Histochemistry (1981) 72:83–90

Histochemistry

Antibody Coated Gold Particles Containing Radioactive Gold in the Demonstration of Cell Surface Molecules

S.P. Kent and F.B. Allen

Department of Pathology, University of Alabama in Birmingham, University Station, Birmingham, Alabama 35294, USA

Summary. Gold particles of varying size which contain either ^{195}Au or ^{198}Au were prepared using white phosphorus or sodium citrate as the reducing agent. After coating with specific antibody to blood group A antigen or human IgG, these particles were used to determine the number of particles binding to the surface of A_1 RBC's or rat RBC's to which human IgG had been attached. The number of particles binding to the surface of cells correlated with the number of antibody coated gold particles in the fluid bathing the cells as well as the number of antigen molecules on the cell surface. That is, the number of particles binding increased as the particle density of the suspension increased and as the cell surface antigen density increased. Under the conditions of the experiments, both blood group A antigen and human IgG appeared to be randomly distributed over the surface of the cells in TEM and SEM preparations. This approach permitted the quantitation of the number of gold particles bound per cell and at the same time, the examination of the distribution of the particles over the surface of the same cell population by TEM and SEM.

Introduction

Colloidal gold has been known and used for various purposes since ancient times (Weiser 1933). Recently gold particles were found to be satisfactory as a marker for specific antibody in EM studies (Horisberger and Rosset 1977). Antibodies apparently bind irreversibly to the surface of gold particles. Further, gold particles can be prepared in a variety of sizes that can be seen quite well in Scanning Electron Microscopy (SEM) as well as Transmission Electron Microscopy (TEM) preparations (Horisberger and Rosett 1977; Frens 1973). We have found that varying amounts of radioactive gold, ^{195}Au or ^{198}Au can be incorporated into the particles when they are formed. This permits the counting of gold particles bound to the surface of the cells. In the same

0301-5564/81/0072/0083/$01.60

cell population the distribution of gold particles can be studied by either SEM or TEM. We have applied this approach to the study of two cell surface antigens, blood group A antigen and human IgG bound to the surface of rat RBC's.

Material and Methods

Preparation of Gold Particles. Suspensions of gold particles were prepared as described by Frens (1973). Five mg of $AuCl_3$ HCl $4H_2O$ were dissolved in 50 ml of distilled water. Forty µl of ^{195}Au containing approximately 1×10^6 counts per minute were added (New England Nuclear, Boston, Mass.). Depending on the size particle desired, varying amounts of a 1% solution of sodium citrate (0.3 ml in experiments 18 and 31 and 2 ml in experiment 37 – Table 1) were added. The solution was boiled for five minutes and allowed to cool. The volume was measured and a 0.1 ml sample set aside for counting in a Packard 5230 Gamma Counter.

Other suspensions of gold particles were prepared using white phosphorus as the reducing agent after the method of Horisberger and Rosset (1977). Varying amounts of ethyl ether saturated with white phosphorus (0.2 ml in experiment 25 – Table 1 and experiment 46 – Table 2) were added to 50 ml samples of previously boiled distilled water that contained 5 mg of chloroauric acid and 40 µl of ^{195}Au. The solution was boiled for fifteen minutes and allowed to cool. The volume was determined and a 0.1 ml sample was set aside for counting. In preparing some batches, ^{198}Au was substituted for ^{195}Au.

Purification of Anti-Sera. Ten ml of hyperimmune human anti-A (Bio-Lab, Birmingham, Ala.) were dialyzed overnight against 0.01 M potassium phosphate buffer pH 8. The serum was then applied to a 30 ml DEAE cellulose column (Levy 1960). The column was washed with 0.01 M phosphate buffer. The first fraction containing the IgG was cycled twice through a 5 ml sepharose-A antigen column (Axén et al. 1967). The column was washed with Tris-HCl buffer 0.05 M pH 7.4 until the effluent gave a 0 reading with an Hitachi spectrophotometer set at 280 mµ. Two ml of 10% N-Acetyl-d-galactosamine in phosphate buffered saline (0.1 M pH 7.2) (PBS) were added and mixed with the column. After 3 h at 4° C the column was washed with 20 ml of Tris-HCl buffer. An equal volume of saturated ammonium sulfate solution was added to the eluate. The antibody was collected by centrifugation and dissolved in 1 ml of 0.005 M saline and dialyzed against changes of saline overnight at 4° C.

Five ml of Rabbit anti-human IgG serum (Hyland Laboratories, Costa Mesa, Ca.) were cycled twice through a 5 ml sepharose-human IgG column (Axen et al. 1967). The column was washed with Tris-HCl buffer until the effluent gave a 0 reading with the spectrophotometer set at 280 mµ. The column was washed with 0.1 M glycine-HCl buffer pH 2.4. After the pH of the effluent began to drop, 30 ml was collected. Sodium hydroxide solution, 1 N, was added to raise the pH to 7. An equal volume of saturated ammonium sulfate solution was added. The precipitate was collected by centrifugation and was dissolved in 2 ml of PBS and dialyzed against three changes of 0.005 M saline overnight at 4° C.

Binding of Antibodies to Gold Particles. A batch of gold particles prepared as described above were added to 150 ml of 0.005 M NaCl. One ml of the antibody solutions prepared as described above was added and mixed thoroughly. The solution was allowed to stand for two minutes before 5 ml of 1% carbowax 20,000 was mixed with the solution. Using pH paper, 0.2 N potassium carbonate was added to raise the pH to 7. A 0.1 ml sample was set aside for counting.

The suspension was centrifuged for 45 min at 40,000 g in a Beckman Model L. Ultracentrifuge. The supernate was removed with a pipet and the gold particles resuspended in 0.005 M NaCl and 1% carbowax 20,000 (1:1). After a second centrifugation the gold particles were resuspended in 5 ml of 0.5 M potassium chloride or 0.05 M Tris buffer containing 0.15 M NaCl and 0.5 mg/ml of carbowax 20-M (Horisberger and Rosset 1977). After sonication for 30 seconds, a 0.1 ml sample was removed for counting.

Preparation of Target Cells. Five ml of heparinized blood were obtained from a blood type A_1 volunteer. The cells were sedimented and washed $\times 5$ with PBS. They were fixed in 15 ml 2.5% glutaraldehyde for three hours. They were washed $\times 4$ in PBS before use. Cells used as controls

in the experiments were obtained from a blood type 0 volunteer. "0" cells were prepared as described above.

Blood from a Sprague Dawley rat was treated as described above. To 100 ml of 1% suspension of the fixed rat cells, enough sodium periodate was added to make a 0.0005 M solution (Kent et al. 1972). After stirring occasionally for 30 min at 22° C the cells were washed $\times 2$ with PBS and suspended in 10 ml of borate buffer containing 20 mg of human IgG. This suspension was stirred occasionally for 30 min at 22° C. The cells were washed with PBS $\times 2$ and resuspended in 30 ml of PBS. Two drops of a 1% solution of sodium borohydride was then added with stirring. The cells were washed $\times 3$ with PBS before use.

In order to vary the number of IgG molecules on the surface of the RBC's the IgG was first labeled with I_{125} (David 1972) and then small amounts of IgG, 1 mg or 0.01 mg were reacted with the rat RBC's as described above. In addition, 1 mg of BSA was included with each reaction to further reduce the amount of IgG binding. After washing the cells in PBS they were counted in a Packard Gamma Counter and the number of IgG molecules per cell calculated (Table 2).

Control cells were prepared as described above except that 5 drops of 30% BSA were substituted for the human IgG.

Experiments. In experiments 18 and 25 gold particles averaging 420 ± 50 and 41 ± 14 Å in diameter respectively were coated with anti-human IgG. 5×10^6 rat RBC's with surface bound human IgG were suspended in 0.5 ml of gold particles coated with Rabbit anti-human IgG. For experiments 19 and 25 the gold suspensions contained 8.9×10^{11} and 3×10^{14} gold particles per ml respectively. The suspensions were rotated at 22° C for 2 h. After centrifugation, the supernate was saved for counting. The cells were washed twice with PBS (experiment 18) or 0.5 M KCl in Tris buffer (experiment 25) and transferred to a new tube. The old tube was saved for counting. The new tube containing the cells was also counted. The controls consisted of substituting rat RBC's with surface bound BSA for the rat RBC's with surface bound human IgG in the experiment above. In experiments 31 and 37 (Table 1) gold particles coated with human anti-A were suspended in either human A_1 RBC's or 0 RBC's (5×10^6). The gold suspension for experiments 31 and 37 contained 8.2×10^{11} and 5.7×10^{12} particles per ml respectively. Suspensions were rotated for 2 h at 22° C, centrifuged and the supernate was saved for counting. The RBC's were washed twice with 0.5 M KCl then transferred to a new tube. The old tube and the new tube containing the cells were counted.

In each part of experiment 46 the size of the gold particles, 54 ± 5 Å and the number of antibody coated gold particles/ml were kept the same. The number of antigen molecules, human IgG, on the surface of the RBC's varied in 46-a, 46-b and 46-c (Table 2). 46-e was the same as 46-a except for the addition of 1 mg of goat anti-human IgG to the gold suspension to see if the binding of gold particles to the RBC-IgG could be specifically blocked. Experiments 46-d and 46-f were two additional controls. Experiment 46-d employed BSA bound to the surface of the rat RBC's rather than human IgG. In experiment 46-f the gold particles were coated with nonimmune goat IgG rather than the goat anti-human IgG.

Results

The thirty-eight batches of gold particles prepared varied in size from 41 ± 13 to 420 ± 50 Å, depending on the reducing agent used and the quantity of agent. White phosphorus was necessary for producing the smaller particles. The size of the particles in a batch was determined by directly measuring the diameter of 50 representative particles in prints of TEM photographs. These data, applied to the formula for calculating the volume of a sphere, yielded the average volume of a particle. The volume of a gold particle multiplied by the specific gravity of gold, 19.3 g per cm^3, gave the grams of gold per particle. The weight of an average particle divided into the total amount of gold particles used to make the suspension yielded the total number of particles in a batch. Counting a sample from each batch in the gamma counter permitted the calcula-

Table 1. Reaction of antibody coated gold particles with cell surface antigens

Exp. No.	Size Gold Particles (Å)	Gold Particles (ml)[a]	Antibody	Antigen	Bound Particles/ Cell	% Non Specific Binding
18	420 ± 50	3.2×10^{11}	Rabbit anti-human IgG	A. human IgG-Rat-RBC's B. BSA-Rat-RBC's	$1{,}300 \pm 33$ 148 ± 18	11.4
25	41 ± 13	3.2×10^{14}	Rabbit anti-human IgG	A. human IgG-Rat-RBC's B. BSA-Rat-RBC's	$660{,}000 \pm 65{,}800$ $50{,}000 \pm 10{,}743$	7.6
31	308 ± 61	8.2×10^{11}	human anti Blood Group A	A. Type A_1 RBC's B. Type 0 RBC's	$12{,}000 \pm 303$ $1{,}480 \pm 77$	13.3
37	161 ± 10	5.7×10^{12}	human anti Blood Group A	A. Type A_1 RBC's B. Type 0 RBC's	$170{,}000 \pm 5{,}313$ $17{,}000 \pm 751$	10

[a] 1×10^7 cells were suspended per ml of gold particles

tion of the number of gold particles per count. In the subsequent reactions of the gold particles with cells, these counts were used to determine the number of gold particles present.

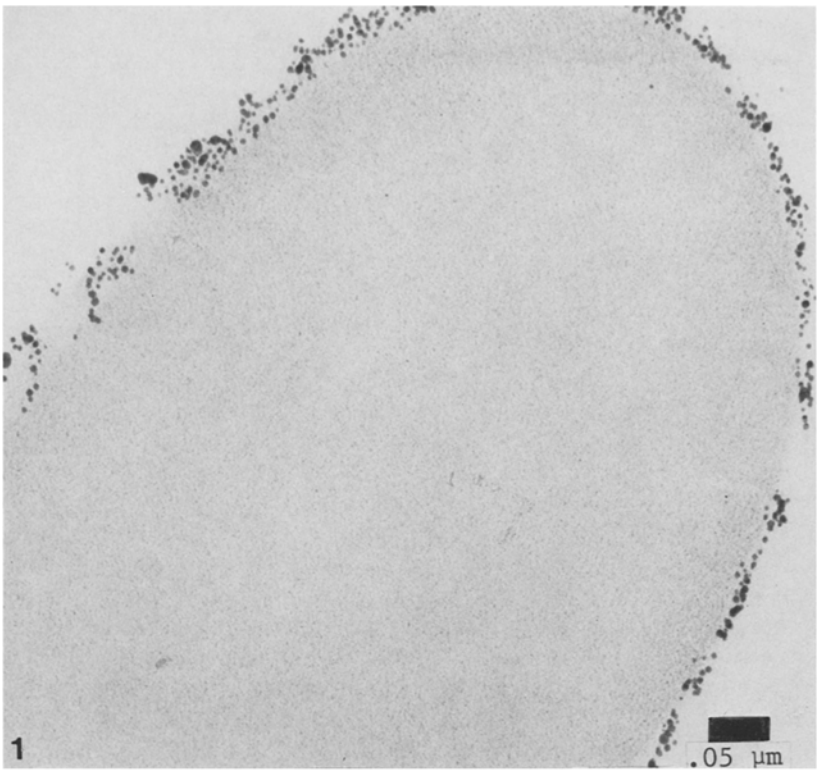
The number of 41 ± 13 Å particles (experiment 25) coated with anti-human IgG that bound to the surface of cells was much greater than in experiment 18 (420 ± 50 Å particles). They were present in a thick coat with occasional skip areas by TEM (Fig. 1). These particles were not visible in SEM. The control cells were largely free of surface particles. The particles present were concentrated in a very occasional cell or in noncell associated aggregates of particles.

The large particles (experiment 18) were readily visible in the SEM as well as by TEM. All the cells had surface particles apparently randomly distributed. The control cells were similar to those seen in experiment 25, i.e., most cells were free of particles. The gold particles in these controls were mostly in noncell associated aggregates or very occasional cells that contain many particles.

Type A_1 RBC's bound gold particles coated with anti-A avidly than the type 0 RBC's used as a control (Table 1). The size of the gold particles and hence the number per ml of suspension profoundly influence the number of particles binding, i.e., the smaller the particle the more binding. SEM revealed particles scattered over the surface of A_1 cells in an apparently random manner (Fig. 2), except for concave areas which appear to be devoid of particles. However, TEM of comparable cells revealed the concave areas to be populated with gold particles. The controls were similar to those seen in experiments 18 and 25. That is, most of the cells were devoid of gold particles. A very occasional

Fig. 1. Rat RBC's with attached human IgG reacted with gold particles coated with anti-human IgG (experiment 25). Note that the thick coat of gold particles over the surface of the cell and the occasional skip area. ×153,000.

Fig. 2. A_1 RBC's reacted with gold particles coated with anti-A (experiment 31). The particles appear to be randomly distributed except in the concave area. TEM of the same preparation showed the particles are also present in the concave area. ×15,400


1
.05 µm

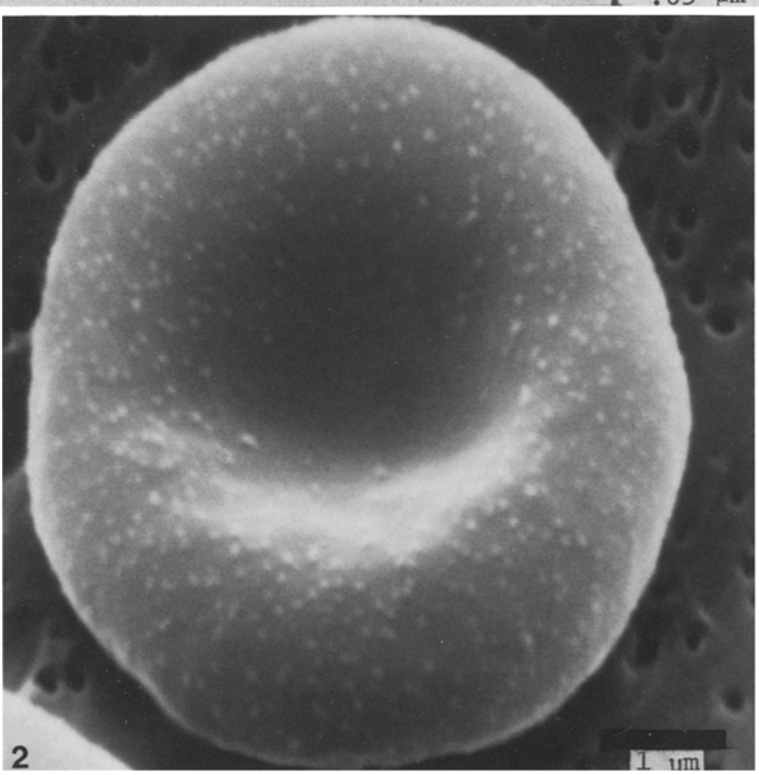

2
1 µm

Table 2. Effect of cell surface antigen concentration on reactions with specific antibody[a]

Experiment Number	IgG Molecules/ RBC	Gold Particles/ RBC
46-a	4.6×10^6	109,000
46-b	3.8×10^5	42,000
46-c	7.1×10^4	15,600
46-d	BSA	10,400
46-e[b]	4.6×10^6	15,600
46-f[c]	4.6×10^6	7,800

[a] Three tubes each containing 5×10^6 rat RBC's coated with human IgG or BSA were reacted with 7.5×10^{13} gold particles/ml coated with goat anti-human IgG

[b] One mg of goat anti-human IgG added per ml

[c] Gold particles coated with normal goat IgG

cell had many particles on its surface and extracellular aggregates of particles were also noted.

In experiments 46-a–c the number of specific antibody coated gold particles in the suspending fluid was kept constant (Table 2). The number of human IgG molecules on the surface of each red cell was the only variable. From this data it is clear that the number of gold particles binding to the red blood cells was proportional to the number of antigen molecules on the cell surface (Table 2). Experiments 46-d–f were controls. When specific antibody was added to the reacting mixture (46-e) it clearly inhibited the reaction of gold particles with the RBC's. When BSA was substituted for human IgG on the cell surface, binding of gold particles was also greatly diminished. A similar decrease in binding was found when nonimmune goat IgG was applied to the surface of gold particles in lieu of the specific antibody (46-f).

Discussion

From the amount of gold used in making a suspension and the average diameter of the particles produced, Frens (1973) reported earlier that the number of particles in a gold suspension could be calculated. If ^{195}Au or ^{198}Au is incorporated into the particle, the number of particles per count can be calculated and used to follow the subsequent surface binding of antibody coated gold particles. This approach provides a quantitative assay of antibody coated gold particles on the surface of the cells as well as the pattern the particles form seen with SEM and/or TEM of the same population. This adds a new dimension to the use of gold particles in the study of cell surface antigens.

The original method for determining cell surface binding by gold particles was indirect (Horisberger and Rosset 1977). That is, after the cells were allowed to react with the gold suspension and had been removed the supernate was assayed for gold particles. The value obtained was subtracted from a similar assay on the original gold suspension. The difference was assumed to represent gold particle binding to the cell surface. With our more sensitive method it is quite clear that under these conditions a good portion of that fraction is in fact bound to the wall of the tubes rather than to the surface of red cells. A further limitation to the original method is that the indirect approach requires

that one be able to measure accurately very small changes in the concentration of the gold suspension. For example, had we been using the indirect method in experiment 18 (Table 1) it would have been necessary to accurately detect a 0.4% change in the gold suspension after the cells had been removed.

Nonspecific binding is an important hazard in all studies of cell surface antigens that involve labeled antibody or antibody coated particles. By adjusting the protein, carbowax, and the electrolyte concentration of the suspending fluid for gold particles, the amount of nonspecific binding can be modest as shown in Table 1. However, Table 1 does not show the unsatisfactory experiments where the binding to control cells approaches binding to experimental cells. A major advantage of our modification of the gold particle technique is that it permitted us to readily recognize the unsatisfactory experiments and make the necessary adjustments to reduce nonspecific binding.

Distribution of the gold particles in the controls was similar regardless of the surface antigen being studied. That is, the surface of most control cells was free of gold particles. The particles present were either on the surface of a very occasional cell or were found in noncell associated aggregates. Care in removing aggregated gold particles from the suspension before placing it with the cells might further reduce the counts attributed to nonspecific binding. As the very occasional cell which binds the particles nonspecifically is obviously different from other cells in the group, some method of removing them prior to the reaction could further reduce the nonspecific binding.

The number of antibody molecules binding to the surface of individual gold particles varies with the size of the particle (Horisberger and Rosset 1977). In an earlier study using similar conditions to those described herein, we found that approximately 3,000 antibody molecules bound to the surface of each 180 Å gold particles (Kent et al. 1979). Despite the large number of antibody molecules on the surface of gold particles, steric conditions should cause each particle to react with only a limited number of cell surface antigen molecules. In our experiments the maximum number of particles present per ml of suspension was 3×10^{14}. If each gold particle acts like an antibody molecule, this is roughly comparable to 0.2 mg of specific IgG per ml. Hence, the degree of saturation of antigen binding sites possible with antibody coated gold particles would be less than with free antibody. This is one of the limitations to the use of gold particles.

References

Axén R, Porath J, Ernback S (1967) Chemical coupling of peptides and proteins to polysaccharides by means of cyanogen halides. Nature 214:1302–1304

Boyde A, Wood C (1969) Preparation of animal tissues for surface-scanning electron microscopy. J Microsc 90:221–249

David GS (1972) Solid state lactoperoxidase: a highly stable enzyme for simple, gentle iodination of proteins. Biochem Biophys Res Commun 48:464–472

Frens G (1973) Controlled nucleation for the regulation of the particle size in monodisperse gold suspension. Nature [Phys Sci] 241:20–22

Horisberger M, Rosset J (1977) Colloidal gold, a useful marker for transmission and scanning electron microscopy. J Histochem Cytochem 25:295–305

Kent SP, Ferrarini M, Coombs RRA, Hockaday AH (1972) Distribution of immunoglobulin determinants and antigen receptors on the surface of rabbit lymphoid cells as shown by peroxidase-labelled antibody and cell rosetting techniques. Int Arch Allergy Appl Immunol 43:563–587

Kent SP, Caldwell TC, Siegel AL (1979) Gold particles coated with anti-A versus ferritin or ^{125}iodine-labeled anti-A in reactions with cell surface antigen. Ala J Med Sci 16:22–25

Levy HB, Sober HA (1960) A simple chromatographic method for preparation of gamma globulin. Proc Soc Exp Biol Med 103:250–252

Sanderson CJ, Wilson DV (1971) Methods for coupling protein or polysaccharide to red cells by periodate oxidation. Immunochemistry 8:163–168

Weiser HB (1933) Inorganic Colloidal Chemistry, Volume I. John Wiley & Sons, New York, p 21

Received April 1, 1981