

Dot-immunoperoxidase assay for the demonstration of soluble Fcγ receptors

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We have developed a sensitive dot-immunoperoxidase assay to demonstrate and characterize the functional activity of soluble Fcγ receptors (sFcγR). Samples containing sFcγR were immobilized on nitrocellulose membrane. Immune complexes of horseradish peroxidase (HRP) and rabbit IgG antibodies to HRP were visualized with an HRP developer. Dot-immunoperoxidase assay is described as a rapid and simple method for the demonstration and characterization of functionally active sFcγR.

Key words: soluble Fcγ receptor, immune complex, dot-immunoperoxidase assay

INTRODUCTION

Several dot-immunobinding assays have been described [1, 2], and the techniques have been useful in the characterization of autoantigens, allergens and infectious organisms [3].

Earlier in our studies the biologically active sFcγR was tested using the EA-rosette inhibition technique [4]. The antibodies obtained against sFcγR inhibited EA-rosette formation. Afterwards we detected its immunochemical characteristics by fluorometric binding assay [5]. These techniques require meticulous procedures, expensive reagents, equipment and are technically laborious. As they employ a variety of different methodologies (chromatography, electrophoresis, fluorescence, agglutination etc.) and generally exhibit high sensitivity and specificity, these tests are quite expensive, a factor that limits their usefulness under a variety of circumstances.

An inexpensive, rapid and reliable test for sFcγR has therefore been developed, which can be performed simply even in the absence of highly sophis-

ticated technical equipment. In the present study we describe a dot-immunoperoxidase assay by which sFcγR at low concentrations are immobilized on a solid support and probed with enzyme-labeled immune complexes. This technique is sensitive, easy to perform, and the results can be analyzed visually.

MATERIALS AND METHODS

Preparation of IgM, IgG, IgG fragments and IgG subclasses. Immunoglobulins G (G1, G2) and M were isolated from the blood of healthy cows by ammonium sulfate precipitation, DEAE cellulose chromatography and gel filtration described by Williams et al. [6]. Bovine F(ab)₂ and Fc fragments were prepared as described by Nisonoff et al. [7]. Aggregated bovine IgG, IgG1, IgG2 were prepared by heating 0.3% IgG, IgG1, IgG2 at 62°C for 20 min. The purity of all immunoglobulins and fragments was verified by polyacrylamide gel electrophoresis in sodium dodecyl sulphate (SDS-PAGE) [8] and immunoelectrophoresis in agar [9] with rabbit antiserum specific for bovine IgG1, IgG2 molecules and F(ab)₂, Fc fragments. The concentrations of Ig and

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fragments were determined by measuring their optical density at 280 nm ($E_{1\text{cm}}^{1\%} = 14.0$) with a Spectromom 204 spectrophotometer (Hungary).

Immune complexes. Antisera to horseradish peroxidase (HRP) (Reanal, Hungary) were raised in rabbit by injecting 5 mg HRP in complete Freund's adjuvant intramuscularly. The rabbits received a booster injection after 3 weeks and were bled 4 weeks later. Antibodies to HRP were purified on HRP-coupled Sepharose 4B (Pharmacia, Sweden) according Axen et al. [10]. The antibodies were eluted with 3 M sodium thiocyanate, dialysed against phosphate buffered saline, pH 7.2 (PBS) and concentrated.

Immune complexes (IC) were prepared by adding antibody dilutions to equal amounts of four-fold dilutions of HRP from 1 mg/ml in PBS. The mixture was incubated for 2 h at room temperature before use.

Preparation of soluble FcR. Staphylococcal protein A (prot A) (Sigma) was used as a positive control for binding IgG by its Fc region. Prot A was dissolved in PBS and titrated as a two-fold dilution in PBS starting from 1 μg in 100 μl PBS.

Soluble bovine Fc γ R (sFc γ R) was obtained by the shedding method in bovine peripheral blood lymphocytes during 1 h incubation at 37 $^{\circ}\text{C}$ [4]. sFc γ R detectable in the medium on their ability to bind IgG specifically. sFc γ R were affinity purified on Sepharose beads with bovine IgG. The sFc γ R preparations were concentrated and stored in small aliquots at -70 $^{\circ}\text{C}$ until use.

The dot-immunoperoxidase assay. ProtA, soluble FcR and a ten-fold value of concentrated serum from bovine blood with chronic leukemia were blotted onto a nitrocellulose membrane (Immobilon-NC HAHY, Millipore). The nitrocellulose membrane was cut into strips of convenient size (e.g., 0.6 \times 5 cm) and placed in a large Petri dish. Five or 10 μl of the soluble preparations were tested applying as a small spots to each strip. The spots were air-dried at room temperature for 2 h. The nitrocellulose strips with air-dried spots were processed for blocking treatment with 2 ml of 3% bovine serum albumin (BSA) prepared in PBS for 30 min at room temperature. This procedure adequately blocked all non-specific protein binding sites. The blocking solution was removed, and the dotted strips were incubated at 4 $^{\circ}\text{C}$ overnight with 2 ml HRP-anti-HRP immune complexes. After a rinse with PBS, bound immune complexes were visualized with a HRP developer 4-chloro-1-naphthol (Merck, Darmstadt) as chromogen. A solution of 10 ml of 4-chloro-1-naphthol in methanol (3 mg/ml) was added immediately before use to a solution of PBS, containing 6 μl of 30% hydrogen peroxide. The sFc γ R dotted strips

were incubated with the enzyme substrate for 15 min at room temperature. After 15 min of incubation in the developing solution in the dark, the intensity of the grey dots reflected the amount of the bound immune complexes, i.e. the amount of FcR. The longer incubation times did not enhance the staining intensity. The strips were then washed in distilled water or dried and stored at 4 $^{\circ}\text{C}$.

Inhibition experiments were performed by mixing four parts of soluble immune complexes with one part of IgG, F(ab) $_2$ or Fc fragments, IgA, IgM or subclasses (all 1 mg/ml at final concentration). Dot pots were also visually interpreted by one of the authors who was unaware of the results obtained on a nitrocellulose membrane.

RESULTS AND DISCUSSION

Protein A was dot-blotted onto nitrocellulose membrane in decreasing amounts. Strong binding of immune complexes to immobilized protA was observed (Fig. 1) and was noted down to 0.06 μg protA. This binding was inhibited by Fc but not by F(ab) $_2$ fragments. Bovine IgG, IgG1 and IgG2 inhibited the binding, whereas only a weak inhibition was observed using IgA and IgM.

Binding of immune complexes was detectable up to dilutions corresponding to approximately 1.5 μg of ten-fold concentrated bovine serum obtained from the blood of bovine with chronic leukemia (Fig. 2). The binding was completely inhibited by bovine Fc fragments, monomeric and aggregated bovine IgG, but not by F(ab) $_2$ fragments, IgA and IgM.

Heat aggregated bovine IgG subclasses IgG1 and IgG2 were used for inhibition studies of binding

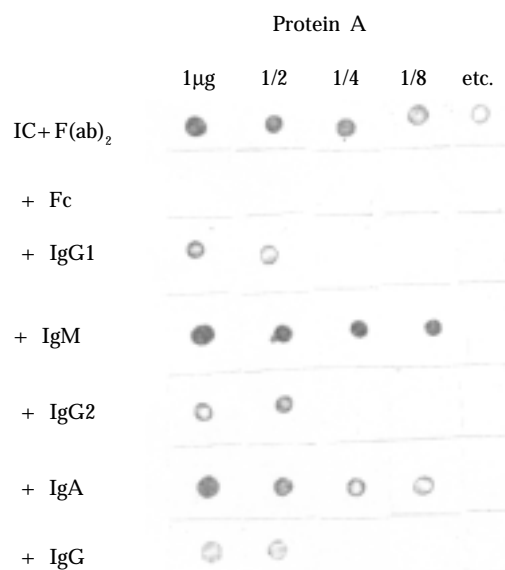


Fig. 1. Binding of the immune complexes to staphylococcal protein A immobilized onto nitrocellulose membrane

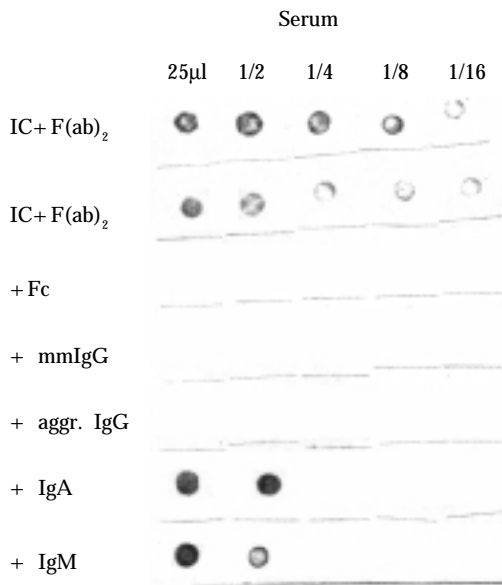


Fig. 2. Binding of immune complexes to tenfold concentrated serum from bovine with chronic leukemia immobilized onto nitrocellulose membrane

immune complexes to tenfold concentrated bovine serum. Interestingly, IgG1 inhibited the binding of immune complexes to ten-fold concentrated bovine serum, whereas IgG2 did not (Fig. 3). Hence, the specificity of this inhibition appeared to be IgG1 > IgG2.

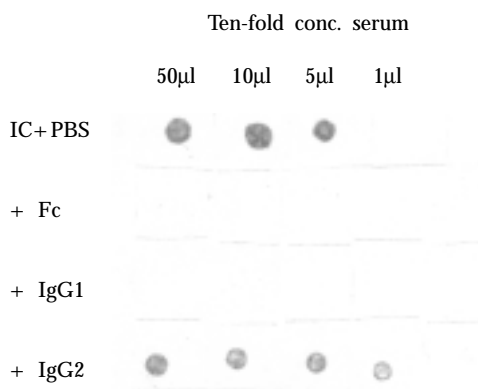


Fig. 3. Binding of immune complexes to tenfold concentrated serum from bovine with chronic leukemia immobilized onto nitrocellulose membrane was inhibited by Fc fragments of IgG, heat aggregated IgG1 but not IgG2

When purified sFcR was blotted onto a nitrocellulose membrane, a strong inhibition was observed with Fc fragments of IgG molecule and aggregated IgG, but not with F(ab)₂ fragments, IgA and IgM (Fig. 4).

A strong hydrophobic association between protein and nitrocellulose membrane forms is the basis of the dot blot assay [11]. Because of this efficient

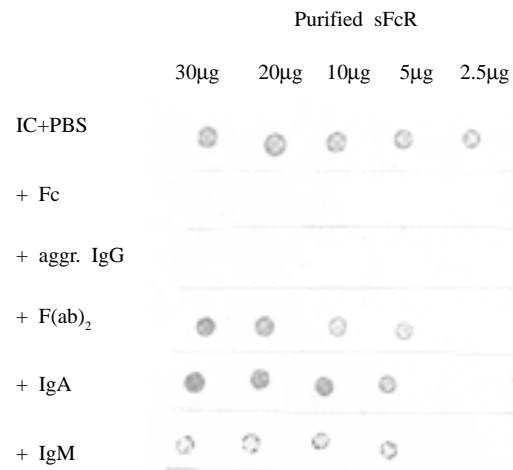


Fig. 4. Binding of immune complexes to purified bovine blood sFcR immobilized onto nitrocellulose membrane

retention of deposited protein, microgram or even nanogram quantities of substances can be used per test. BSA was used after sample application to block non-occupied sites of the NC and also in the reagent dilutions to prevent non-specific binding. This blocking solution can be stored frozen and repeatedly re-used.

In the present report we describe a method for the detection and functional characterization of sFcR by a dot-immunoperoxidase technique. Our data show that material blotted onto a nitrocellulose membrane can retain the functional ligand binding capability. Binding and inhibition experiments using IgG, Fc and F(ab)₂ fragments indicate that binding of immune complexes to protA and to tenfold concentrated serum is Fc-mediated. Soluble forms of low affinity Fc receptors (FcγR), also called IgG-binding factors (IgG-BF), have been shown to play a regulatory role in immune responses. By use of immunodot assay with the anti-mouse FcγR MoAb, 2.492, the levels of IgG-BF have been measured in the sera of mice bearing syngeneic tumors of lymphoid and non-lymphoid origin or in mice injected with high doses of murine IgG [12]. These sera contained large amounts of IgG-BF as compared to controls. The enhancement of serum IgG-BF level was independent of the expression of FcγR by the tumor cells, suggesting that the majority of IgG-BF secreted in response to tumor was produced by the host rather than by the tumor (cells). Therefore, in our situation concentrated serum from bovine with chronic leukemia is a convenient model to demonstrate sFcR.

The binding of subclasses of bovine IgG to protA showed the same specificity as reported elsewhere (Bjork and Kronwall [13]). A dot immunobinding assay on nitrocellulose membranes has been deve-

veloped for the quantification of human IgG subclasses using subclass-specific monoclonal antibodies [14]. The high sensitivity combined with the small sample size required makes this technique suitable for the analysis of IgG subclass levels in serum and other body fluids obtained from patients. Dot immunosay techniques are widely used to detect a variety of substances such as antibodies [11] and antigens [15]. These studies showed that FcγR mediate the binding of IgG to material immobilized on nitrocellulose membrane.

The specificity of bovine IgG has been reported to IgG1 and IgG2 [16]. Direct fluorometric binding studies indicated that IgG1 bound the same average number of FcR (3×10^5) on bovine leukemic lymphocytes as IgG2 with approximately the same affinity. It appeared that IgG1 and IgG2 were bound at the same sites with similar affinity. However, there are no data on FcR responsible for these interactions.

Three subclasses of human IgG have been described previously [17], and two of these, IgG2 and IgG3, occur in two allelic forms. The half-life of the two major IgG isotypes (IgG1, IgG2) has been reported in several studies, but the values are extremely divergent among the publications (for review, see Butler [18]). However, the data indicate that they both fall in the range of 10–22 days, with a longer half-life for IgG2. Based on the positive correlation between binding affinity and half-life, these data may suggest that IgG2 binds more effectively to the FcγR than does IgG1. In a sense this contradicts our results. Indeed, it is not known whether FcRI, -II or -III retain their Fc-binding ability when immobilized to nitrocellulose membrane or whether the Fc-binding observed is mediated by other FcRs.

The sensitivity and the accuracy of the assay, the care of performance and extremely stable color reaction on the nitrocellulose membrane also permit semi-quantitative analysis by naked eye. The results could be interpreted visually without any absorbance measurement and stored as a permanent record. All the reagents in the assay were commercially available thus minimizing the technical time. It allows avoiding the use of radioactive probes, too.

In conclusion, the dot-immunoperoxidase assay described a rapid and simple method for the demonstration and characterization of subclass specificity of sFcγR. Moreover, our findings suggest that the dot-immunoperoxidase assay may be a useful tool for rapid detection of active sFcR in serum or in other body fluids. For example, sFcγRIII serum levels may be of clinical interest. They can be modified in patients with multiple myeloma and AIDS [19]. It is a practical technique for the detection of

FcR molecules in solutions ranging in volume from a few μl to 1–2 ml, depending on the affinity of FcR.

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References

1. Joi-Van-der Zijde CM, Labadie J, Vlug A, Radl J, Vossen JM, Van Tol MJD. *J Immunol Methods* 1988; 108: 195–203.
2. Renner SW. *Arch Pathol Lab Med* 1988; 2: 780–84.
3. Stott DJ. *J Immunol Methods* 1989; 119: 153–7.
4. Miliukienė VV, Dikiniene NP. *Ukrainian Biochemical Journal* 1988; 60 (N2): 7–14.
5. Miliukienė VV, Dėdiniene J, Aleksienė A. *Ukrainian Biochemical Journal* 2001; 73 (N 6): 84–8.
6. Williams MR, Maxwell DAG, Spooner RL. *Res Vet Sci* 1975; 18: 314–21.
7. Nisonoff A, Wissler FC, Lipman LN. *Arch Biochem Biophys* 1960; 89: 230–3.
8. Laemmli UK. *Nature (Lond.)* 1970; 227 (5259): 680–5.
9. Grabar P. *J Immunol Methods* 1975; 7: 305–26.
10. Axen R, Porath J, Ernback S. *Nature* 1967; 214: 1302–7.
11. Xu J-I, Gorny MK, Zolla-Pazner S. *J Immunol Meth* 1989; 120: 179–83.
12. Lynch A, Tartour E, Teiland JL, Asselain B, Fridman WH, Sautes C. *Clin Exp Immunol* 1992; 87: 208–14.
13. Bjork L, Kronvall G. *J Immunol* 1984; 133: 969–71.
14. Jol-Van der Zijde CM, Labadie J, Vlug A, Vossen JM, Radl J, Van Tol MJD. *J Immunol Methods* 1988; 108: 195–203.
15. Van Vooren JP, Turneer M, Yernault JC, Bruyn JD, Burton E, Legros F, Farber CM. *J Immunol Methods* 1988; 113: 45–9.
16. Dikiniene NP, Miliukienė VV, Tamošiūnas VJ. *Vet Immunol Immunopathol* 1986; 13: 111–20.
17. Kacs Kovics I, Wu Z, Simister NE, Frenyo LV, Hammarstrom L. *J Immunol* 2000; 1889–97.
18. Butler JE. *Prog Vet Microbiol – Immunol* 1986; 2: 1–34.
19. Astier A, De la Salle H, Moncuit J, Freund M, Cazenave JP, Fridman WH, Hanau D, Teillaud JL. *J Immunol Methods* 1993; 166: 1–10.

Valė Miliukienė, Audronė Pilinkienė, Genė Bizilevičienė DOT-IMUNOPEROKSIDAZINIS METODAS TIRPIAM Fcγ RECEPTORIUI NUSTATYTI

S a n t r a u k a

Mes siūlome jautrø dot-imunoperoxidazės metodà tirpiam Fcγ receptoriui (sFcγR) nustatyti bei jo funkciniam aktyvumui ištirti. Tiriamuose mėginiuose esantys sFcγR, imobilizuoti ant nitroceliuliozinės membranos, buvo nustatyti remiantis jø sàveika su imuniniais kompleksais, sudarytais ið krienø peroksidazės (KP) ir triuðio IgG antikūnø prieš KP. Imuniniø kompleksø panaudojimas apraðytame dot-imunoperoxidaziniame metode leidþia ne tik greitai ir paprastai nustatyti sFcγR funkcinà aktyvumà, bet ir poklasinà IgG1 ir IgG2 specifiðkumà.