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USE OF COLLOIDAL GOLD AND NEUTRON ACTIVATION IN CORRELATIVE MICROSCOPIC LABELING AND LABEL QUANTITATION

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Abstract

Albumin was conjugated to 16 nm gold particles (Alb-Au_{16}) and infused into anesthetized pigs to determine if plasma, tissue and bronchoalveolar lavage (BAL) fluid concentrations of gold could be quantitated by neutron activation (Au^{198}). Additionally, transmission electron microscopy (TEM) of lung and liver samples was evaluated for sites of gold distribution and morphological changes. The minimum concentration of gold detected by neutron activation ranged between 1.4 and 1.9 ppb (ng/gm of sample). No gold was detected in the plasma of pigs prior to Alb-Au_{16} infusion, while mean post infusion concentrations were 1.037 $\pm$ 0.69 ppm (μg/gm plasma, $\pm$SD). The concentrations in the lung and liver were 274.4 $\pm$ 0.03 and 88.3 $\pm$ 0.04 ppm, respectively. There was no measurable Alb-Au_{16} in the BAL fluid. TEM showed gold particles within phagolysosomes in pulmonary and hepatic intravascular macrophages. No morphological changes were observed within the two populations of macrophages and no gold particles were observed within the alveolar space. Neutron activation of blood, tissue and BAL fluid samples from pigs administered intravenous Alb-Au_{16} was sensitive to the ppb concentration. The capability of neutron activation to detect very low concentrations of Au^{198}, combined with the freedom from contamination, make neutron activation an excellent technique for the study of the distribution and metabolism of a substance *in vivo*.

Key Words: Colloidal gold, intravascular macrophages, neutron activation, pig, electron microscopy, correlative microscopy.

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Introduction

Colloidal gold has been used extensively to study structural and ultrastructural cellular components and functions [1, 4, 8, 10, 14, 19, 29]. Colloidal gold particles are easily observed by scanning and transmission electron microscopy (SEM and TEM, respectively). Video-enhanced light microscopy (LM) is useful to detect larger gold particles (> 15 nm) or groups of smaller particles on flat thin specimens, due to the inflated diffraction image of the colloidal gold [2, 3, 15]. With respect to endotoxin-mediated lung injury, immunogold labeling has proven to be a useful marker of endotoxin-macrophage receptor binding and endocytosis and neutrophil-endothelial cell adhesion, activation and migration [5, 12, 17, 21, 23, 24, 27, 31]. Albumin is largely responsible for the oncotic pressure of plasma and interstitial fluid and is transported through pulmonary capillaries by receptor-mediated transcytosis; consequently, albumin gold may be a useful indicator for the morphological assessment of altered permeability [15, 34, 35, 36]. Albumin-gold, taken up by various organs and tissues after being administered intravenously, can be quantitated by neutron activation ($Au^{197} + \gamma \Rightarrow Au^{198}$) [7, 18, 22, 28, 32, 33].

The present study was designed to determine whether systemic administration of albumin colloidal gold could be quantitated in the plasma, liver, lung and bronchoalveolar lavage and correlated with liver and lung morphology. Our previous preliminary studies in ponies (Woods, Ginther and Albrecht, unpublished) demonstrated the potential of this approach to determine compound distribution in large animals. Since the neutron activation step is performed on tissue or fluids subsequent to their collection, no exposure of the animals to radioactivity or radioactive compounds is necessary.

For this work, we employed a previously described model system used to investigate gram-negative septic shock in pigs [20, 25]. Gram-negative septic shock is associated with life-threatening hemodynamic and metabolic decompensation, which is mediated, in part, by inflammatory mediators released in response to bacterial endotoxin [16, 30]. The lung is particularly susceptible

to acute injury in septic shock because it produces inflammatory mediators from intravascular and alveolar macrophages and pro-thrombin factors from both endothelial cells and macrophages which potentiate neutrophil infiltration and microvascular permeability, respectively [6, 11]. In this study, we investigated the feasibility of correlating bulk concentrations of albumin colloidal gold in plasma, liver, lung and bronchoalveolar lavage fluid with tissue morphology to determine the exact location of the colloidal gold labeled albumin relative to tissue and cell structure changes in normal animals. If successful, this technique would be applied to a porcine model of acute lung injury in order to evaluate the distribution and fate of gold labeled albumin during endotoxemia [23, 25, 39].

Materials and Methods

Albumin gold preparation

Colloidal gold particles were prepared by reducing chloroauric acid with tannic acid and sodium citrate [26, 37]. The colloidal gold sol was pH adjusted with 0.2 N K_2CO_3 to pH 7.0, basic to the isoelectric point of bovine serum albumin (BSA; Sigma, St. Louis, MO) and filtered through a 0.45 μm filter. A stable conjugate of Alb-Au_{16} was achieved at 180 μg of BSA/ml of gold, as determined by the salt flocculation test. Twelve liters of Alb-Au_{16} were conjugated at one time to minimize conjugate variability. Prior to use, the albumin-gold was concentrated 80 fold by centrifugation (15,000 *g*, 30 minutes) and 50 ml of concentrate, extended to 60 ml with phosphate buffered saline, was infused into each pig.

Animal preparation

Two eight- to twelve-week-old pigs were anesthetized and maintained using standard procedures employing pentobarbital sodium, as previously described [25]. The pigs were placed in supine position and were mechanically ventilated via tracheostomy at a constant tidal volume and rate (Edco Scientific Inc., Chapel Hill, NC). Pigs were instrumented for standard cardiopulmonary measurements, allowed to stabilize for a minimum of 30 minutes and administered Lactated Ringers solution (Baxter-Travenol, Deerfield, IL) at 5 ml/kg/hr through a jugular vein to help maintain circulating blood volume and pressure for 6 hours. At 5.25 hours, 60 ml of concentrated Alb-Au_{16} was infused at a rate of 2 ml/min for 30 minutes. Blood samples were obtained at hours 5 and 6 for quantitation of plasma Alb-Au_{16}. At 6 hours, bronchoalveolar lavage (BAL) fluid, and lung and liver tissue samples were obtained from the left cardiac and right central lobes, respectively. The BAL fluid was collected by infusing 80 ml of phosphate buffered saline into each bronchus and gently massaging the lobes while aspirating the BAL fluid. Neutron activation samples were weighed in 2/5 dram polyethylene flip-top vials for Alb-Au_{16} quantification. The intermediate lobe was fixed with 10% phosphate buffered formalin for 5 minutes by airway and vascular perfusion. Sagittal sections were then collected caudal to the major bronchus, fixed for 2 hours at room temperature (22°C) in Karnovsky's solution (2% paraformaldehyde and 1.25% glutaraldehyde) with 0.1 M cacodylate buffer and then rinsed and stored under refrigeration in 0.1 M cacodylate buffer. The tissues were routinely prepared for TEM (i.e., post-fixed in osmium tetroxide, serially dehydrated and embedded in Epon Araldite). Semi-thin sections (1.0 μm) were stained with 0.1% toluidine blue for orientation and evaluation of lesions. Thin sections (60-70 nm) were cut on a Reichert Ultracut E Ultramicrotome fitted with Diamond Knife (Diatome, Bienne, Switzerland), placed on copper grids (E. Fullam, Latham, NY), stained with 1% uranyl acetate, followed with lead citrate, and examined on a Phillips 410 transmission electron microscope.

Neutron activation

Neutron activation studies were performed at the University of Wisconsin, Nuclear Reactor Laboratory, in a 1 M TRIGA open pool reactor, in the department of Nuclear Engineering and Engineering Physics. Vials without contents (blanks) and vials containing samples and standards of liquid Au solution were sealed by friction welding. The blank vials were analyzed to determine the gold content of the polyethylene. In batches of 8 unknown samples and one standard, the vials were exposed for 60 minutes to the same thermal neutron flux (1×10^{13} neutrons/cm^2-sec) by rotating the sample carrier about its axis during irradiation. Four days post irradiation, the samples were counted for 3600 seconds at 2 cm on the vertical axis of a γ-ray detector (170 cm^3, aortic intrinsic germanium detector, #GEM 40190, Ortec, Oak Ridge, TN) coupled to a PC based multichannel γ-ray spectrometer using a PAC-II MCA board (Oxford Analytical Systems, Oak Ridge, TN). Photopeak areas were corrected to saturation activity and corrected for any known interfering gamma rays. Neutron flux was calculated based on the selected element (Au^{197}) in the standard, using a library of element data and detector efficiencies. Conversion factors in this library were standardized to the standard samples (10 and 100 ppm, μg Au^{198}/gm of sample) run with the unknown samples. Neutron flux calculations, which assume only thermal neutron activation, facilitate the comparison of standards from group to group to assure they were counted in approximately the same flux.

For each sample, the decay time (the time from the end of the irradiation to the start of the count), the live

time (the number of seconds the counter was available to process data during the counting period), and the fractional dead time (a measure of the counting time lost while processing pulses) were determined [9, 13]. The decay and live times were used to correct for radioactive decay and compute counts/second, respectively. The fractional dead time was used to correct for loss of pulses from the spectrum while processing pulses. For each element measured, the energy (keV) and counts/second for that γ-ray, and the standard deviation of the measured count rate were calculated. If an element was detected in the sample, and the material was certified for that element in the standard, the micrograms of element/gram of material was calculated from the count-rate date. To determine the precision of the photopeak area (which yields counts per second) the percentage standard deviation (%SD) was calculated from Poisson statistics using the net photopeak area and the background on which the photopeak is riding, and subtracting any interfering elements contributing to the photopeak. The mass of gold present in each sample was expressed in micrograms, and the fractional portion of gold in the unknown samples was expressed in micrograms per gram of sample (parts per million, ppm). For liquid samples (plasma and BAL fluid), the fractional portion was expressed as micrograms per milliliter. The amount of gold in each unknown sample was determined by subtracting the mean gold value of the blank vials from the gold quantitated for the unknown.

Results

Neutron activation

The mean amount of gold detected by neutron activation in the empty (blank) vials was 1.6 ng/gm of sample (ppb) (range 1.4 - 1.9 ppb), and neutron activated liquid gold standards were quantitated at 10 and 100 ppm (μg Au^{198}/gm of sample) (Table 1). There was no detectable gold in the plasma of the pigs prior to the Alb-Au_{16} infusion, while mean post infusion concentrations were 1.037 $\pm$ 0.69 ppm (μg/gm plasma $\pm$ SD). The concentrations of Alb-$Au_{16}{}^{198}$ in the lung and liver were 274.4 $\pm$ 0.03 and 8.83 $\pm$ 0.04 ppm, respectively. There was no measurable amounts of Alb-Au_{16} in the BAL fluid.

Transmission electron microscopy

On TEM, Alb-Au_{16} was observed within phagolysosomes in pulmonary and hepatic intravascular macrophages (Figs. 1 and 2). The gold particles were observed within phagolysosomes. No observable morphological changes to the pulmonary and hepatic microvasculature and macrophages were observed. Additionally, gold particles were not observed in the alveolar space.

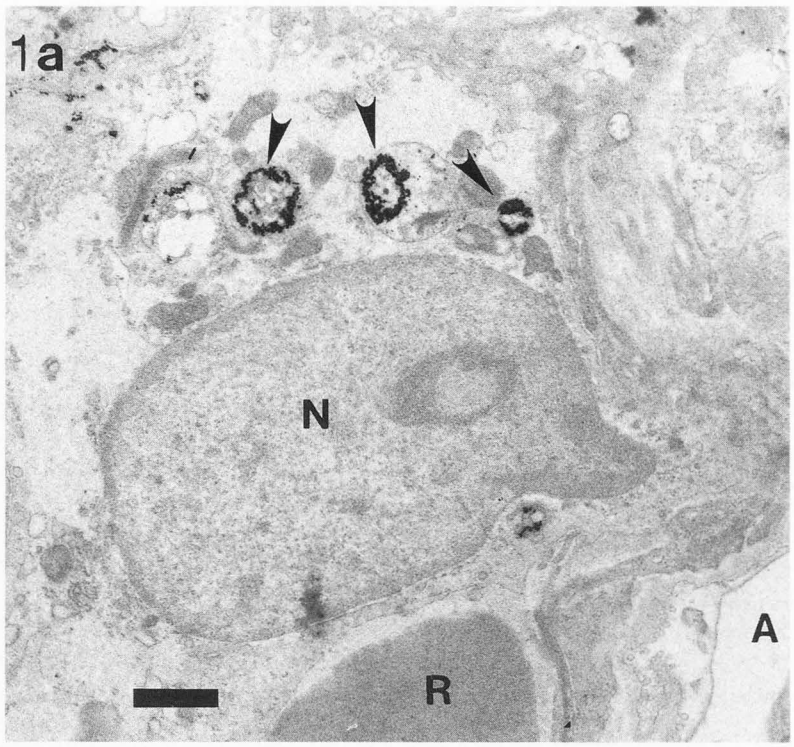
Table 1. Quantitation of neutron activated colloidal albumin-gold (Alb-$Au_{16}{}^{198}$) in the plasma, lung, liver and bronchoalveolar lavage (BAL) fluid. Value = total μg Au^{198} minus background in polyethylene vial (1.6×10^{-3} ppm).

Sample	Neutron activated Au^{198} ppm (μg/gm sample $\pm$ % SD)
Plasma (pre Au^{198})	0.0 ($\leq$ background)
Plasma (post Au^{198})	1.037 ppm $\pm$ 0.69
Lung	274.4 ppm $\pm$ 0.03
Liver	88.3 ppm $\pm$ 0.04
BAL fluid	0.0 ($\leq$ background)

Discussion

Neutron activation analysis is the measurement of radioactive isotopes produced from the bombardment of an element (i.e., gold, Au^{197}) by neutron or other high-energy nuclear particles. We activated gold with a flux of thermal neutrons, thus creating the reaction $Au^{197}(n,\gamma) \Rightarrow Au^{198}$, where Au^{198} is the radioactive isotope. Au^{198} is a nuclide with a relatively short half-life (2.7 days) and emits a γ ray and β particles with energies of 411 keV and 963 keV, respectively. If the bombardment conditions are kept constant at the time the standards and unknown samples are activated, then the resultant nuclide is directly proportional to the weight of the target material in the unknown sample [38]. Rotating the sample carrier during the irradiation process ensured that all samples received the same amount of thermal neutron flux. The thermal neutron activation cross section of gold is large (σ_{ac} = 96 $\pm$ 10 barn), a fact which favors its use by neutron activation, and Au^{198} has a detector efficiency which lends itself to easy and accurate analysis, therefore making quantitation extremely specific. Additionally, because quantitation of Au^{198} is very sensitive (1×10^{-9}g Au^{198}/gm of sample, ppb), it is very useful in the detection of an extremely small amount of substance, thus making the technique suitable for studying the fate and distribution in animals weighing up to 20 kg and more.

The advantage of irradiating samples post mortem is that it eliminates introducing a fairly strong radionuclide in studies of distribution and metabolism of a substance *in vivo*. It is also possible that what is observed in an *in vivo* study with a radioactive substance may be different from that which would have occurred if a non-radioactive substance was administered [18]. Relative freedom from contamination and the very low detection limits of neutron activation make it an



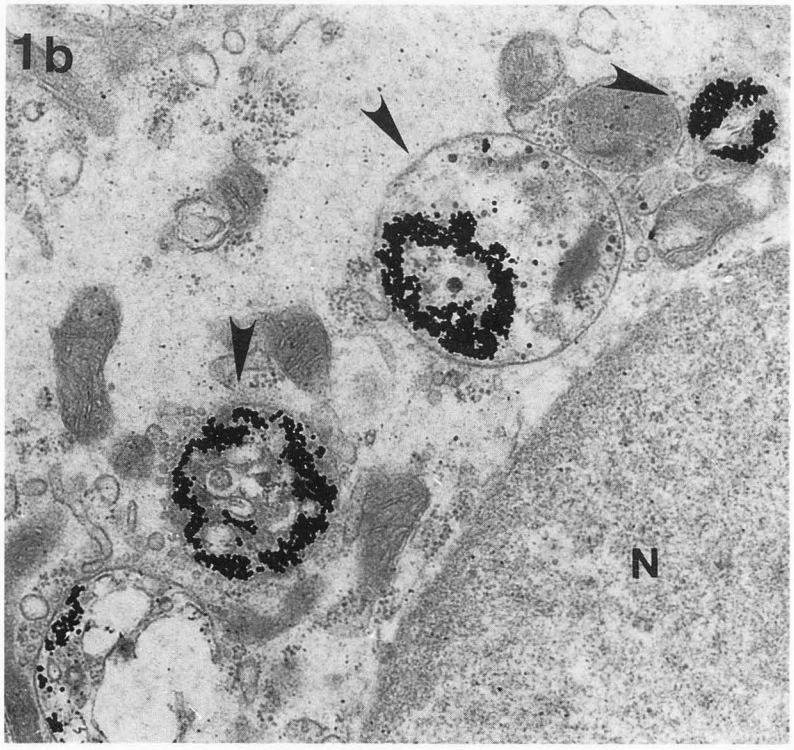
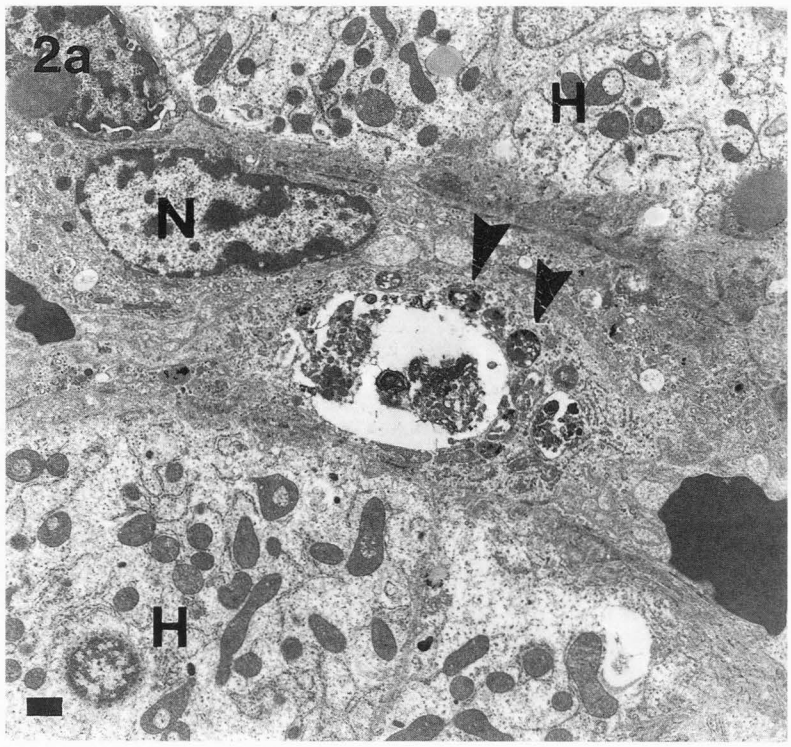


Figure 1. (a) Alveolar septal capillary containing erythrocyte and pulmonary intravascular macrophage (PIM). Arrow heads mark phagolysosomes. N = nucleus of PIM; R = red blood cell; A = alveolus. Bar = 0.1 μm. (b) Higher magnification of Figure 1a; arrowheads point to phagolysosomes which contain numerous gold particles.



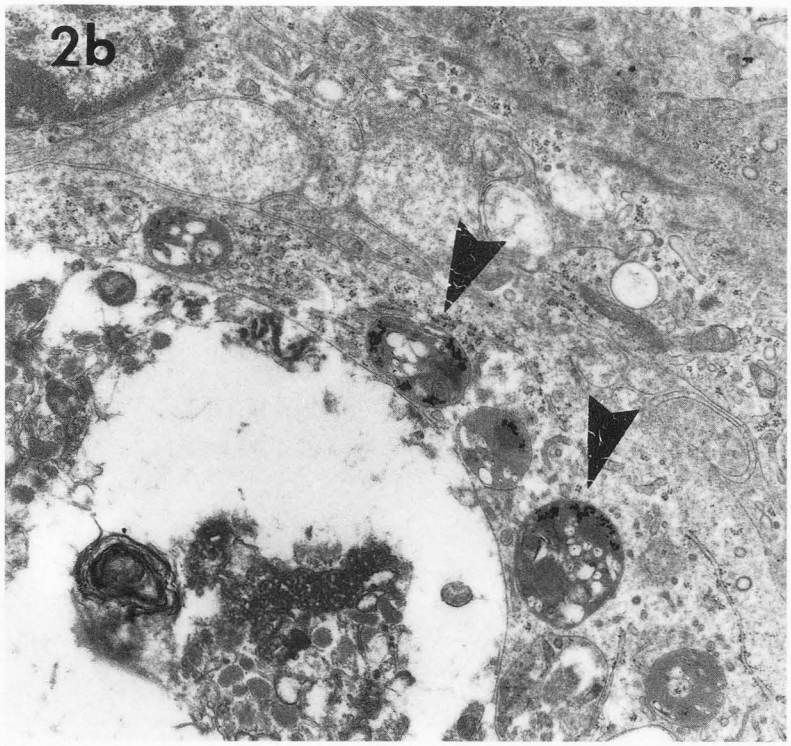


Figure 2. (a) Hepatocytes marginating sinusoid containing erythrocyte and Kupffer cell. Arrowheads mark phagolysosomes. N = nucleus of Kupffer cell; H = hepatocyte. Bar = 0.1 μm. (b) Higher magnification of Figure 2a; arrowheads point to phagolysosomes which contain numerous gold particles.

excellent technique for the *ex vivo* analysis of gold as well as other selected elements [19, 36].

The molecular species can be produced in several forms. Individual molecules or active fragments can be conjugated to very small, 2-5 nm, gold particles which are smaller than the molecules being evaluated. Larger gold particles, 15-30 nm, can be used to form larger probes by condensing the gold particle with one or more active molecules attached to it [36]. While the larger gold particles are readily visible by electron microscopy, the smaller gold particles can be enlarged, following labeling, with silver enhancement to improve their visualization in SEM, TEM, or LM. Tissue samples can be divided and a portion prepared for electron microscopy analysis prior to neutron irradiation. Additionally, for inorganic samples, the small amount of radioactive material, short half-life, and α and β emission properties do not preclude examination of irradiated samples. Results of this study demonstrate the feasibility of performing sensitive quantitative assays of the amount of gold label present in a given tissue or location and correlative electron microscopy observations of the tissue in order to determine the precise location of label relative to tissue, cellular and subcellular structures [40].

During endotoxemia, there is profound immune suppression and impaired peritoneal and mononuclear phagocytic clearance of bacteria, which predisposes the host to increased susceptibility to bacterial dissemination and to the risk of developing acute respiratory distress syndrome and multiple organ failure [12, 21, 23]. Neutron activation of tissue (liver and lung), plasma and BAL fluid following endotoxemia may provide a means by which to study two dynamic pathophysiological events, specifically, mononuclear phagocytic dysfunction and microvascular injury and permeability. Concomitant with changes would be the opportunity to evaluate morphologic changes by SEM, TEM or LM.

It should be noted that, as indicated in **Materials and Methods**, the colloidal gold was synthesized and conjugated to albumin in our laboratory. While we routinely prepare our own gold and gold conjugates for a number of antibody and ligand labeling procedures, it is of added importance in studies such as these. The total cost, materials and time, to prepare the 12 liters of gold-albumin was approximately $100. If the materials were purchased commercially, the cost would be considerably more.

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Discussion with Reviewers

M. Jacques: Would you expect similar results if unconjugated gold particles were infused into pigs? What is the effect of albumin on gold distribution?
Authors: Colloidal gold is a hydrophobe sol which would quickly aggregate in the presence of physiological salt concentrations in blood. This would probably result in large aggregates which would become coated with available serum proteins, including albumin. These large aggregates would ultimately be phagocytized. Albumin is used to coat the gold particles so they remain as individual particles in the circulation. Albumin was chosen because it is nonadhesive, has a relatively long half life, and while there may be localized cell binding sites which facilitate albumin uptake, albumin is not thought to have receptor mediated signal transducing activity.

M. Jacques: Your rationale is to use gold conjugates to study microvascular injury and permeability. The size of the probe will certainly influence its diffusion. You should have compared gold particles of different diameters (e.g., 3-5 nm, 16 nm, and 25-30 nm). Why did you use 16 nm gold particles?
J. Hobot: Is there any advantage in using smaller gold particles (2-3 nm or less) than those of 15-30 nm, in terms of easier passage or permeability through body or cellular channels?
Authors: The purpose of the present study was to show that neutron activation had sufficient sensitivity to quantitate the extremely small amount of gold used and also to demonstrate neutron activation could be correlated with electron microscopy to show the location of the gold particles, in addition to their bulk concentration. We are presently testing various sized particles. It is also important to note that the overall probe size is the gold core plus the shell of adsorbed protein. With the small gold particles, a single particle intercalates into the molecule such that the probe size is usually equivalent to the size of a single protein molecule. This is best for studies of the fate of soluble molecules, whereas larger gold particles with multiple bound protein molecules are better for studying the fate of aggregates. We used the 16 nm particle because the final probe size with adsorbed albumin is roughly 20 - 22 nm, equivalent of small aggregates which can leave through spaces in permeable vascular endothelium but are also normally phagocytosed.

J. Hobot: What other proteins can be conjugated to colloidal gold and used in the neutron activation studies? Have other proteins been tried? Is the sensitivity of neutron activation of particles independent of particle size?
Authors: In theory, any protein can be used. Most proteins, under appropriate conditions, bind very strongly by hydrophobic bonding to the gold and are as stable *in vivo* as the unconjugated protein. To date, we have not used other proteins *in vivo*, although many have been used in *in vitro* studies. Quantitation by neutron activation is only related to the amount of gold, not the adsorbed protein or particle size.

J. Hobot: When gold is conjugated to albumin (or perhaps another protein of interest) and injected into an animal, would not the animal's immune system treat the gold as an unwanted invader and react accordingly, such that the distribution of albumin-gold is adversely affected?
Authors: In the present study, the time periods are far too short for the host to mount an immune response unless prior sensitization has occurred. Foreign proteins of any kind will trigger a specific immune response whether or not it is conjugated to gold. For "self" proteins to be recognized as foreign, new "non-self" epitopes must be created. Binding of "self" proteins to various metals, such as nickel, is thought to be a basis for change in antigenicity and possibly subsequent immune mediated skin sensitivity. In the case of colloidal gold, various molecular species bind to gold via random hydrophobic bonding, although preferred locations in proteins do exist, due to highly charged or hydrophobic areas, etc. The activity and antigenicity appear relatively unaffected. Although the potential to trigger an immune response by epitope modification may exist, so far data is insufficient to predict the outcome.

B. Humbel: First of all, it should be proven that albumin gold follows the same pathway as uncoupled albumin. In the **Discussion**, the authors state "It is also possible that what is observed in an *in vivo* study with a radioactive substance may be different from that which would have occurred if a non-radioactive substance was administered." There is no reason why a gold particle bound to many BSA molecules should be more reliable than albumin with a radioactive tracer. In fact, this control experiment is missing.
Authors: Our intent was to demonstrate first that colloidal gold coupled molecular species, in this case albumin, could be quantitated in bulk samples of different tissues or in blood by the neutron activation procedure. Second, we wished to show that correlative electron microscopy could be performed to see qualitatively where in the tissues the gold was located. We did not intend to imply that Alb-Au_{16} (roughly 22 nm total diameter) would behave as soluble albumin. In fact, we chose the larger particles to demonstrate differences in phagocytic clearance of particulates. The use of 2 or 3 nm gold, which would result in single albumin molecules conjugated to a smaller gold particle, would be expected to behave more like soluble albumin but, control studies using radiolabeled albumin would be important to verify this.