A Solid Phase Urease-Linked Cellular Immunosorbent Assay for Circulating Polymorphonuclear Binding Immunoglobulin*

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ABSTRACT

A cellular enzyme linked immunosorbent assay (CELISA) is reported for the detection of circulating polymorphonuclear granulocyte binding immunoglobulin (PBG) in patients' sera. The assay features a solid phase microtiter method in which the enzyme urease is fixed to the antihuman globulin conjugate reagent and uses 0.25 percent glutaraldehyde fixed normal human polymorphonuclear neutrophils (PMNs) as target cells. The assay gave positive results in four of 13 (31 percent) cases of idiopathic neutropenia in which an autoimmune etiology was suspected and one typical case of isoimmune neonatal neutropenia. In a group of 15 patients receiving multiple blood transfusions for chronic anemia, five (33 percent) showed significantly higher levels (p < 0.001) of PBG than non-transfused normal donors. The PBG-CELISA appears potentially useful for the detection of autoimmune and isoimmune PMN antibodies and PMN binding IgG immune complexes.

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INTRODUCTION

A number of assays for polymorphonuclear neutrophils (PMN) isoimmune and autoimmune antibodies have been developed utilizing agglutination, cytotoxic-
ity,13,14,15,16,17,35,36 and functional assays1,6,8,19,29 for the detection of PMN associated IgG (PBG). Recent efforts have focused on the development of sensitive suspension phase microtiter methods for detecting circulating PMN binding immunoglobulin (PBG) using fluorescein tagged38 and radiolabelled24,32,33 antibodies to human IgG or Staph protein A binding.25 However, there have been some difficulties with these assays in terms of a requirement for viable PMNs,1,4,5,6,8,9,10,11,12,13,14,15,16,17,19,29,31,34,35,36 false positivity,21,22,26 and lack of correlation2 indicating a need for the development of a more reliable and simpler assay for PBG2 and one that does not require viable PMNs.

The enzyme linked immunosorbent assay (ELISA) for IgG is highly reproducible and has been successfully adapted for the detection of HLA antibodies to viable lymphocytes in a microtiter assay28 and for the detection of small amounts of IgG in lymphocyte culture.23 Moreover, a reliable solid phase cellular assay for lymphocyte antibodies has been developed using glutaraldehyde as fixative.33

One difficulty in adapting the above CELISA methods to the detection of PBG on PMN is the presence of abundant isoenzymes in the PMN, such as myeloperoxidase and alkaline phosphate.18 The presence of these enzymes in the PMN may lead to false positivity. In the present study an analytically sensitive phase CELISA is reported for the detection of PBG based on the use of a urease indicator system.

Methods

PMNs

Polymorphonuclear granulocytes (PMNs) were taken from normal laboratory volunteers. Forty milliliters (ml) of whole blood were drawn and mixed with four ml anti-coagulant citrate dextrose solution (ACD) and eight ml of dextran (six percent dextran in 0.9 percent sodium chloride).

The white blood cell rich plasma9 was gently expressed into a clean centrifuge tube. The tube was centrifuged for 10 minutes at 2,000 RPM. After discarding most of the plasma, the pellet was resuspended in four ml of the autologous ACD plasma. This was carefully overlayed onto a Percoll* double gradient. To make the double gradients, the stock Percoll was first made isotonic by mixing 15 ml of nine percent (10×) normal saline with 135 ml of the stock Percoll reagent. For 1.100 sp.gr. Percoll mix use 80 ml isotonic Percoll and 20 ml 0.9 percent saline. For the 1.075 sp.gr. Percoll mix, use 60 ml isotonic Percoll and 40 ml 0.9 percent saline. Four ml of the 1.100 sp.gr. Percoll was placed in the bottom of a clean 15 ml centrifuge tube and four ml of the 1.075 sp.gr. Percoll was placed carefully over this. The plasma overlayed on the double gradient Percoll solutions was spun for 30 minutes at 2,000 RPM. The PMNs are clearly layered at the interface of the 1.100 sp.gr. and the 1.075 sp.gr. This layer was carefully removed and placed into a clean 15 ml plastic centrifuge tube and washed three times in 0.9 percent saline.

Using this technique, the yield is approximately 40 percent. Thus, on any given run, starting with 40 ml of whole blood, there are enough PMNs to provide $1 \times 10^5$ cells per well for 800 to 1,000 wells (or eight to 10 microplates). Viability was not assessed since the cells were fixed routinely in glutaraldehyde. However, unfixed aliquots of cells had normal O$_2$ consumption values.10

Using U-Bottom polystyrene microplates (NUNC-262170), $1 \times 10^5$ cells were added to each well. Plates were spun for five minutes at 750 g. The

* Pharmacia #17-0891-01.
supernatant of each was then carefully removed. Two hundred microliters (µl) of 0.25 percent glutaraldehyde in PBS made fresh daily and kept on ice, was carefully pipetted into each well. This was incubated for five minutes at 4°C. The plates were then quickly turned and the glutaraldehyde solution was discarded, leaving the cells fixed to the bottom of the plates. The plates were washed three times with 0.9 percent saline. If plates were not used immediately, 200 µl of PBS with one percent ovalbumin and 0.2 percent sodium azide were added to each well. Plates were covered with parafilm and stored at 4°C. The plates gave reproducible results for up to three weeks.

**IMMUNOGLOBULIN BINDING**

**Serum.** Fifty microliters (µl) of test or control serum diluted with one percent ovalbumin in PBS (ovalbumin used in accordance with the Ureiase manufacturer's protocol) were covered with parafilm and incubated for one hour at room temperature.

**Urease Conjugates.** After serum incubation, the plates were washed three times with 0.9 percent saline. Rabbit anti-human IgG conjugated to urease (Ureiase Kit),† was diluted at 1:1000 using PBS, one percent ovalbumin, and 0.2 percent sodium azide solution. One hundred µl were added to each well and the plates were covered with parafilm. They were incubated at 37°C (moist) for half hour. After incubation, the plates were washed using 0.9 percent saline.

To each well were added 100 µl of the urea substrate solution† with a pH indicator which yields a color reaction from bright yellow to shades of purple. The plates were incubated for 15 minutes at room temperature. The reaction was stopped with 10 µl of a one percent merthiolate/distilled water solution and the supernatant transferred to flat bottom polystyrene plates (NUNC-262170) and the optical density read with a spectrophotometer‡ at 590 nm.

**Results**

**REACTIVITY WITH IMMUNE COMPLEXES**

Immune Complexes (IC) formed by heating convert negative sera to positive in the lymphocyte CELISA.³⁷ Similarly, heating four normal serum to 56°C for half an hour produced a significant increase (p < 0.001) in the PBG-CELISA (figure 1). Freezing IC specimens (−20°C) for up to five days did not produce any increase or decrease reactivity in the PBG-CELISA. Fifty µl of IC run in triplicate were used to standardize the assay. A heated sample stored at −20°C and thawed at various

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† Allelix, Inc., Mississauga, Ontario, Canada.

‡ Dynatech—ELISA reader.
times over a five day period gave the following values: 77, 61, 34, 68, 48, 77, and 57.

**Reactivity of Sera from Normal and Multiply-Transfused Patients**

The mean and standard deviation of 10 normal laboratory volunteers' sera against a panel of 33 normal donors' cells was 4.9 ± 3.8 units using 16 O.D. units as the cutoff for the upper limit of normal (×35 D.S.). Of 15 multiply transfused patients' sera (figure 2), five were higher (p < 0.001) than normal controls. Seven specimens (four negatives and three positives) were analyzed blindly using the immunofluorescence assay which gave a direct correlation to the PBG-CELISA technique (all positives, positive, and vice versa). No correlation was observed for positivity and degree of HLA mismatch. The NA (neutrophil antigen) specificity was not determined.

**Reactivity in Patients with Acquired Neutropenia**

The results of the PBG-CELISA on 13 patients with acquired neutropenia are shown in figure 2. Four gave positive results and the remaining nine cases were not significantly different than normal. In addition, sera from a woman who had a history of children with transient neonatal neutropenia gave a positive result (35 O.D. units).

**Discussion**

The present report describes an analytically sensitive urease based solid phase CELISA for the detection of PMN associated immunoglobulin. Morris et al. previously described a cellular enzyme linked immunospecific assay that detected antibodies to human lymphocyte cell surface antigens utilizing a suspension phase method and an alkaline phosphatase linked immunoglobulin conjugate. In the present study, urease was used rather than alkaline phosphatase since alkaline phosphatase is present in the human PMN. Concerns were raised in the previous report that a solid phase method based on glutaraldehyde fixation of cells to plastic could not be successfully developed because of high background, but unusual difficulty was not encountered by the present authors with background (see Results).

Our results with glutaraldehyde fixed human PMNs were consistent with those of Stocker and Heusser. Fixed (0.25 percent glutaraldehyde) and non-fixed murine leukocytes gave similar values in a solid phase radioimmunoassay. Solid phase 0.25 percent glutaraldehyde fixed
cells also gave comparable positive values to a fixed suspension phase radioimmunoassay when reacted with murine antisera and were technically easier to perform.\textsuperscript{33} Use of this assay on samples stored longer than one month may not be advisable since Morris et al\textsuperscript{28} showed that negative samples stored longer than six months show an increase in nonspecific immunoglobulin binding.

The urease based CELISA for PMN associated IgG was positive in one-third of chronically transfused dialysis patients. This percent is in approximate agreement with the 37 percent positivity of transfused renal dialysis patients in granulocyte toxicity assays.\textsuperscript{27} The presence of a strongly positive assay in a women with a history of neonatal isoimmune purpura (see Results) and in four of 13 (31 percent) other patients with "autoimmune" neutropenia, adds support to the concept that anti-PMN IgG antibody in the patients' sera may have been the source of the IgG binding to the target PMNs. However, since heating serum also produced a positive result (figure 1), a positive result in a given serum may be due to IgG containing circulating immune complexes (IC). It is possible to remove IC with polyethylenglycol (PEG);\textsuperscript{3} with adaptation of this methodology to the CELISA, a positive result owing to IC from that owing to anti-PMN IgG could be made. However, PEG IC extraction methods thus far have not altered IC reactivity prepared by heating in our assay.\textsuperscript{*} Another potential problem in interpreting positive results is the finding of Sears et al\textsuperscript{32} that in non-neutropenic patients who have high titers of rheumatoid factor, immunoreactivity against PMN as measured by a solid phase radioimmunoassay can occasionally be demonstrated. Accordingly, at this time, a positive CELISA in the setting of neutropenia can be viewed as suggestive of an autoimmune etiology for the neutropenia. Only a positive test in the setting in which isoimmune sensitization is the only source of PMN binding globulin should be interpreted as proof of the demonstration of a PMN antibody.

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References


* Preliminary observations


