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Distribution of Colloidal Gold Tracer Within Rat Parasternal Lymph Nodes After Intrapleural Injection

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ABSTRACT ***Background:*** **Tracer studies are an important tool to obtain information about the processes involved in the immunological response. Colloidal gold is widely used as a tracer, but its small size of label can cause some difficulty during low-resolution analysis. To overcome this difficulty, we developed a new method to follow the route of tracer movement within lymph nodes.**

Methods: **We applied conventional X-ray analysis, X-ray fluorescence analysis (XFA) subtractional microscopy using synchrotron radiation (SR) beams, light mycroscopy, and ultrastructural analysis to study the distribution and quantity of colloidal gold coupled with albumin within rat parasternal lymph node 2, 4, 6, 8, and 10 h after intrapleural injection of the tracer.**

Results: **At all the time points XFA-SR revealed that tracer formed a circle with a maximum concentration in the node periphery. XFA-SR measured colloidal gold concentration in the nodes reached its maximum (0.5–0.75 weight %) in 6–8 h. Substractional microscopy revealed superficially located groups of cells filled with colloidal gold tracer. Light microscopy and ultrastructural analysis confirmed that the tracer was concentrated in the reticular cells, situated in the sinuses of the node. Sinusoidal reticular cells concentrated tracer at much higher rates than sinusoidal macrophages. Four hours after injection, gold appeared in the lysosmes of the follicular reticular cells. At the same time point, evidence of antigen presentation was obtained. Antigen presentation proved to be an extremely rare event since only one ultrastructural incident was found in 150 analysed grids.**

Conclusion: **SR is a valuable tool for the analysis of gold tracer passage within the living organism.** © 1995 Wiley-Liss, Inc.

Key words: Lymph node, Colloidal gold, X-ray

Colloidal gold is widely used as a tracer in vivo to study the passage of certain proteins because many of these proteins can be adsorbed on the gold particles without any major alteration of their biological properties (De Roe et al., 1987). The high contrast of colloidal gold particles makes it easy to identify them in ultrathin sections, but the small size of the particles causes some difficulty with the identification of the tracer in low resolution analysis (light microscopy, X-ray). To overcome this, in the present study we used synchrotron radiation (SR) generated by a storage ring. SR is characterized by a highly monochromatic wavelength, and this fact, together with some other unique features of SR, give researchers an advantage over traditional X-ray methods. X-ray fluorescence analysis using SR (XFA-SR) is based on the ability of the SR to activate atoms of elements to emit fluorescence radiation. Their fluorescence is captured by the detector and enables one to evaluate the quantity and distribution of certain elements within tissues (Dementiev et al., 1991; Kulipanov et al., 1983). Though very quick and exact, this method has some limitation in spatial resolution. To overcome this problem, a method of subtractional microscopy using SR has been developed.

It is known that every element has a very specific K-adsorbtion edge. When the energy of SR is close to the K-adsorption edge of the certain element, its adsorption changes dramatically. The idea of the subtractional analysis is to expose two images at different energies of monochromatic SR. When the energies for

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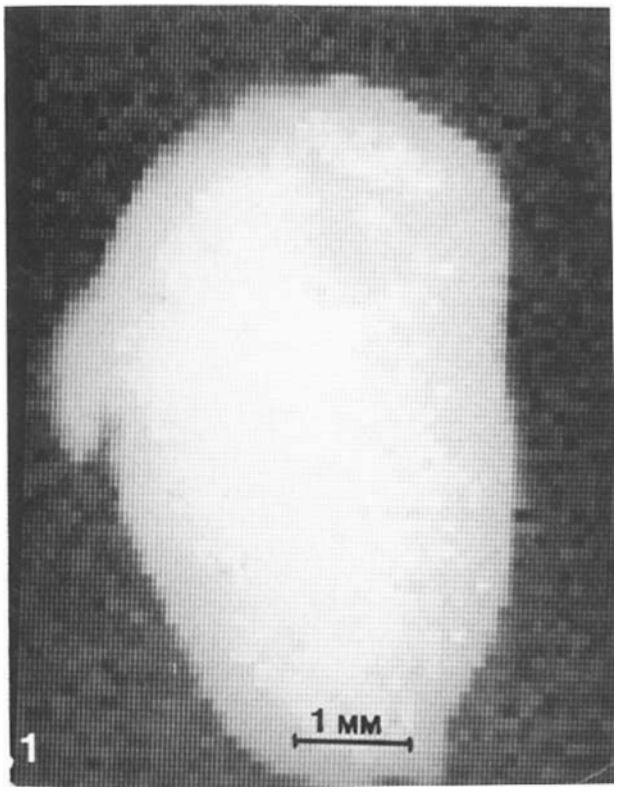


Fig. 1. X-ray scanning analysis of the rat parasternal lymph node 4 h after tracer intrapleural injection. Empty dark fields correspond to the subcapsular sinuses.

exposure are close but one of them is higher and the other lower than the K-adsorption edge of a certain element, they will be differently adsorbed by this very element. Adsorbtion in soft tissues changes insignificantly for these small variations in energy, and, hence, they are not seen in the subtractional image. Subtraction of the images with the help of the computer results in an image corresponding to the distribution of a certain element (like gold) in the tissue (Dementiev et al., 1991).

XFA-SR and subtractional analysis with SR enables direct and highly specific measurement of very low concentrations of certain elements within biological specimens (Dragun et al., 1989). Gold is a suitable contrast agent for this technique both because it is a heavy metal and because it is nearly absent in biological specimens. The aim of this investigation was to study the distribution of colloidal gold labelled albumin within lymph nodes in time after intrapleural injection of the tracer.

MATERIALS AND METHODS

Colloidal gold (10 nm) was produced according to Frens (1973). The flocculation point for human albumin had been determined, and albumin was added to the colloidal gold solution in a concentration 100 times higher than the stabilization point. This solution was then cleared of unbound protein by centrifugation (10,000*g*, 1 h) and the resulting sediment resuspended in a minimal possible amount of the supernatant. This solution was diluted with physiological solution to an optical density of 0.5 (wavelength 525 nm), and 0.3 ml was then injected into the pleural cavity of 25 Wistar rats (aged 1–1.5 years: weight 300–350 g). Thus, each animal received 0.01 mg of metallic gold. Five animals were sacrificed at every time point at two-hour intervals after injection (2, 4, 6, 8, 10 hours), and the medi-

Fig. 2. XFA-SR analysis of the distribution of gold within rat parasternal node 6 h after tracer injection. Dark fields represent gold. Tracer forms the ring corresponding to the subcapsular sinuses.

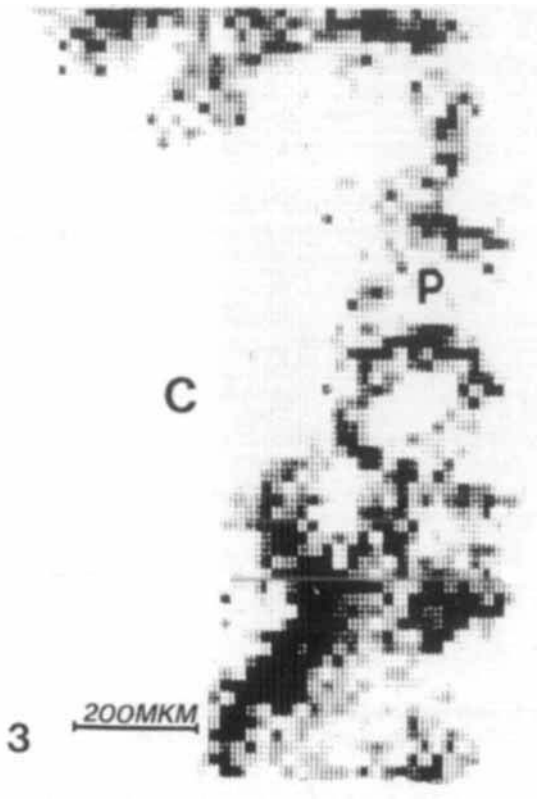


Fig. 3. Microscopic subtractional X-ray analysis of the peripheral part of the parasternal lymph node 6 h after injection. Dark dots represent gold, concentrated inside the group of superficially located cells. C, central; P, peripheral part of the node.

astinal lymph nodes (4–5 from each animal) were collected.

Some nodes were fixed in 10% neutral buffered formalin and further studied by means of conventional, XFA-SA, and subtractional analysis. For conventional and XFA-SR analysis, the SR from VEPP-3 storage ring (Institute of Nuclear Physics, Novosibirsk, Russia) was used. The SR beam, focused by single crystal graphite monochromator, was directed to the sample. The type of monchromator used allowed one to obtain an X-ray line within the energy range of 10–45 KeV, with 1% bandwidth. Induced X-ray fluorescence from the sample was captured by Si(Li) detector with energy resolution about 180 eV (in 5.9 keV line). The signal from the detector was proportional to the concentration of gold in the lymph node. A two-dimentional scanner permitted movement of the sample with a 1 μm step in 50×50 mm^2 field, thus making it possible to study the distribution of the gold within different parts of the node. Computer analysis yielded the final picture with a spatial resolution of scanning analysis of about 200 mkm.

To perform subtractional microscopic X-ray analysis, SR first had to be monochromatized by an X-ray monochromator based on two Ge(111) crystals, which could quickly change the X-ray energy about the value close to the gold L_3 adsorption edge. The beam was formed by two slits—horizontal and vertical—arranged in series. The scanner moved the node in the field 50×50 mm^2. Two images at a photon energy higher and lower than the critical adsorption L_3 edge of gold were registrated with the use of scintillation detector counting quanta flux passed through the specimen. Computer analysis provided the final pictures and subtractional image, characterizing the distribution of gold in tissue. X-ray microscopic images of the sample were taken with a spatial resolution of 15 μm.

Other nodes were fixed in 4% paraformaldehyde and 1% OsO_4 and embedded in Epon-Araldit. Semithin sections were stained with toluidine blue, and ultrathin sections were counterstained with uranyl acetate and lead citrate.

RESULTS

Scanning X-ray analysis demonstrated certain areas of diminished adsorption in the region of the lymph node sinuses (Fig. 1). XFA-SR analysis revealed colloidal gold in the mediastinal lymph nodes at 2 h; increased concentration of the tracer was revealed at 4 h after intrapleural injection, and concentration reached its maximum (0.5–0.75 weght %) after 6–8 h, with slight decreasing of the concentration at 10 h. Gold formed a ring or ellipse structure displaying maximum concentration in the peripheral regions of the nodes (Fig. 2). Microscopic subtraction analysis by SR revealed certain areas with a higher concentration of gold within the peripheral regions of the node corresponding in size to a group of a few cells (Fig. 3). Gold was more concentrated in superficial sinuses rather than the cortex and medulla.

Light microscopic analysis confirmed that most of the gold particles were concentrated in the subcapsular sinus of the node, in the reticular cells, which branched across the lumen of subcapsular sinus and lined the lymphatic follicles and blood vessels inside the subcapsular sinuses. From 2–8 h after injection, reticular cells of the subcapsular sinus did not display any visible difference in the concentration of colloidal gold particles. Reticular cells of the radial and medullary sinuses reached a peak concentration of gold particles at 6 h which was maintained up to 10 h (Fig. 4). Concentration of colloidal gold particles in medullary and radial sinuses was less than in the subcapsular sinuses. Different lymph nodes displayed great variability in the concentration of colloidal gold. Histologically we could not find gold in the follicles.

Fine structural analysis revealed that most of the particles were situated in the reticular cells of the subcapsular sinus (Fig. 5). Reticular cells were rod-like in shape and contained inclusions of connective tissue collagen. Gold particles formed groups on the membranes of the reticular cells, lined the membranes of the transportation vesicles, and finally were concentrated inside the secondary lysosomes. Some of the secondary lysosomes were filled with colloidal gold particles, and in other lysosomes colloidal gold lined the walls. Sometimes exocytosis of gold particles to the collagen fibers inside the reticular cells could be observed.

Sinusoidal macophages defined on the basis of their round shape, absence of connective tissue collagen in cytoplasm, numerous lysosomes including giant heterophagosomes, and numerous projections from the cell wall carried many surface-bound particles, but inside the macrophages very few gold granules could be

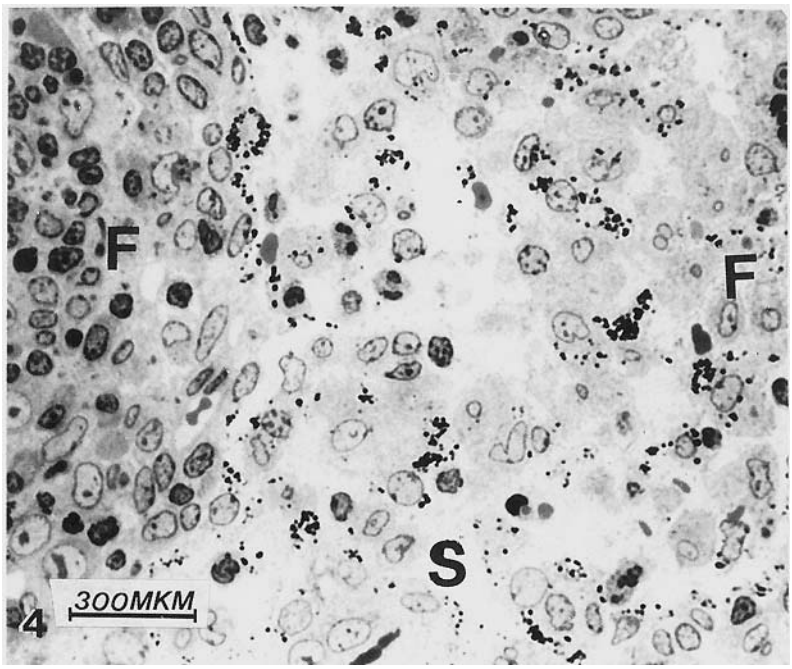


Fig. 4. Semithin section of the parasternal lymph node 6 h after tracer injection. Gold particles are concentrated inside the lysosomes of the sinusoidal reticular cells. Counterstaining with toluidine blue. F, follicles; s, trabecular sinus.

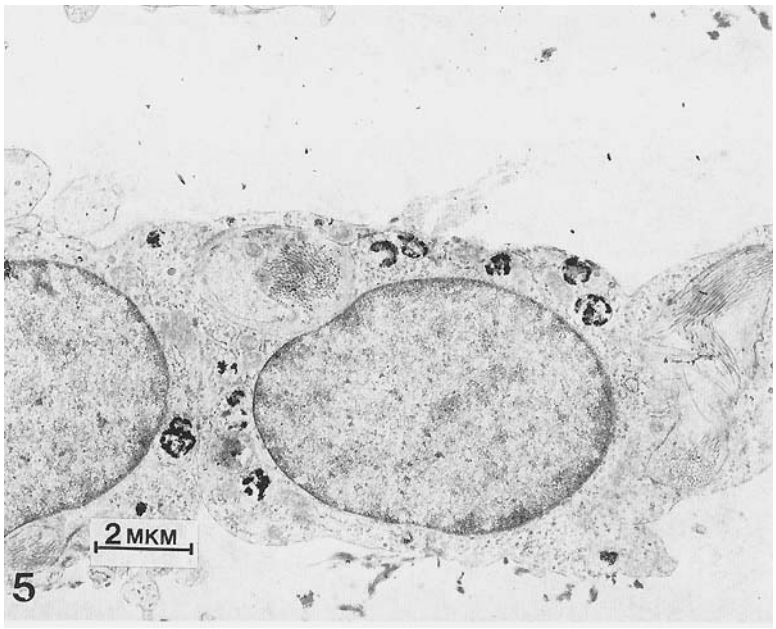
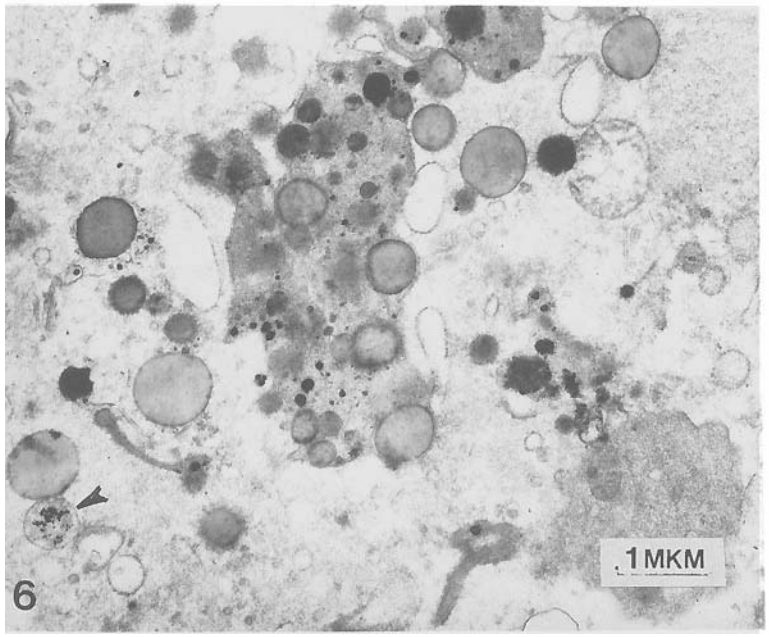
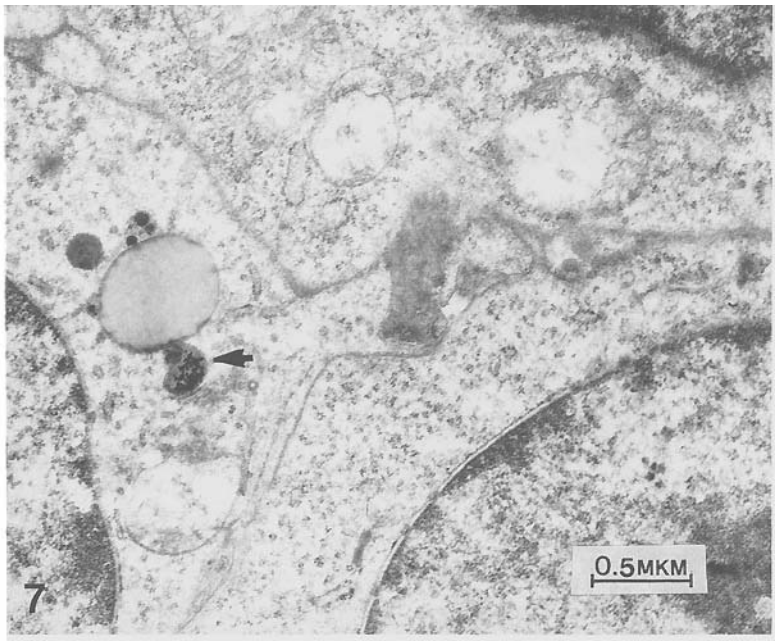


Fig. 5. Subcapsular sinus of the lymph node. Secondary lysosomes of the sinusoidal reticular cells with characteristic collagen fibers inside are filled with gold granules. Four hours after tracer injection.

found. Even big multivesicular bodies contained just a few colloidal gold particles (Fig. 6). Macrophages with empty secondary lysosomes were a striking contrast to reticular cells whose secondary lysosomes were filled with tracer. Great variability in the concentration of collodal gold particles in sinuses between different nodes and different regions of the same node makes it difficult for histologists to compare the concentration of the tracer at different time points. At 2 h we did not find colloidal gold particles inside the follicles of the





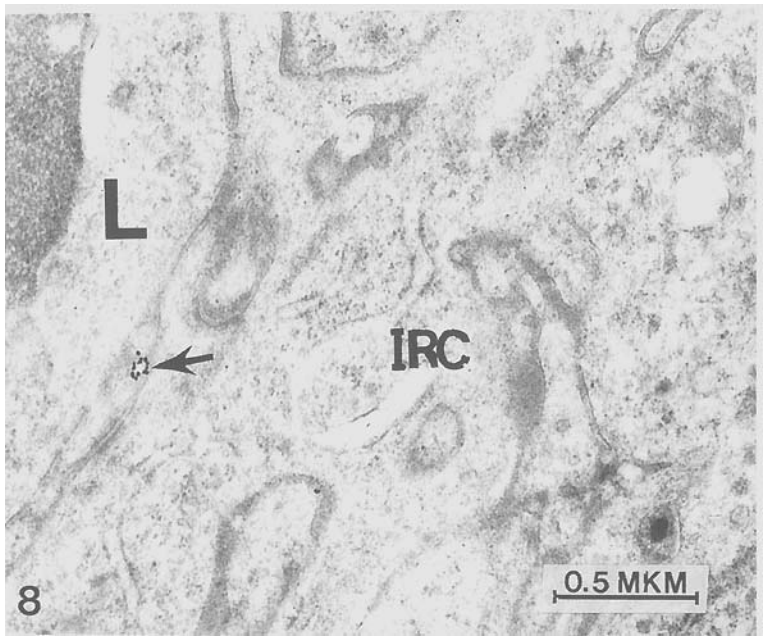


Fig. 6. Cytoplasm of the giant macrophage, situated inside the sinus, 4 h after tracer injection. Only one secondary lysosome contains gold particles (arrowhead). Other lysosomes are void of labelling.

Fig. 7. Follicle of the lymph node 4 h after tracer injection. Follicular reticular cell with lymphocytes. Inside the lysosome of the reticular cell is a group of gold particles (arrowhead).

Fig. 8. Follicle of the lymph node. Interdigitating reticular cell presents on its membrane a group of tracer particles to the lymphocyte (arrow). Four hours after tracer injection. IRC, folding of the memebrane of interdigitating reticular cells; L, lymphocyte.

lymph node, but at 4 h tracer appeared in the secondary lysosomes of the interdigitating reticular cells of the lymphatic follicles (Fig. 7). Interdigitating reticular cells were defined on the basis of their irregulary shaped cell nucleus, wide cytoplasm, cytoplasmic interdigitations, and numerous tubules and vesicles in the cytoplasm (Kaiserling and Wolburg, 1988). Up to 10 h the concentration of the gold particles in follicles did not change. The particles could be found in the secondary lysosomes of interdigitating cells and sometimes in secondary lysosomes of macrophages. The concentration of gold particles in follicles was always very low in comparison to the sinuses. Only once, after examining about 150 grids, did we find evidence of the presentation of colloidal gold on the membrane of interdigitating reticular cells to the lymphocyte (Fig. 8).

DISCUSSION

The initial aim of this study was to develop a new contrast agent for experimental lymphography, which can be simultaneously used in X-ray and histological analysis. This new tracer had to demonstrate passage not of the contrast agent but of the specific protein coupled with a metal. We chose collodal gold because it could be easily coupled with different proteins and further detected by all the methods used. But certain experiments performed in our laboratory cast some doubts on the specifity of this approach. We found that albumin–collodal gold solution, when mixed with oxidized glutathione, acquires new staining properties (Glazyrin et al., 1993). So, albumin seemed to be replaced on the gold surface by some other compounds, and, hence, we probably cannot say what protein (or proteins) are coupled with colloidal gold particles at any given moment.

Still the method used in this present report effectively demonstrates the speed and direction of protein adsorption from the pleural cavity. The role of macrophages in the phagocytosis of foreign proteins in the lymph node has been traditionally emphasized (Griffiths et al., 1989). Our data show that most active phagocytic cells inside the lymph node are reticular cells. It proves that most foreign proteins are transported in the lymph node not by migratory macrophages but with the lymph. This paper demonstrates that morphologic immunological activity in the follicles of the regional lymph node starts 4 h after injection of antigen. Antigens seem to be transported to the immune cells situated deeply in the follicle within the processes of the follicular reticular cells, similar to biologically active compound transportation within the processes of neuronal cells. Also, our data demonstrate that antigen presentation to immune cells is a comparatively rare event. Most of the antigen molecules are concentrated in the secondary lysosomes of phagocytic cells.

Our data about the speed of adsorption through the lymphatic system are in accordance with results of studies, utilizing I^{131}-labelled albumin to check the speed of protein passage from the pleural cavity (Cartfedge et al., 1988). Differences in tracer concentration in superficial sinuses vs. cortex and medulla demonstrated by microscopical SR analysis seem to reflect a higher number of reticular cells in the superficial sinuses.

The present study demonstrates that employment of SR gives some advantages over traditional methods. Different elements can be analyzed with much higher sensitivity and without constant x-ray radiation. Exact quantity analysis is another advantage of this technique. Morphologically one could not find differences in the concentration of gold in the sinuses at different time points; but by means of XFA-SR these differences could be evaluated. SR probably will have broad medical and biological applications not only for the analysis of tracer passage, but also to study the quantity and dispersion of certain elements in biological structures. Potentially XFA-SR can detect very low concentrations of different elements, while microscopical SR analysis can potentially demonstrate differences in elemental concentrations within single cells.

LITERATURE CITED

Cartfedge, Y.T., D.J. Martin, C. Guintini, and D.E. Taylor 1988 Readsorption kinetics of albumin from pleural space of dogs. Am. J. Physiol., *255*:375–385.

De Roe, C., P.J. Courtoy, and P. Baudhuin 1987 A model of protein–colloidal gold interaction. J. Histochem. Cytochem., *35*:1191–1198.

Dementiev, E.N., I.P. Dolbnya, S.G. Kurylo, N.A. Mezentsev, V.F. Pindyurin, and M.A. Sheremov 1991 Experimental station of X-ray microscopy and micrography at the storage ring VEPP-3. Nucl. Instr. and Methods A., *308*:352–355.

Dragun, G.N., E.V. Zelentsov, K.V. Zolotarev, Y.A. Zorin, L.F. Krylova, G.N. Kulipanov, and V.N. Gorchakov 1989 Nontraditional contrast agents for mediastenal lymphography using synchrotron radiation. Nuclear Instruments and Methods in Physics Research, A., *282*:493–494.

Frens, G. 1973 Controlled nucleation for the regulation of the particle size in monodisperse gold suspensions. Nature (Phys. Sci.), *241*: 20–23.

Glazyrin, A.L., S.I. Kolesnikov, and A.J. Safronov 1993 Histochemical localization of oxidized glutathione-binding enzymes in human term placenta. Histochem. J., *25*:45–50.

Griffiths, G.D., A.G. Leith, M.D. Leek, and M.A. Green 1989 Distribution of ricin within the mammalian para-aortic lymph node. 2. Comparison of the localization, after intramuscular dosage of colloidal gold–labeled ricin in vivo, with in vitro binding characteristics of the native toxin. Histochem. J., *21*:387–392.

Kaiserling, E., and H. Wolburg 1988 Interdigitating reticular cells in dermatopathic lymphadenitis: Freeze-fructure and ultrathin-section morphology. Virch. Arch. B. Cell Pathol., *55*:19–30.

Kulipanov, G.N., N.A. Mesentsev, V.F. Pindurin, A.N. Skrinsky, M.A. Sheemov, A.P. Ogirenko, and V.M. Omigov 1983 Applicaion of synchrotron radiation to the study of man's circulatory system. Nuclear Instrum. Methods in Physics Res. A *208*:677–683.