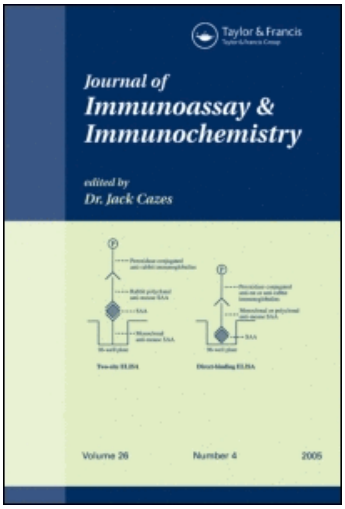

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Characterization of Antibody Labelled Colloidal Gold Particles and Their Applicability in a Sol Particle Immuno Assay (SPIA)

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CHARACTERIZATION OF ANTIBODY LABELLED COLLOIDAL GOLD PARTICLES AND THEIR APPLICABILITY IN A SOL PARTICLE IMMUNO ASSAY (SPIA)

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ABSTRACT

This study describes the characterization of antibody labelled colloidal gold particles and their applicability in a sol particle immuno assay (SPIA) to quantify murine monoclonal antibodies of all IgG isotypes. Two physical methods (transmission electron microscopy (TEM) and dynamic light scattering (DLS)), were used to obtain information about particle size and morphology of the gold sols, but only with DLS antibody labelling could be detected. In addition, electrophoretic methods like agarose electrophoresis and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) showed also that antibody labelling was successful.
The biological activity of the antibody gold conjugates was determined by using them in a SPIA and as an electrondense marker in an immunogold labelling procedure to visualize meningococcal surface exposed outer membrane proteins labelled with monoclonal antibodies. The SPIA was applicable to determine murine monoclonal antibodies of all IgG isotypes with a sensitivity of 20 - 80 ng/ml and a coëfficiënt of variation of 6.7 ± 2.2%.

(KEY WORDS: colloidal gold, antibody labelling, SPIA, monoclonal antibodies).

INTRODUCTION

Since Köhler and Milstein (1) introduced the hybridoma technique monoclonal antibodies are being used in a wide range of applications e.g.

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for in vitro and in vivo diagnostics, purification of antigens and as therapeuticum (2). For these purposes the demand for large quantities of monoclonal antibodies has increased dramatically over the last few years. So large scale cultivation of hybridomas has become a widely used method to produce monoclonal antibodies (3-6). The enzyme-linked immunosorbent assay (ELISA) has become a broadly used technique as an in process control to determine monoclonal antibody concentrations during cultivation and downstream processing (7, 8).

Another possibility to quantify monoclonal antibodies is based on agglutination of complexes of monoclonal antibodies and antibody labelled colloidal gold particles resulting in colour reduction of the gold sol. An agglutination immuno assay using conjugates consisting of antibody coated colloidal gold particles was earlier described by Leuvering et al. (9, 10) for the quantification of human chorionic gonadotrophin. Also Wielaard et al. (11) described an agglutination immuno assay for the quantification of anti-rubella antibodies. The technique based upon this agglutination principle is termed sol particle immunoassay (SPIA). Conjugates of antibody coated gold particles have also found a wide acceptance as cytochemical marker for TEM and scanning electron microscopy (SEM) (12-17).

In the present study we describe the characterization of antibody labelled colloidal gold particles and their applicability for the quantitative determination of murine monoclonal antibodies.

MATERIALS AND METHODS

Antisera

Affinity purified goat anti-mouse IgG (Fc-specific) (GAM-IgG) was purchased from Cooper Biomedical, Malvern PA. The GAM-IgG was dialysed overnight against 0.002 M borax (Merck, Darmstadt, FRG), pH 9.0.

Preparation of colloidal gold

Colloidal gold with an average particle diameter of 50 nm (G 50) was prepared by controlled reduction of a boiling solution of 0.01% chloroauric acid (250 ml) (Merck, Darmstadt, FRG) with 1% sodium citrate (2.5 ml) (Merck, Darmstadt, FRG) (18). All the glassware was siliconized prior to use with a silicon solution (Serva, Heidelberg, FRG). The solutions of the reagents were prepared in double distilled water and filtered through a 0.2 μm membrane filter (Sartorius, Göttingen, FRG).

Gold sols with an average diameter of 40 nm (G 40) were purchased from Janssen Pharmaceutica, Beerse, Belgium.

Preparation of antibody-gold

The pH of the gold sol was adjusted to 9.0 with 0.2 M K_2CO_3 (Merck, Darmstadt, FRG). The dialyzed GAM-IgG solution was added until an IgG-concentration of 6 μg/ml gold sol was obtained (determined by constructing a concentration variable adsorption isotherm (14, 15)). After 10 min. incubation at room temperature the conjugate was stabilized either with bovine serum albumine (BSA) (Boseral Dubos, Organon Teknika, Oss, The Netherlands) or polyethylene glycol (PEG) (20,000 Mw, Serva, Heidelberg, FRG). If BSA was used for stabilization, a 10% BSA solution was added to a final concentration of 1%. The mixture was then incubated for 1 hr at room temperature and subsequently centrifuged (15,000 x g, 10 min.). The supernatant was discarded and the pellet was resuspended in a 1% BSA solution, pH 8.2. This was repeated two times. Concentrated conjugate was stored at $4^{\circ}C$ (A_{540} = 2.5). In case of stabilization with PEG, a solution containing 1% PEG and 5 mM sodium chloride (Merck, Darmstadt, FRG), pH 9.0, was added to a final concentration of 0.02% PEG. After every washing cycle, of a total of three, the pellet was resuspended in 0.02% PEG solution containing 5 mM sodium chloride, pH 8.2, and stored at $4^{\circ}C$ (A 540 = 2.5).

Transmission electron microscopy and dynamic light scattering

The average particle diameter of the gold particles and antibody gold conjugate (GAM-G conjugate) was determined by TEM (EM 400; Philips, Eindhoven, The Netherlands) at 80 kV and DLS (19) (system 4600; Malvern Instruments, Ltd., Worcestershire, U.K.). For TEM observation carbon-stabilized formvar-coated copper grids were used, supplied with 1% poly-L-lysine (MW 30,000 to 70,000; no. P2636; Sigma, St.Louis, MO). Grids were incubated for 15 min. by floating them on drops of colloidal gold or GAM-G conjugate placed on Parafilm, followed by jet-washing with aquadest and air-drying.

Sol Particle Immuno Assay

The concentrated, BSA stabilized, GAM-G40 conjugate was used for the SPIA and whole mount incubations. Monoclonal antibody samples were stepwise diluted in phosphate buffered saline (PBS), pH 7.3, containing 0.05% Tween 20 (Merck, Darmstadt, FRG) and 0.5% BSA. Series of eight two-fold dilution steps were prepared in polystyrene microtitre plates (Flow Laboraties, Woodcock Hill, U.K.) (100 μl/well). The GAM-G40 conjugate was added, 100 μl to each well, and then incubated for 30 min. at room temperature. The absorbance at 540 nm was measured with a microtitre plate reader (Flow Laboratories, Woodcock Hill, U.K.).

Enzyme-linked Immunosorbent Assay

Wells in polystyrene microtitre plates (Flow Laboraties, Woodcock Hill, U.K.) were coated, overnight at room temperature, with sheep anti-mouse IgG (SMuG 73; 28 μg/ml in PBS, pH 7.3, National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands). The plates were washed three times with PBS, containing 0.05% Tween 20 (Merck, Darmstadt, FRG). Monoclonal antibody samples were stepwise diluted in PBS-Tween-BSA. Series of eight two-fold dilution steps were prepared in

polystyrene microtitre plates (Flow Laboraties, Woodcock Hill, U.K.) (100 µl/well). After 1 hr at 37°C the wells were washed three times with PBS containing 0.05% Tween 20 (PBS-Tween). Peroxidase-labelled GAM-IgG (Cooper Biomedical, Malvern, PA) was added (100 µl 1:2,000 in PBS-Tween-BSA) to each well. After incubation for 1 hr at 37°C the wells were washed three times with PBS-Tween and 100 µl of the enzyme-substrate, 300 mg 3,3',5,5'-tetramethyl benzidine (Sigma, St.Louis, MO) in 50 ml dimethyl sulfoxide (Merck, Darmstadt, FRG) was added and incubated for 3-7 minutes. The enzyme reaction was stopped by adding 100 µl 2 M H_2SO_4. Absorbance was measured at 450 nm with a microtitre plate reader (Flow Laboratories, Woodcock Hill, U.K.).

Whole-mount incubations

To determine the biological activity of the antibody gold conjugate, whole bacteria were incubated in suspension as recently described by Pâques et al. (16). Briefly, 500 µl of a suspension of Neisseria meningitidis (strain H44/76, serosubtyped as B:15:P1.16) was incubated for 30 min. with 50 µl polyclonal antibodies (dilution 1:10 in PBS) with binding specificity for surface antigens, obtained by immunizing NIH mice intraperitoneally with outer membrane vesicles of the meningococcal strain H44/76 containing 50 µg of protein per ml and 2 mg of $AlPO_4$ per ml as an adjuvant. As a negative control a 1:10 dilution of normal mouse serum was used. After this primary incubation the cells were suspended in 50 µl undiluted GAM-G and incubated for 30 min.. Between and after the incubations the cells were washed thoroughly with PBS. To stabilize antigen-antibody complexes, the cells were fixed with a mixture of 2% paraformaldehyde and 0.5% glutaraldehyde in PBS for 5 min.. Finally, the cells were adsorbed to carbon-stabilized formvar (Schuckardt, München, FRG) coated copper grids (Stork Veco, The Netherlands) supplied with 1% poly-L-lysine (Mw 30-70,000; no. P2636; Sigma, St.Louis, MO). The cells were

negatively stained for 15 sec. with a 0.5% aqueous potassium phosphotungstate (Merck, Darmstadt, FRG) solution, pH 6.5, and examined in a TEM (EM 400, Philips, Eindhoven, The Netherlands).

Agarose electrophoresis

Agarose electrophoresis was performed in 2% agarose (FMC Corporation, Marine Colloids Div., Rockland, ME) in barbital buffer pH 8.6. Gels were poured on the hydrophilic side of 7 x 10 cm gel bond. After cooling, wells were punched out the solidified gels in which the samples were placed. Electrophoresis was run at a field strength of 3.5 V/cm for 120 min. Since the GAM-G conjugates have an intrinsic bright red colour, the results were immediately apparent.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SDS-PAGE was performed according to Laemmli (20) in a vertical electrophoresis system. Briefly, the running gel consisted of 12.5% acrylamide (Merck, Darmstadt, FRG) cross-linked with 0.1% bisacrylamide (Merck, Darmstadt, FRG) in 4.5% Tris hydrochloric buffer (pH 8.8) (Merck, Darmstadt, FRG) containing 0.1% sodium dodecyl sulfate (SDS) and 0.1 M urea (Merck, Darmstadt, FRG). The gel was polymerized with 0.036% ammonium persulfate and 0.005% N,N,N',N' tetramethyl ethylenediamine (TEMED). The stacking gel consisted of 4% acrylamide, 0.1% bisacrylamide and 0.1% SDS in 1.5% Tris hydrochloric buffer (pH 6.8). Samples were diluted into a sample buffer containing 0.76% Tris hydrochloric buffer, 2% SDS, 1.5% dithiothreitol, 0.002% bromophenol blue, and 6% urea, and were loaded into the slots and then overloaded with running buffer (0.3% Tris hydrochloric acid, 1.4% glycine (Merck, Darmstadt, FRG) and 0.1% SDS, (pH 8.3).

Electrophoresis was carried out overnight at 50 V. The gel was fixed in 50% methanol (Merck, Darmstadt, FRG). The protein bands were visualized by silver staining, according to Wray et al. (21).

TABLE 1

Mean particle sizes of G40 colloidal gold particles and G50 colloidal gold particles and their respective GAM-G conjugates. Particle sizes were determined with transmission electron miscroscopy (TEM) and dynamic light scattering (DLS).

	TEM	DLS	
Sample	Mean particle size ± SD (nm)	Mean particle size ± SD (nm)	Polydispersity index
G40	42 ± 6 (n = 100)	44 ± 2 (n = 6)	0.2
GAM-G40	42 ± 6 (n = 100)	50 ± 1 (n = 3)	0.5
G50	58 ± 10 (n = 100)	60 ± 6 (n = 6)	0.6
GAM-G50	60 ± 7 (n = 57)	54 ± 2 (n = 3)	0.5

RESULTS

Characterization of the colloidal gold sol and the antibody gold conjugate

TEM and DLS. Table 1 shows the results of particle size measurements of our gold sol (G50) and a gold sol obtained from Janssen Pharmaceutica (G40) and the corresponding antibody gold conjugates (GAM-G50 and GAM-G40, respectively). The measurements were performed with TEM and DLS. The values obtained with the two different techniques agreed very well. Mean particle sizes of unlabelled gold particles differed less then 5%. For the conjugated particles larger deviations were found: 16% for the G40 gold particles and 10% for the G50 particles. The polydispersity indices for the G40 gold sol and the G50 gold particles were about 0.2 and 0.6, respectively. This indicates that G50 sols were more heterogeneous when compared to the G40 sols. This was confirmed by the TEM studies, which showed that the colloidal G40 gold particles had spherical morphology and no aggregates were found, whereas colloidal G50 gold sol contained more pleomorph shaped particles and aggregates. Also twinned colloidal gold

particles were observed. These larger non-uniformly shaped particles also contribute to the higher polydispersity index.

Agarose and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. To estimate if the IgG adsorption onto the gold particles has been successful, two electrophoretic techniques were performed: agarose electrophoresis and SDS-PAGE. Electrophoretic mobilities of the labelled and unlabelled gold particles in agarose gels differed dramatically. Unlabelled particles did not leave the well, whereas the labelled ones had a good electrophoretic mobility. The mobility of the GAM-G conjugates was also depending on the particle size: smaller particles had a greater electrophoretic mobility than larger gold particles. SDS-PAGE was performed under reducing conditions. The proteins were desorbed from the surface of the gold particles and migrated into the gel, while the gold particles were retained in the sample slot. The GAM-G conjugate stabilized with PEG showed a characteristic SDS-PAGE pattern of IgG, i.e a heavy and a light chain. The GAM-G conjugate stabilized with BSA showed a broad band in the SDS-PAGE pattern. This was due to the high ratio BSA-to-IgG (> 1,000).

Effect of the stabilizer on the SPIA. Figure 1 shows the relation between the steepness of the dose response curves and the stabilizer used in the SPIA. When PEG was used as stabilizer instead of BSA, the linear part of the dose response curve turned out to be much steeper. For both stabilizers, the slopes of the standard curve (purified monoclonal antibodies of the IgG2a isotype) corresponded to those of sample curves (concentrated culture supernatant of the IgG2a isotype). Thus, a quantitative interpretation of the data is possible, resulting in the same monoclonal IgG concentration for both stabilizers. The GAM-G conjugates stabilized with BSA were stable for at least eight months, whereas the GAM-G conjugates stabilized with PEG were stable for only one week. Therefore we used the BSA stabilized GAM-G conjugates for further experiments.

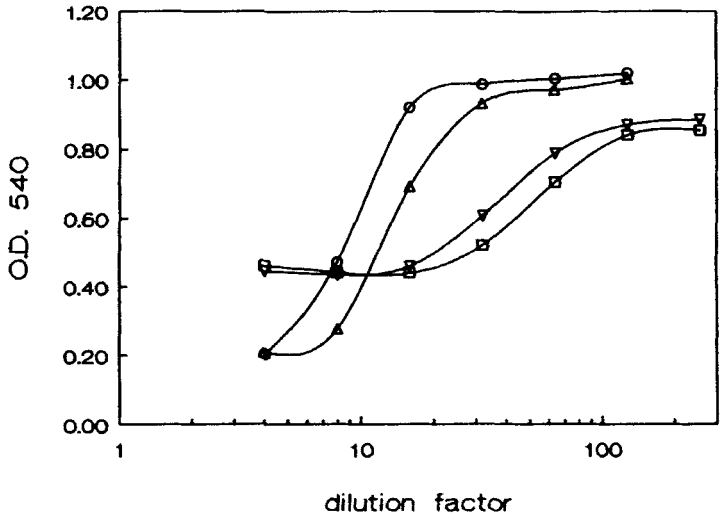

FIGURE 1. Effect of the stabilizers BSA (▽, □) and PEG (Δ, o) on the dose response curves of purified monoclonal antibodies of the IgG2a isotype (o, ▽) and concentrated culture supernatant of the IgG2a isotype (Δ, □), incubated for 30 min..

Determination of the biological activity of the GAM-G conjugate. Whole mount incubation of meningococcal cells with the polyclonal antibodies (anti-outer membrane proteins of meningococci) followed by GAM-G conjugate resulted in moderate labelling densities (Fig. 2a). Control incubations with negative mouse serum resulted in cells that were virtual free of label (Fig. 2b). The results are indicative for the biological activity of the GAM-G conjugate. All obtained results of whole mount incubations and SPIA agreed with each other.

Sol Particle Immuno Assay

Effect of the IgG isotypes. For studies with the SPIA, BSA stabilized GAM-G40 conjugate was used. The effect of the murine isotypes IgG1, IgG2a, IgG2b and IgG3 on the dose response curve in the SPIA is shown in figure 3. The murine isotypes IgG1, IgG2a and IgG2b have similar slopes,

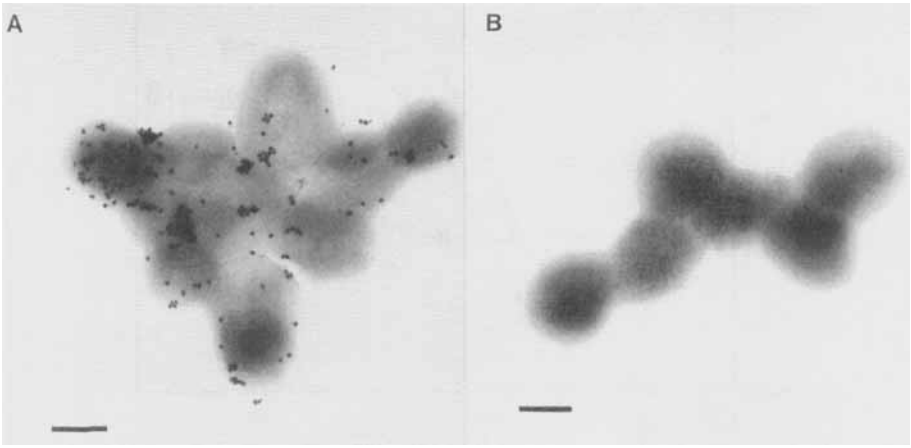

FIGURE 2. Whole mount incubation of meningococcal cells. Incubation of whole N.meningitidis bacteria (strain H44/76) in suspension with polyclonal antibodies against outer membrane proteins of meningococci followed by the GAM-G conjugate (Fig.2A), and negative mouse serum followed by the GAM-G probe as a negative control (Fig.2B). Bars: 400 nm.

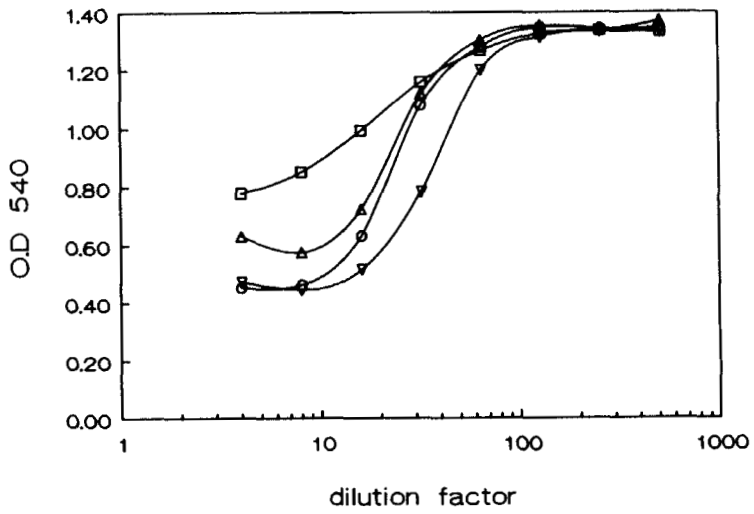

FIGURE 3. Dose response curves of purified monoclonal antibodies (1 mg/ml) of the murine isotypes IgG1 (Δ), IgG2a (o), IgG2b (∇) and IgG3 (□).

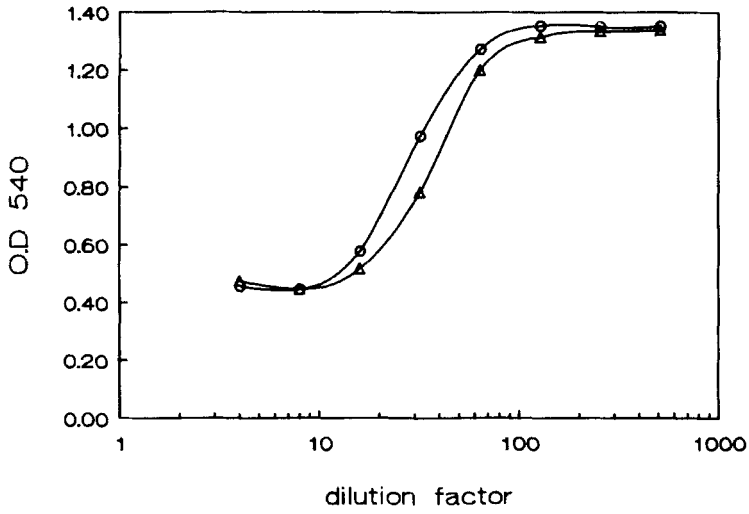

FIGURE 4. Effect of the origin of the sample on the dose response curves. Concentrated culture supernatant of the IgG2a isotype (Δ) and protein A purified concentrated culture supernatant of the same isotype (o).

which are steeper than the slope of the murine IgG3 isotype. For murine IgG3 it was necessary to increase the incubation time to 60 min. to obtain a significant sigmoid dose response curve.

Comparison of SPIA and ELISA. The SPIA showed to be suitable to quantify murine monoclonal antibodies of all isotypes in contrast to the ELISA, which could not be used to detect murine monoclonal antibodies of the IgG3 isotype. Figure 4 shows the effect of the origin of the sample (clarified concentrated culture supernatant and protein A purified clarified concentrated culture supernatant of the IgG2a isotype) on the dose response curves. The SPIA shows the same slope steepness in the linear part of the dose response curve. This was confirmed for the other murine isotypes (not shown). To determine the correlation between SPIA and ELISA, the concentration of monoclonal murine IgG of the IgG1, IgG2a and IgG2b isotypes in eleven samples have been determined and the mean was

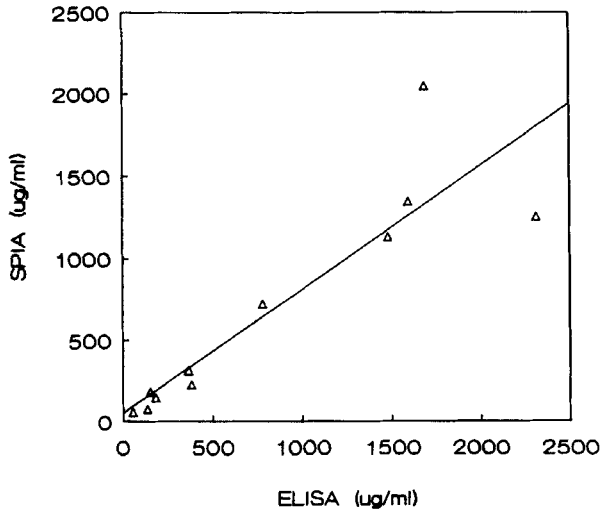

FIGURE 5. Correlation data of eleven samples determined with SPIA and ELISA.

calculated. The results of the correlation study are shown in figure 5. The correlation coefficient was 0.90. Each sample was determined four times in SPIA and ELISA. The mean coefficient of variation of the eleven samples was 6.7% for the SPIA and 6.6% for the ELISA. The linear part of the dose response curves in the SPIA as well as in the ELISA was between 20 and 80 ng/ml.

DISCUSSION

In this study we describe the characterization of antibody labelled colloidal gold particles and their use in a SPIA to determine the concentration of monoclonal murine IgG in hybridoma culture supernatants, as concentrates and as purified antibodies.

We have labelled colloidal gold particles with affinity purified goat anti-mouse polyclonal antibodies at pH 9.0 (14, 15). The labelling of antibodies to colloidal gold particles is a not well understood complex phenomenon depending on the stability of the colloid itself, the concentration, conformation and isoelectric point of the antibody, and the ionic strength, pH, and temperature of the suspending medium (12, 15).

Several methods were used to characterize the gold particles and the GAM-G conjugate. By TEM observation the particle size was determined, but could not give information about antibody adsorption on its surface, which should increase the particle size with about 30 nm, as the size of an antibody molecule is about 15 nm (17).

On the other hand, DLS measures the fluctuations in intensity of a scattered laser beam caused by the Brownian motion of the particle. So this method should detect antibody adsorption. The size of the G40 gold particles increased after labelling, which was not found for the G50 colloidal gold particles. The polydispersity index of the G50 gold particles was high (0.6), which made it more difficult to interpret the data with respect to the G40 gold particles, which had a lower polydispersity index (0.2). This is indicative for the wider variation in particle diameter of the G50 sol. TEM pictures also showed the presence of non-uniformly shaped gold particles. Also twinned colloidal gold particles were observed, as described earlier by Dhere et al. (22). Although the BSA stabilized GAM-G40 and GAM-G50 conjugates showed morphological differences, this did not influence the SPIA (data not shown).

Two electrophoretic methods, agarose electrophoresis and SDS-PAGE were also used to determine if protein adsorption on the surface of the gold particles was successful. In the case of agarose electrophoresis the unlabelled gold particles did not migrate into the gel in contrast with labelled particles. Depending on the particle size the particles migrated more or less into the gel. It can be concluded that the electrophoretic

mobility of colloidal gold is influenced by the antibodies adsorbed on its surface. SDS-PAGE under reducing conditions in a 12.5% gel showed for the GAM-G conjugate stabilized with PEG a typical IgG pattern, while the GAM-G conjugate stabilized with BSA showed a broad band of BSA. This was due to the high ratio BSA-to-IgG (> 1,000), which made the simultaneous detection of both proteins impossible. Although these two methods are well suited to determine whether the conjugation was successful or not, they do not give any information about the activity of the GAM-G conjugate.

Two methods, which were indicative for the biological activity, were the whole mount incubations of meningococci (Figure 2) and the SPIA. All the tested gold GAM-G conjugates, which were active in the whole mount incubations, were also active in the SPIA.

There are several reports describing the determination of monoclonal antibody by an ELISA (7, 8). ELISA includes two incubation steps of at least one hour and is followed by an enzyme-substrate reaction. With SPIA it takes only one incubation step of 30 min. for IgG1, IgG2a and IgG2b. For IgG3 the incubation time should be increased to 60 min.. The linear part of the dose response curves show parallellity for all tested samples. These two advantages make this assay attractive as an alternative for the ELISA.

The effect of two stabilizers (BSA and PEG), the isotypes of the murine IgG's and the origin of the sample were studied in the SPIA. The correlation between SPIA and ELISA was also investigated. The aim of the stabilizer is to minimize possible aggregation and to enhance the stability of the gold antibody conjugates. It also stops further antibody adsorption on the surface of colloidal gold and adsorbs to uncoated parts still present on the surface of colloidal gold. The uncoated parts could be arised during preparation or storage of the antibody gold conjugate and causes instability of the conjugate and effectuates aggregation. The GAM-G conjugate stabilized with PEG showed steeper dose response curves than the one stabilized with BSA (Fig. 1). Comparing both dose response curves with a reference resulted in the same IgG concentration.

Figure 3 shows the effect of murine isotypes on the dose response curves. It can be concluded that the slopes of the isotypes are identical, except for isotype IgG3, which is less steep. Comparing this with the dose response curves of the isotypes in an ELISA, all the slopes were different (data not shown), and with this ELISA system no murine monoclonal antibodies of the IgG3 isotype could be detected. The IgG concentration of the samples was 1 mg/ml. This would implicate that the dose response curves of the isotypes IgG1, IgG2a and IgG2b should be identical. The fact that this was not observed, could be due to differences in the affinity of the goat anti-mouse serum for the different isotypes.

This study shows that the SPIA can be applied successfully for the detection of all IgG isotypes, whether or not purified, in the 20-80 ng/ml range (linear part of the dose response curves) with a coefficient of variation of 6.7%. The detection limit and the coefficient of variation are comparable with those of the ELISA. The SPIA showed also a good correlation with the ELISA. The advantages of the SPIA are its simplicity and time saving.

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