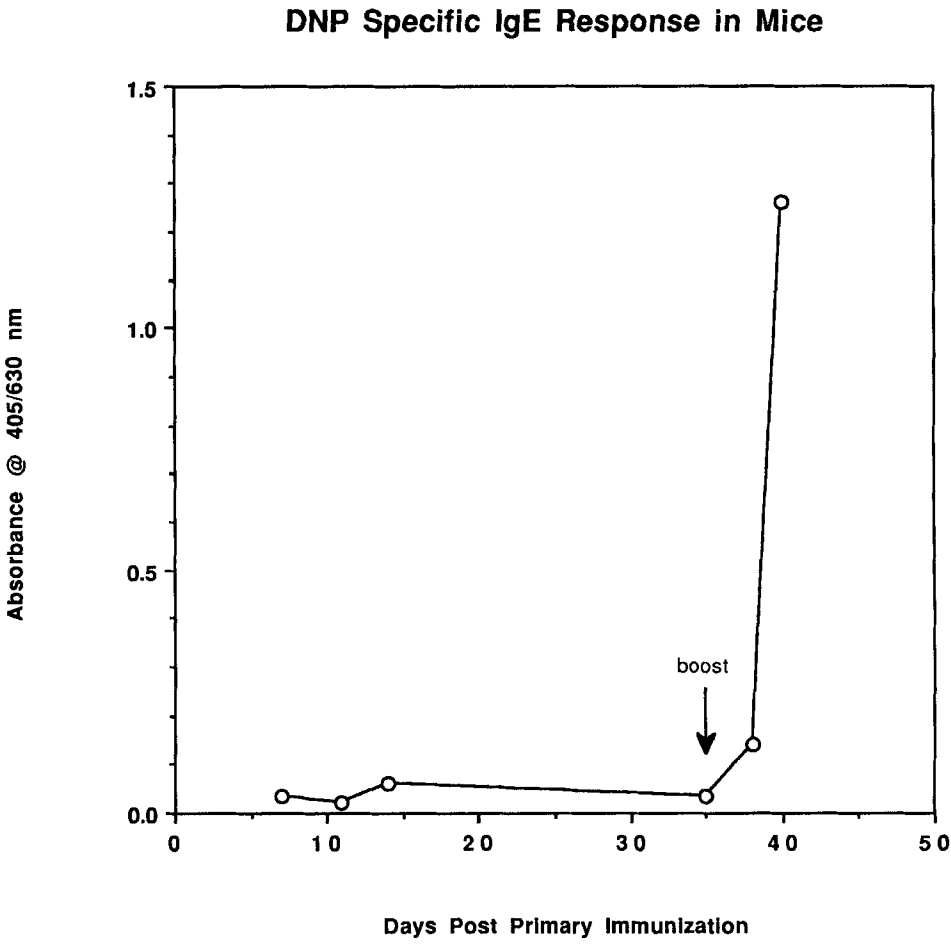

FIGURE 3. Measurement of IgE_{DNP} in Immunized CAF_1 Mice.

various mouse immunoglobulins at concentrations ranging from 1000 ng/ml through 15.6 ng/ml. Results showed that there was no loss of IgE binding as compared with non-competed IgE (see Table 3), thus indicating that the capture antibody is specific for IgE.

Figure 2 shows that there is minimal interference from normal mouse serum in this ELISA. The slightly reduced readings are probably a result of non-antigen specific IgE levels in the normal mouse serum.

We have shown the utility of this assay for measuring antigen specific IgE using alum precipitated antigen. Figure 3 shows the results of this assay used to monitor the IgE response in immunized CAF_1 mice.

DISCUSSION

This IgE capture ELISA is ideal for screening large numbers of sera samples for antigen-specific IgE, and gives a highly specific, sensitive and reproducible quantitative measure of specific IgE. Also, by changing the capture antibody, one can quantitate other antigen specific isotypes or subtypes (e.g. IgG_{2b}, IgM) so long as the capture antibody is specific. Currently, we use this assay to detect specific IgE and IgG. ELISA technology eliminates the long time periods, as well as the technical expertise and variability associated with passive cutaneous anaphylaxis assay (PCA).

During the preparation of this manuscript a reverse IgE-capture fluorometric assay was described by Sakaguchi [4]. Our assay offers comparable sensitivity and equal specificity and has several important advantages. First, our assay requires less time to run, since plates can be pre-coated, blocked and stored at 4°C for at least one week. This allows our assay to be completed in less than 3 hours, while the fluorometric assay requires 2 days to complete. Our equivalent sensitivity can be attributed to using an avidin-biotin-peroxidase complex as the detector, since more enzyme per site can be bound than by conjugating avidin to the enzyme directly, and so shorter incubation times can be used. Second, equipment needed for our assay is more commonly available and less expensive than that associated with fluorometric assays. Third, our assay diluent does not require the incorporation of normal mouse serum as reported by Sakaguchi [4]. Lehrer [9] reported normal mouse serum does contain circulating IgE which would effectively reduce assay sensitivity . The reduced absorbance values when purified DNP-specific IgE is added to normal mouse serum suggest that this may occur (see Figure 2). Our assay uses no normal mouse serum in the diluent, and mouse sera in concentrations as high as 10% do not result in appreciable background or interference (see Table 2).

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TRIIODOTHYRONINE UPTAKE MEASUREMENT IN SERUM BY TIME-RESOLVED FLUORESCENCE IMMUNOFLUOROMETRY

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ABSTRACT

A solid phase competition-type fluoroimmunoassay for triiodothyronine (T3) uptake in serum is described. In the assay, exogenous free T3 binds to the unoccupied binding sites on serum thyroxine binding proteins while the remaining unbound T3 competes with immobilized T3 for binding to a soluble biotinylated anti-T3 monoclonal antibody. The bound biotinylated antibody is quantitated by the addition of streptavidin labeled with the europium chelator 4,7-bis(chlorosulfophenyl`-1,10 phenanthroline-2,9-dicarboxylic acid (BCPDA) in the presence of excess europium. The fluorescence signal of the final complex, which is directly proportional to the number of unoccupied binding sites on thyroxine binding proteins, is then measured on the dried solid-phase with a pulsed-laser time-resolved fluorometer. The assay requires a 10 µl serum sample and a total incubation time of 90 minutes. The coefficients of variation for within-run and between-run assays ranged from 2.0 to 5.7%. Results obtained by the present method compared well with those determined by two commercial radioimmunoassays ($r > 0.9$). (KEY WORDS: time-resolved fluorescence immunoassay; fluorometer; T3 uptake; thyroid tests; monoclonal antibody; europium chelate)

INTRODUCTION

Thyroxine binding globulin (TBG) is primarily responsible for the storage and transportation of thyroid hormones in serum (1). Because of the relatively high

binding capacity and affinity of TBG for thyroxine (T4) and triiodothyronine (T3), the total levels of these hormones are influenced by changes in serum concentration of TBG which occurs in response to a host of physiological and pharmacological stimuli (2-6). To correct for alterations in TBG levels, T4 values have been normalized by calculating the Free Thyroxine Index (FTI) which relies on simultaneous measurement of total T4 and an estimate of T4 binding capacity of serum proteins. The latter has been traditionally performed by a variation of the T3 uptake test originally introduced by Hamolsky et al. (7). A number of investigators have recently questioned the value of the T3 uptake test for its being an indirect measure of TBG and for its reported inability to correct the serum T4 for the effects of high TBG concentrations (8-11). However, despite these controversies, the FTI is still one of the most widely used methods of thyroid assessment and it has been shown to correlate well with both the clinical status of the patients and the free T4 concentration (12-17).

Methods for the measurement of T3 uptake have involved exposure of the test serum to a fixed amount of radiolabeled T3 and to a variety of solid-phase materials including ion exchange resins, coated charcoal, silicate, macroaggregated albumin and solid-phase anti-T3 antibodies (18-22). Newer methodologies have incorporated enzymes, chemiluminescent, or fluorescent probes as labels in a variety of homogeneous and heterogeneous assay designs (23-26).

The fluorescent europium complexes have a number of advantages as immunological tracers compared with isotopic and other nonisotopic labels (27-30). Because the fluorescence emitted from these complexes is long lived, the tracer can be selectively detected by a gated fluorometer after the background fluorescence is allowed to resolve. The synthesis of 4,7-bis(chlorosulfophenyl)-1,10 phenanthroline-2,9-dicarboxylic acid (BCPDA), a new europium chelator, has been described recently (31). BCPDA exhibits a number of attractive properties which