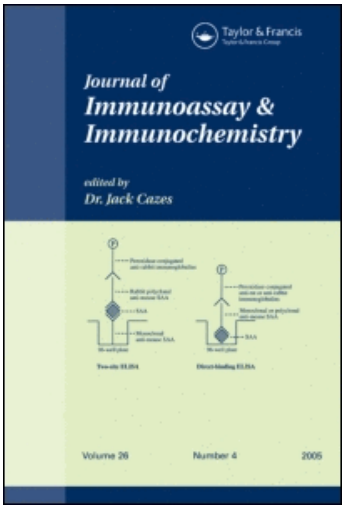

Journal of Immunoassay and Immunochemistry

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

Slide Immunoenzymatic Assay (SIA): Improving Sensitivity to Measure Antibodies when Samples are Very Small and Dilute, and Antigen is Scarce

E. Conway de Macario[a]; R. J. Jovell[a]; A. J. L. Macario[a]
[a] Department of Health and School of Public Health Sciences State University of New York at Albany, Wadsworth Center for Laboratories and Research New York State, Albany, New York

To cite this Article de Macario, E. Conway , Jovell, R. J. and Macario, A. J. L.(1987) 'Slide Immunoenzymatic Assay (SIA): Improving Sensitivity to Measure Antibodies when Samples are Very Small and Dilute, and Antigen is Scarce', Journal of Immunoassay and Immunochemistry, 8: 4, 283 — 295
To link to this Article: DOI: 10.1080/15321818708057028
URL: http://dx.doi.org/10.1080/15321818708057028

JOURNAL OF IMMUNOASSAY, 8(4), 283-295 (1987)

SLIDE IMMUNOENZYMATIC ASSAY (SIA): IMPROVING SENSITIVITY TO MEASURE ANTIBODIES WHEN SAMPLES ARE VERY SMALL AND DILUTE, AND ANTIGEN IS SCARCE

E. Conway de Macario, R.J. Jovell, and A.J.L. Macario

Wadsworth Center for Laboratories and Research
New York State Department of Health
and
School of Public Health Sciences
State University of New York at Albany
Albany, New York 12201

ABSTRACT

Modifications to the slide immunoenzymatic assay (SIA) using the PAP reagent (SIA-PAP) were developed which increase sensitivity considerably. These SIA modifications are particularly useful for measuring speedily antibodies in dilute samples available only in µl-volumes when antigen is scarce, whether a molecule, or a prokaryotic or eukaryotic cell.

(KEY WORDS: Slide immunoenzymatic assay (SIA); Peroxidase anti-peroxidase (PAP); Antibody limitation; Fibronectin; Methanococcus vannielii; Methanogenium cariaci; Toxoplasma gondii; Antibody measurements; Increased sensitivity; Monoclonal antibodies).

INTRODUCTION

The slide immunoenzymatic assay (SIA) (1) uses enzyme-labelled immunoreagents (2,3). It is a quantitative micromethod based on the principles of the enzyme-linked immuno-sorbent assay (ELISA) (2). It is performed on a flat circular reaction area rather than in conventional vessels (e.g. the well

of a microtitration plate). The advantages of using a circle instead of other solid-phase shapes and standard containers have been discussed elsewhere (1, 4).

SIA has proven useful for a variety of purposes, particularly for measuring antibodies in dilute samples available only in small volumes. Because of this, and because it requires comparatively small quantities of antigen, SIA is very helpful in hybridoma technology (4).

There are, however, extreme situations of antibody (or antigen) limitation which fall beyond the sensitivity of SIA. The sample available may be very dilute and too small to allow for routine concentrating procedures, or the nature of the work may not permit any delays that might be caused by these concentrating procedures if applied before testing by SIA. Moreover, the antigen may be scarce precluding attempts at increasing sensitivity by augmenting antigen concentration in the assay.

We set out to increase the sensitivity of SIA without increasing sample volume or antigen demands. Experiments were designed using solutions of antibody concentrations so low that they were barely detected, or not detected, by SIA. Steps were added to the standard SIA procedure, so that a very low or negative reading might become measurable at reliable levels.

MATERIAL AND METHODS

Antigens, antisera, and monoclonal antibodies. Fibronectin and Toxoplasma gondii, and rabbit antisera against them were kindly

provided by Drs. E. Greenfield and W. Stahl, respectively, of this Center. Methanococcus vannielii SB and Methanogenium cariaci JR1c were kindly provided by Drs. M.J. Wolin and T. Miller of this Center. Rabbit antisera against Mc. vannielii SB and Mg. cariaci JR1c, and murine monoclonal antibodies against the latter were prepared by us. Hybridomas were constructed by standard procedures (5) using Sp2/0-Ag 14 myeloma cells. Monoclonal antibodies were produced and tested in mouse ascitic fluid and tissue culture medium. Corresponding controls were ascitic fluid and medium harvested from mice and cultures, respectively, in which Sp2/0-Ag 14 cells were growing. Protein and immunoglobulin determinations were done by published procedures (6) (see also pertinent articles in Refs. 4 and 5), and using a commercial kit (ICN Immuno Biologicals, Lile, IL).

Other reagents. Other reagents used were: unlabelled and peroxidase labelled goat anti-rabbit and anti-mouse IgG immunesera (Cooper Biomedical, Inc., Malvern, PA); horseradish peroxidase (HRP)-rabbit anti-HRP and HRP-mouse anti-HRP (PAP reagents (7)) (EY Laboratories, Inc., San Mateo, CA, and Jackson Immunoresearch Laboratories, Inc., Avondale, PA); formaldehyde 10% ultra pure TEM grade (Tousinis Research Co., Rockville, MD); calf serum (Gibco, Grand Island, NY); o-phenylenediamine (Sigma Chemical Co., St. Louis, MO); hydrogen peroxide (30%) (J.T. Baker Chemical Co., Phillipsburg, NJ). Citric acid 0.1 M buffer (pH 4.5); distilled water; ethanol 95%; tissue culture medium (CM); phosphate buffered

saline (pH 7.2); normal rabbit serum (NRS); and normal goat and mouse sera were prepared by us or provided by this Center.

SIA and modifications. Antigen, antisera, monoclonal antibody solutions, PAP, and other reagent solutions were delivered onto the circles of the SIA slides (designed by us and manufactured by Cel-Line Associates, Inc., Newfield, NJ) by means of an Electronic Digital Pipette (Rainin Instruments, Co., Inc., Woburn, MA). Dilutions of all antibody and control solutions were done in CM containing 10% calf serum (CM+). SIA was performed as described (4), and with modifications using PAP (7). The main modifications were as follows: antigen-coated SIA-slide circles were incubated first with the sample in which antibody was to be measured (antiserum or monoclonal antibody solution); second with unlabelled goat anti-rabbit IgG immuneserum (or goat anti-mouse IgG when the primary antibody was monoclonal); and third with PAP before adding substrate for peroxidase (SIA-PAP); or antigen-coated circles were incubated with primary antibody, anti-rabbit (or anti-mouse) IgG immuneserum and PAP, as above, and then with peroxidase-labelled goat anti-rabbit (or anti-mouse) IgG immuneserum before substrate addition (SIA-PAP+). In between the first and second incubations the circles were covered with normal goat serum diluted 1:3 for 10 minuntes. Experiments were also done in which sera, ascitic fluids or culture media devoid of antibodies substituted for primary antibody, and peroxidase-labelled substituted for unlabelled goat anti-rabbit

IgG, in the second step of SIA-PAP or SIA-PAP+. All incubations and washings were done as per SIA methodology (4). Enzymatic reactions were read at 450 nm using a vertical beam spectrophotometer (Dynatech Instruments, Alexandria, VA).

Calibration of the system. A series of preliminary experiments were done to calibrate the system. Antisera, and monoclonal antibodies in ascitic fluids or tissue culture media, were titrated by SIA using different antigen concentrations to define conditions of extreme limitation of antigen and antibody. For a given concentration of antigen, extreme limitation of antibody was the end of the titration curve including the highest antiserum dilution, or lowest monoclonal antibody concentration, giving a measurable reading and up to four higher, subsequent dilutions. SIA modifications were run with these dilutions to measure how much, if at all, the modifications would increase very low readings, and would detect antibodies at concentrations which standard SIA had not.

Unlabelled and peroxidase-labelled goat anti-rabbit and anti-mouse IgG immunesera and the PAP reagents were used at various dilutions to find the optimal ones, i.e. those that would enhance antibody measurements without increasing the background (which is negligible for standard SIA). The results reported here are those obtained with these optimal dilutions, which for the batches of reagents used were 10^{-1} for the unlabelled anti-rabbit and anti-mouse second antibodies, 10^{-3} for the perox-

idase-labelled anti-rabbit and anti-mouse second antibodies, 10^{-2} for the rabbit PAP reagent, and 2×10^{-3} for the mouse PAP reagent.

RESULTS

SIA vs. modifications: antibodies against non-particulate antigens. Results obtained with non-particulate antigens are typified by those obtained with fibronectin and rabbit antiserum against it, Fig. 1. SIA-PAP gave 2 to 5-fold higher readings than SIA at all high antiserum dilutions ($\geq 10^{-4}$) tested, Fig. 1A. Negative controls in which antigen or antiserum were replaced by unrelated antigen or by normal (non-immune) serum gave readings no higher than 0.05. It is interesting to notice that the enhancing effect of SIA-PAP becomes clearer as the antiserum dilutions increase, i.e. as SIA readings decrease. Addition of PAP increased the sensitivity of the assay and enhanced measurements under conditions of extreme limitations of antibody and antigen, Fig 1B. Using only 0.5 µg of fibronectin/ circle, antibodies were detectable by SIA at a serum dilution of 10^{-4} but not at dilutions of 10^{-5} or greater. SIA-PAP, however, detected antibodies up to a serum dilution of 10^{-6}. Addition of peroxidase-labelled second antibody after PAP (SIA-PAP+) increased readings at all these antiserum dilutions, and made possible the detection of antibodies at a serum dilution of 10^{-7}.

Experiments were also carried out to determine whether SIA-PAP+ would noticeably increase readings under non-extreme

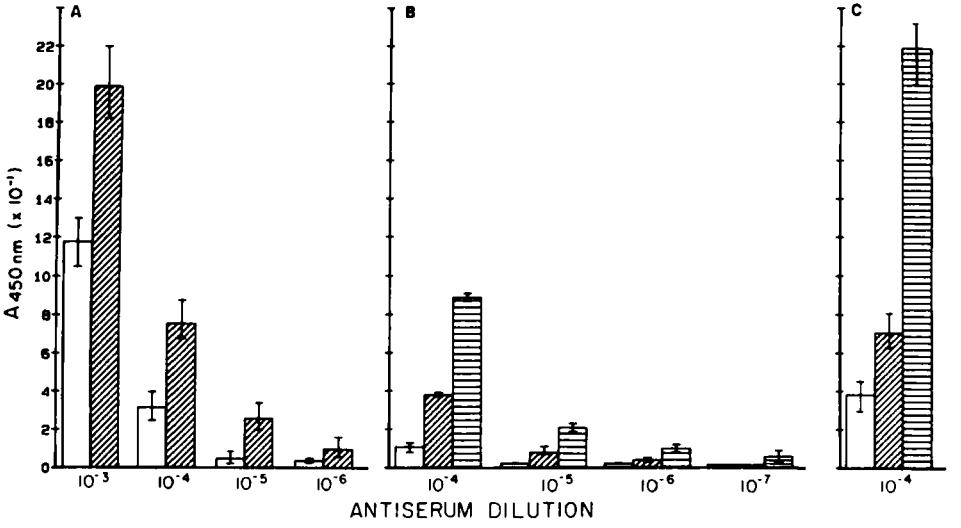

FIGURE 1. Comparison of absorbance readings using SIA (open bars), SIA-PAP (diagonally shaded bars) and SIA-PAP+ (horizontally shaded bars) for measuring antibodies against a non-particulate antigen, fibronectin (1 µg/circle, A and C; or 0.5 µg circle, B). Readings were done 30 minutes after addition of substrate for peroxidase; negative control values (0.01 [0-0.02] for SIA; 0.02 [0.01-0.02] for SIA-PAP; and 0.03 [0.03-0.03] for SIA-PAP+; n=3 in all cases) have been subtracted from values shwon. (Arithmetic mean ± range; n=3).

conditions of antigen limitation. Illustrative results are shown in Fig. 1C. SIA-PAP+ increased readings by a factor of ∿6 above those given by SIA, and by a factor ∿3 above those obtained with SIA-PAP.

Experiments in which unlabelled second antibody was replaced by its peroxidase-labelled counterpart in the second step of SIA-PAP and SIA-PAP+, produced the same results (not shown).

Antibodies against particulate antigens. The results obtained with prokaryotic and eukaryotic cells are illustrated by those

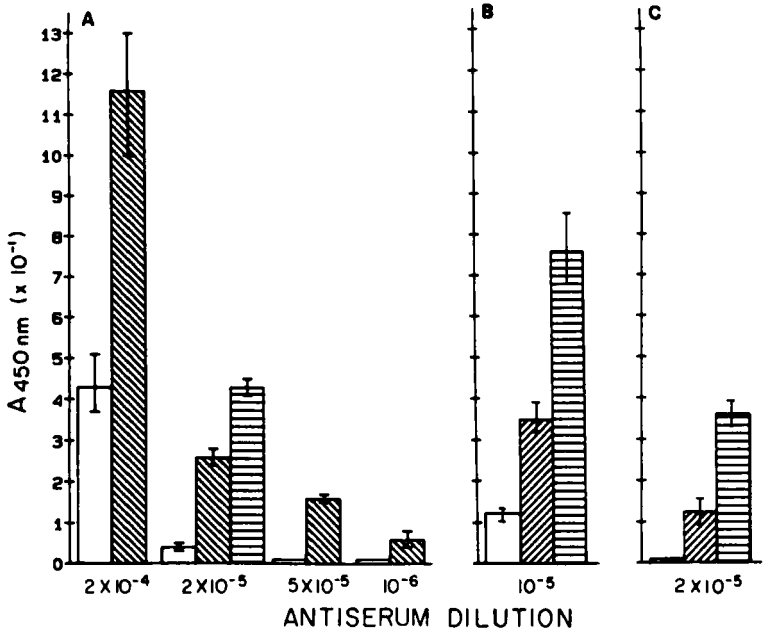

FIGURE 2. Comparison of absorbance readings using SIA (open bars) SIA-PAP (diagonally shaded bars) and SIA-PAP+ (horizontally shaded bars) for measuring antibodies against particulate antigens: Mc. vannielii cells (A and B) or T. gondii cells (C). Readings: same as in Fig. 1.

obtained with Mc. vannielii SB (Fig. 2A,B) and T. gondii (Fig. 2C), respectively, using rabbit antisera. Again, extreme dilutions (i.e. those giving minimal readings by SIA, and dilutions immediately following them) of the antisera were used. SIA-PAP increased readings by at least a factor of 2 when Mc. vannielii and its antiserum were tested; also SIA-PAP made antibody detection possible at antiserum dilutions that appeared devoid of antibodies when tested by SIA, Fig. 2A. Addition of second antibody labelled with peroxidase after PAP (SIA-PAP+),

TABLE 1. Comparison of monoclonal antibody measurements by SIA and modifications.

Antibody in ascitic fluid[a] (μg/ml)	Absorbance 450nm		
	SIA	SIA-PAP	SIA-PAP+
10	0.40 [0.36-0.45][b]	0.94 [0.90-0.99]	1.61 [1.37-1.82]
	(0.40)[c]	(0.92)	(1.57)
1	0.11 [0.08-0.14]	0.41 [0.37-0.45]	0.83 [0.75-0.92]
	(0.11)	(0.39)	(0.79)
0.1	0.01 [0-0.02]	0.11 [0.09-0.13]	0.48 [0.42-0.54]
	(0.01)[d]	(0.09)	(0.44)
0.01	0 [0-0]	0.05 [0.04-0.07]	0.23 [0.20-0.26]
	(0)[d]	(0.03)[d]	(0.19)

[a]Monoclonal antibody against Mg. cariaci.

[b]Arithmetic mean [range]; n=4

[c]Values within parenthesis are mean reading given by antibody minus mean reading given by negative controls. Negative control readings were: SIA: 0 [0-0]; SIA-PAP: 0.02 [0.01-0.03]; and SIA-PAP+: 0.04 [0.03-0.05]. Arithmetic mean [range]; n=4.

[d]These values were considered negative, i.e., no antibody was detected.

increased the sensitivity of the assay still further, Fig. 2A and B. The same observations were made using the system T. gondii - rabbit anti-T. gondii immuneserum, Fig. 2C, and monoclonal antibodies against Mg. cariaci JR1c, Table 1. These data, obtained with antibody in ascitic fluid, were corraborated by

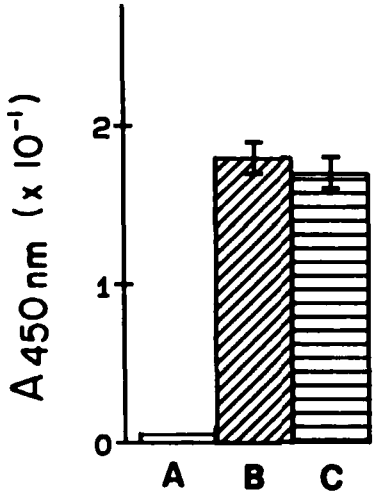

FIGURE 3. Comparison of absorbance readings using SIA (A), SIA-PAP without circle precoating (B) or with circle precoating with normal goat serum (C) under conditions of limitation of antibody against Mc. vannielii (rabbit antiserum diluted 1:500,000). Readings: same as in Fig. 1.

testing the same antibody in culture medium, and by assaying other monoclonal antibodies (results not shown).

The increase in readings obtained by adding PAP, or PAP followed by second antibody labelled with peroxidase was not due to the binding of these reagents to the supporting solid phase (SIA- slide circles). Concerning the factor of increase in readings it made no difference if prior to adding PAP for example, the circles were coated with normal goat serum, Fig. 3. These data, and the results obtained with positive and negative controls indicated that the increased readings observed using SIA-PAP and SIA-PAP+ were due to reproducible antigen-antibody reactions.

DISCUSSION

The need for ultrasensitive assays for measuring antibodies and antigens is widespread. In many occasions in clinical laboratory practice, field studies, epidemiologic surveys, and research, problem samples must be dealt with which are very dilute and available only in small volumes. Confronting these difficulties lead us to the development of SIA. Standard microtitration plates requiring relatively large volumes of reagent solutions could not be used. We also decided to avoid the use of radioactive materials.

To measure antibodies in dilute, small samples one may have to resort to increasing the antigen concentration in the assay, and the time of incubation of the antigen-antibody mixture. However, lengthening incubation times is impractical, and augmenting antigen in the assay may be impossible. This impossibility occurs when antigen is not available in the amounts necessary to increase significantly the sensitivity of the assay, especially when many samples have to be tested. To deal with this type of problem we developed SIA modifications. Adding PAP (SIA-PAP) consistently increased SIA readings. Further addition of second antibody labelled with peroxidase after PAP (SIA-PAP+) augmented still more SIA readings. These increases were particularly meaningful when SIA readings were very low due to extreme limitation of antibody and antigen. Keeping antigen concentration at the same low levels used for SIA, SIA-PAP measured antibodies in samples in which SIA had failed to detect

them. Considering the antiserum dilutions at which SIA and SIA-PAP were able to detect antibodies, the latter was up 100-fold more sensitive (see Fig. 1B). Along the same lines, SIA- PAP+ was 1,000-fold more sensitive than SIA.

When SIA did barely detect antibodies, giving very low readings, SIA-PAP increased such readings by at least a factor of two, which was sufficient to yield readings falling within the spectrophotometer's most reliable range. SIA-PAP+ improved readings by factors of 4 or greater above those given by SIA. Thus SIA-PAP+ despite its requiring additional steps as compared with SIA-PAP, is the method of choice in situations of extreme antibody (and antigen) limitation, in which even SIA-PAP fails to yield reliable readings.

Both SIA-PAP and SIA-PAP+ worked well for measuring antibodies against non-particulate (molecules available in solution) and particulate (prokarytic and eukaryotic cells) antigens.

ACKNOWLEDGEMENT

We thank the colleagues mentioned in the text for providing antigens. We also thank Dr. Jose Rubiolo and Mr. Morris Laster for their assistance during part of this work. This work was supported in part by Grant No. DE-FG02-84ER 13197 from the U.S. Dept. of Energy.

REFERENCES

1. Conway de Macario, E., Macario, A.J.L. and Jovell, R.J. Quantitative slide micro-immunoenzymatic assay (micro-SIA) for antibodies to particulate and non-particulate antigens. J. Immunol. Methods 1983: 59, 39-47.

2. Engvall, E. and Perlmann, P. Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. Immunochemistry 1971: 8, 871-874.

3. van Weemen, B.K. and Schuurs, A.H.W.M. Immunoassay using antigen-enzyme conjugates. FEBS Letters 1971: 15, 232-236.

4. Conway de Macario, E., Macario, A.J.L. and Jovell, R.J. Slide immunoenzymatic assay (SIA) in hybridoma technology. In: Langone, J.J. and Van Vunakis, H., eds. Methods in Enzymology, Vol. 121. Orlando, Academic Press, 1986: 509-525.

5. Oi, V.T. and Herzenberg, L.A. Immunoglobulin-producing hybrid cell lines. In: Michell, B.B. and Shiigi, S.M., eds. Selected methods in cellular immunology. San Francisco, W.H. Freeman and Co., 1979: 351-372.

6. Kumar, S.A. and Macario, A.J.L. Comparative analysis of rat uterine estrogen-induced creatine kinase with monoclonal antibodies. Hybridoma 1985: 4, 297-309.

7. Sternberger, L.A., Immunocytochemistry. New York: John Wiley and Sons Inc.1979.