Hen Egg Immunoglobulin Y in Colloidal Gold Agglutination Assay: Comparison With Rabbit Immunoglobulin G

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Conjugation of immunoglobulin G (IgG) from rabbit serum with colloidal gold for sol agglutination assay was conducted. Effects of various buffer systems on conjugation and stability of sols were studied. Colloidal gold assay for detection of human myoglobin and C-reactive protein was developed. It was shown that rabbit IgG had a poor stabilizing effect on colloidal gold sol and therefore that it

cannot be effectively used in such assays. In contrast, hen egg immunoglobulin Y (IgY) had excellent stabilizing properties used in various buffer systems and in a wide range of concentrations. IgY was considered as a useful alternative for rabbit antibodies in gold sol agglutination assay. *J. Clin. Lab. Anal.* 19:124–127, 2005. © 2005 Wiley-Liss, Inc.

Key words: immunoglobulin Y; colloidal gold; immunoassay

INTRODUCTION

Particles labeled by antibodies are widely used in clinical practice. In particular, polystyrene latex beads covered by antibodies are applied for detection of appropriate antigens (1–3). This approach permits the conduction of not only semiquantitative but also quantitative analysis of various molecules (4–6). However, it is well known that such latex agglutination systems give more false-positive results than erythrocytes conjugated by the same antibodies (7). Moreover, quantitative analysis supposes measurement of turbidity, which demands special equipment; in addition, the turbidity of the probes themselves can give less than adequate results. Colloidal gold particles conjugated with antibodies are a useful alternative in immunoassay. The principle of the assay is based on the fact that colloidal gold has different colors dependent on its size. Small particles are purple but an increase in their size shifts their color from purple to blue (8). Thus, after the binding of the appropriate antigen with antibody-gold particles, an aggregation takes place with a change of sol color that can be easily detected by photometry. This approach permits the detection of antigens with high specificity and sensitivity. The sensitivity of this assay is comparable with traditional Enzyme Immune Assay (EIA) but, in contrast to the latter process, gold sol agglutination assay is one-stage, easily performed, and does not demand complex equipment. This procedure was successfully applied for quantitative determination of various antigens (9–11). But there are also some

disadvantages of this process. Conjugation of the proteins with colloidal gold takes place effectively at a pH near the isoelectric points of the proteins; it is not always possible to provide such conditions since gold sols have limited stability at $\text{pH} \leq 7.0$. On the other hand, some antibodies (rabbit and human immunoglobulin G (IgG)) have poor stabilizing properties and their conjugates with colloidal gold have a tendency toward spontaneous agglutination (12). This limits the wide employment of this technology to a certain extent. Therefore, it is useful to study the stabilizing influence of other antibodies on colloidal gold. Such an alternative could be the hen egg yolk immunoglobulin Y (IgY). It has some advantages in comparison with serum immunoglobulins. The antibodies can be stored in eggs at 4°C for at least 1 year and large amounts of antibodies can be obtained at a relatively low cost (13). Moreover, IgY is more resistant to high temperature, ionic strength, and more importantly, it does not cross-react with human rheumatoid factors; this is advantageous in many immunological tests (14). This article presents data on the stabilizing properties of hen egg yolk IgY on colloidal gold and a comparison of its

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properties with the properties of human and rabbit immunoglobulins.

MATERIALS AND METHODS

Human C-reactive protein (CRP) was purified from pleural fluid according to (15), myoglobin taken post-mortem from human heart was prepared by cation exchange chromatography on carboxymethyl (CM) cellulose followed by chromatography through G-50. Polyclonal antibodies to these proteins were developed in rabbits by the procedure described in Ref. 16. The titer of antibodies was determined by double immunodiffusion assay as described in Ref. 17, and it was not less than 1:8. Rabbit IgG fractions were prepared as described in Ref. 18. Concentrations of immunoglobulins were determined from extinction at 280 nm $E_{1\text{cm}}^{1\%} = 14.5$. The IgY from hen egg yolk was prepared according to Ref. 19, without thiophilic chromatography. Concentration of the IgY was determined from extinction at 280 nm $E_{1\text{cm}}^{1\%} = 15.1$. The purity of the antibody preparations was at least 90%, judged from electrophoresis in 6% Polyacrylamide gel (PAAG). Colloidal gold particles with sizes of 50 nm for application in agglutination reactions were prepared according to Ref. 9. All solutions used for preparation of the sol were filtered through 0.22- μm membrane. Buffers used for agglutination reactions were prepared as concentrated solutions (Tris-HCl and glycine-NaOH as 1 M and borate buffer as 0.25 M). Stability of protein-gold conjugate was tested photometrically by the absorbance decrease at 530 nm after the addition of 0.5 volume 2 M NaCl to the conjugate.

For the assay procedure, 1 mL of conjugate was added to 0.1 mL standard antigen solution (CRP or myoglobin). The contents of the test tube were mixed thoroughly. After an incubation time of 1.5 hr at room temperature, the absorbance at 530 nm was measured. For the control test, 0.1 mL of buffer solution was added to gold conjugate. The practical measuring range was defined as the part of the dose-response curve that can be approximated by a straight line; the part of the dose-response curve with absorbance within three standard deviations (SDs) of the average absorbance of the blank was excluded. To investigate the reproducibility of the dose-response curves, we repeated the procedure on 5 days. The analytical detection limit was defined as the concentrations of analytes that can be discriminated with 99% confidence from control (0 mg/mL of standard). The within-run coefficients of variation (CVs) were calculated from three measurements of CRP and myoglobin in four different samples. Concentrations of the myoglobin were as follows: 100, 345, 520, and 1,000 ng/mL. Concentrations of the CRP were as follows: 15, 25, 40, and 50 $\mu\text{g/mL}$.

RESULTS

For preparation of the antibody-gold conjugate to 10 μL of glycine-NaOH buffer (pH 8.8), various amounts of antibody preparations were added and to them 1 mL of colloidal gold sol was added. After incubation for 10 min (22%) BSA in concentration of 22% was added, to a final concentration of 0.2%; after additional incubation for 10 min, concentrated buffer solution and 2 M NaCl were added, to a final concentration of 0.1 M and 0.15 M, respectively. Similar conjugations were conducted in other buffer systems, only in the case of borate buffer, 40 μL of buffer was added to the various concentrations of antibodies. Figure 1 demonstrates the effects of increasing concentrations of rabbit IgG on the stability of colloidal gold in the buffer systems noted. As can be seen, the increasing concentrations of rabbit IgG bring the colloid to spontaneous agglutination. Tris-HCl buffer at pH 7.1 is very unfavorable for stabilization of gold sol by these antibodies. In particular, antibodies at a concentration of less than 2 μg cause spontaneous agglutination. In this case, the addition of only IgG is sufficient for irreversible agglutination. The situation with Tris-HCl buffer (pH 8.5) and glycine-NaOH buffer (pH 8.8) is somewhat better. In this case, a concentration of antibodies of 2–4 μg causes spontaneous agglutination. Better results were obtained in the borate buffer system. Moreover, it should be noted that any attempt to centrifuge conjugate to remove the unbound protein was unsuccessful. Residue after centrifugation cannot be dissolved in buffer solution. Thus, in contrary to a latex agglutination system, rabbit antibodies have a destabilizing action on colloidal gold particles. Figure 2 shows the stabilizing properties of hen egg IgY. As can be seen, an excellent stabilization takes place in a wide range of concentrations and in various buffer systems. It is also

Fig. 1. The effects of rabbit immunoglobulin G concentrations on agglutination of colloidal gold in various buffer systems. ◆: glycine-NaOH buffer, pH 8.8. ●: Tris-HCl buffer, pH 8.5. ▲: Tris-HCl buffer, pH 7.1. ■: borate buffer, pH 8.5.

Fig. 2. The effects of hen egg immunoglobulin Y concentrations on agglutination of colloidal gold in various buffer systems. $\blacklozenge$: glycine-NaOH buffer, pH 8.8. $\bullet$: Tris-HCl buffer, pH 8.5. $\blacktriangle$: Tris-HCl buffer, pH 7.1. $\blacksquare$: borate buffer, pH 8.5.

Fig. 3. Gold sol agglutination assay for detection of human myoglobin (A) and C-reactive protein (B). The assay was conducted by adding 0.1 mL of appropriate antigen to 1 mL of gold antibody conjugate. After incubation for 1.5 hr, differences in the optical density at 530 nm of control and probe were measured. The control assay was conducted similarly, only the antigen was replaced by physiological solution. A: The concentrations of antibodies for 1 mL sol were: 3 μ g ($\blacklozenge$) and 6 μ g ($\blacksquare$). B: The concentrations of antibodies for 1 mL sol were: 8 μ g ($\blacksquare$) and 15 μ g ($\blacklozenge$).

important to note here that centrifugation to remove the unbound material does not bring the gold-protein conjugate to irreversible agglutination. Figure 3 demonstrates the examples for determination of C-reactive protein and myoglobin by colloidal gold immunoassay with rabbit antibodies. As can be seen, the concentra-

tion of antibodies applied for sensitization of gold particles strongly influences the assay. So, the linearity of the assay is limited and is extended for myoglobin up to about 1,000 ng/mL at an antibody concentration of 3 μ g/mL of sol. Increasing the concentration of the antibodies up to 6 μ g/mL of sol shifts the linearity to higher concentrations, disturbing the linearity at low concentrations. In this assay, the CV was about 7% for lower concentrations of myoglobin (100 ng/mL) and about 4% for the upper range (1,000 ng/mL). For CRP, assay linearity is observed in only a narrow scale, and here coefficients of variations were higher, at 9% and 5%, respectively. Storage of conjugates for 1 month or more impaired the analytical characteristics of these systems. So, some spontaneous agglutination takes places during storage and as result, the sensitivity of the test also is decreased. Therefore, calculation of CVs in between-run assays after storage were not conducted. The analytical characteristics of these systems were worse than colloidal gold assays for ferritin (9) and chorionic gonadotropin (11), in which mice monoclonal antibodies were applied as sensitizers. It should be noted that any attempt to increase the concentration of antibodies for sol sensitization causes spontaneous agglutination. Further, it was impossible to centrifuge conjugate to remove any unbound material and to concentrate sol for increasing sensitivity of the assay.

DISCUSSION

In particle-enhanced immunoassay, stability of sensitized particles is critical for reliable assay. Decreased stability is a cause for false-positive results and for impairment of sensitivity. In polystyrene latex bead immunoassay, these particles in general are stabilized by antibodies and in this case the problem can be resolved by the addition of large amounts of the antibodies to these beads. On the other hand, it causes increased sensitivity of the assay (16). In contrary, in colloidal gold immunoassay, sensitization of beads by rabbit antibodies causes spontaneous irreversible agglutination of gold particles. The data presented demonstrate that stable conjugation of rabbit antibodies to colloidal gold depends on various factors, namely the buffer system, pH, concentration of antibodies, and their titer. Since the increasing concentrations of these antibodies cause spontaneous agglutination, it is difficult to achieve the desired results. Moreover, failure to remove any unbound material by centrifugation creates additional problems. It can be supposed that deviation from linearity at high concentrations of antibodies is associated with the increasing amounts of the unbound material in medium. Some approaches were developed to overcome this problem. In particular, the preliminary

modification of the gold surface by some agents prevents spontaneous agglutination after consequent immobilization of biomolecules (20, 21). On the other hand, hen egg IgY has excellent stabilizing effects on gold sol without any modification. It permits the use of any amount of antibodies necessary for sensitization. Further unbound material can be removed by centrifugation. The isoelectric point of hen egg IgY is below that of rabbit IgG and it is possible to speculate that conjugation of IgY with colloidal gold increases the total surface negative charge of particles, which causes their repulsion and thus their stabilization. In this aspect the data presented demonstrate that egg yolk IgY might be considered as a very useful alternative for such assays. Moreover, there are large phylogenetic differences between birds and mammals, and antibodies to human antigens can be developed more easily in bird than in any mammal.

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