Survey of Radioactive Agents for In Vitro Labeling of Phagocytic Leukocytes. II. Particles

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When various radioactive particles are incubated and tumbled in concentrated suspensions of blood phagocytes at body temperature for 1 hr, they bind to the phagocytic cells with a labeling yield of 30–40%. In vitro experiments show that, for some radioactive colloids, a sizeable fraction of the total cellular binding results from nonspecific surface adsorption to other cells and from reversible surface adsorption to phagocytes without engulfment. No completely satisfactory in vitro methods have been found for separating leukocytes with completely engulfed particles from those with surface-adherent particles; nonetheless, surface adherence can be partially reversed by 20% acid citrate dextrose (ACD) solution or by an excess of nonradioactive colloid. Gelatinization of colloidal particles tends to increase their binding to phagocytic cells but also increases the degree of nonspecific adherence to other cells. Technetium-99m-millimicrospheres, 0.5–2 μm in diameter, are optimal in size for phagocytosis by neutrophils, and their nonspecific adherence to other cells is minimal. Because of the microspheres' poor stability in aqueous suspension, however, it is technically difficult to separate free from phagocytosed radioactivity after cell incubation. The highly stable small-particle colloids (<0.1 μm), such as ^{198}Au-colloid or ^{111}In-colloid without iron carrier, are phagocytosed poorly or not at all by neutrophils, although they are engulfed by mononuclear cells.

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Recently the phagocytosis of radioactive colloids has attracted considerable interest as a simple method of labeling monocytes and granulocytes with gamma-emitting radionuclides for clinical studies. Ever since the discovery of this phenomenon by Haeckel in 1862 (*1*), phagocytosis has been observed for a variety of particles (*2*), often accompanied by marked changes in leukocyte metabolism (*3*). The in vivo labeling of leukocytes with a radioactive colloid (^{198}Au) was first noted in dogs by Ganz et al (*4*). After colloidal gold was added to blood in vitro and incubated over 24 hr, the plasma activity decreased as the activity in the leukocyte fraction increased. In experimental granulomas, Spector and Lykke (*5*) found that colloidal carbon particles localized not only within inflammatory cells, but also penetrated the walls of abnormally permeable small venules at inflammatory sites. Recently, phagocytosed ^{99m}Tc-sulfur colloid was used by Charkes et al (*6*) to localize thrombi in dogs by radionuclide imaging. For the localization of experimental abscesses, the same colloid was employed by English et al (*7*) and ^{99m}Tc-labeled millimicrospheres were used by Fisher et al (*8*). This report describes in vitro experiments that evaluate the labeling of blood phagocytes by various readily available radioactive particles suitable for in vivo use.

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MATERIALS AND METHODS

Technetium-99m-sulfur colloid was prepared from two commercial kits (Radiochemical Centre, Amersham, U.K., and E. R. Squibb & Sons, New Brunswick, N.J.) using pertechnetate eluant in saline from a Radiochemical Centre generator. Indium-111-chloride (Radiochemical Centre) was incorporated into two colloids, one with and one without ferric chloride, using a modified form of Colombetti's method (*9*). Commercial millimicrospheres, 0.5–2 μm in diameter (CIS Radiopharmaceuticals, Bedford, Mass.), were labeled with 5 ml of pertechnetate generator eluant, with a labeling yield invariably greater than 99.5% at 15 min. These particles were used also after heating in a boiling-water bath for 10 min, and also after similar heating following the addition of 0.1 ml of 2% gelatin solution per milliliter of suspension.

Five milligrams of zymosan particles (Sigma Chemical Co., St. Louis, Mo.) suspended in saline were allowed to settle for 2–3 min. The supernatant, containing approximately 3 mg of particles, was withdrawn and labeled with ^{99m}Tc-pertechnetate and stannous chloride in hydrochloric acid at pH 2.5. After heating the particles in the boiling-water bath for 10 min, a labeling efficiency greater than 99% was achieved. These particles also were gelatinized using the same procedure as for the millimicrospheres. Phytate, which is known to form colloids in the presence of plasma calcium (*10*), was labeled with ^{99m}Tc and stannous chloride, using the method of Dr. G. Subramanian.

One-milliliter aliquots of red blood cells, obtained from the same donor as the white cell suspensions, were washed and suspended in 0.9% saline, labeled with either carrier-free ^{203}Pb or ^{111}In-acetylacetone, and then denatured either by heating at 50°C for 1 hr in a water bath, or by soaking in tannic acid (25 μg/ml) at room temperature for 20 min (*11*). Following denaturation the labeled red cells were washed twice in saline prior to their use as phagocytosable particles. Liposomes (*12,13*) containing ^{111}In-bleomycin ($<<0.1$ μm) were prepared and supplied by Dr. G. Gregoriadis.

Cell-rich supernatant plasma was obtained after red cell sedimentation of 50 ml of freshly drawn heparinized blood, as described in Part 1 (*14*). Two milliliters of this supernatant and the radioactive particles were added to 5-ml cylindrical polystyrene tubes and sealed with polystyrene caps. Incubation was carried out for 1 hr (and occasionally at other time intervals) at 36°C. The tubes were positioned horizontally on a wheel and rotated mechanically and vertically 12 times per minute. Sometimes, for comparison, phagocytosis was carried out using 2-ml aliquots of heparinized whole blood.

After incubation, 0.5 ml of the tube contents were pipetted as a counting standard. The remaining contents were then centrifuged at 500 g for 5 min to sediment the cells, and 0.5 ml of the supernatant fluid was pipetted and counted. This supernatant radioactivity was expressed as a percentage of the standard. The remaining tube contents were then transferred to a siliconized 10-ml glass centrifuge tube. The cells were resuspended in Hank's balanced salt solution (HBSS) and recentrifuged, the supernatant was removed, and the cell sediment was counted. This washing procedure was repeated five times, as described previously (*14*), and the cell-bound radioactivity was again expressed as a percentage of the total radioactivity originally added to the incubation tube.

Cell-bound activity was also measured by the iron particle method (*15*). The incubation of cell suspensions with radioactive particles was briefly interrupted at 30 min for the addition of 0.15 mg of iron carbonyl particles suspended in 0.1 ml of saline, and the incubation was then continued for an additional 30 min.

Nylon-fiber columns (300 mg) also were tried for separating cell-bound from free supernatant particles, since only the granulocytic, monocytic, and platelet components of blood adhere to these fibers (*14*). We observed, however, that the types of radioactive particles that became bound to leukocytes also invariably adhered to the nylon column when suspended in cell-free plasma. Moreover, these particles readily eluted from the columns after the addition of 20% acid citrate dextrose (ACD) solution in plasma. Thus their behavior on nylon columns mimicked that of the phagocytic cells. On the other hand, those radioactive particles that did not bind to leukocytes did not adhere to nylon fibers. Hence this method could not be used for the assay of cell-bound radioactive colloids.

Theoretically, particles should bind only to the phagocytic cells of blood (granulocytes, monocytes, and platelets). Nevertheless, we observed nonspecific surface adherence of labeled particles to other cellular components, particularly erythrocytes. Consequently for each radioactive particle studied, "control" tubes were prepared, either containing (A) 2 ml of cell-rich supernatant more than 24 hr old and passed through a nylon-fiber column to remove residual viable phagocytes, or (B) 2 ml of plasma with the addition of 0.1 ml of outdated blood-bank blood. The separation and assay procedures carried out with these tubes were identical to those containing fresh viable phagocytes. Additional tubes were prepared with radioactive colloids suspended in only 2 ml of plasma to measure nonspecific adherence to the

incubation tubes and the degree of settling of the particles on centrifugation.

To differentiate between phagocytosis of the particles and surface adherence, additional tubes containing viable leukocytes, washed and suspended in HBSS and free of opsonins, were incubated. Incubation was carried out with cells in plasma also at room temperature (20–22°C), and occasionally at 5°C, without tumbling the tubes. At this refrigeration temperature, the cells had a tendency to clump and became difficult to resuspend. Reduction of surface adherence was attempted also by adding 20% ACD solution or relatively large quantities of nonradioactive colloid, for a further incubation period of 30 min at room temperature, prior to the cell separation procedures.

RESULTS

Table 1 lists radioactive particles exhibiting no significant leukocyte binding, including those previously reported. Other particles that did bind to the cells are listed in Table 2. The percentages of the added radioactivity that became cell-bound after 1 hr of incubation at 36°C are given both for fresh cell-rich supernatant and for cell suspensions containing no viable phagocytes in plasma. The values listed should be regarded as only semiquantitative. Although values were reproducible within about 2% in repeated experiments using fresh blood from one individual, variations in binding of 10–20% were encountered using blood from different individuals. The values obtained using filtered whole blood without phagocytes were even more variable.

No significant difference in cell-binding behavior was noted between the two different commercial preparations of ^{99m}Tc-sulfur colloid. In eight experiments, the average cell binding with fresh supernatant was 42%, but it ranged in different individuals over 21–65%. In old supernatant, the average cell binding of this colloid was 15% but was sometimes as low as 5%. When this colloid was suspended in plasma, centrifugation at 500 g for 5 min resulted in an 8% loss of radioactivity in the plasma supernatant and an even greater loss at higher speeds. When this colloid was incubated in fresh heparinized whole blood, 51% of the activity was cell bound. In whole blood after granulocyte filtration through a nylon column, the cell-associated activity was 44%. Hence, most of the binding in whole blood is probably nonspecific adsorption. Using the iron-particle separation method, 65% of the activity was cell bound for fresh supernatant, compared with 52% for old supernatant. In tubes of fresh supernatant incubated with colloid, the addition of ACD solution decreased the cell binding by 7%, and the addition of 1 ml of nonradioactive colloid reduced the cell-bound activity by 15%. Incubation of tubes at room temperature without tumbling had an average of 36% of the total activity cell bound, and those incubated at 5°C showed erratic and occasionally very high degrees of cell labeling. When the amount of colloid suspension was increased from 0.1 to 0.7 ml, there was only a slight reduction in the percentage of cell binding; likewise, using as little as 10 μl of the colloid suspension produced little difference in the degree of cell binding in fresh supernatant, but it

TABLE 1. LABELED PARTICLES NOT BOUND BY NEUTROPHILS

	Dose per 2 ml of cells
^{99m}Tc-phytate	50 μl
^{111}In-colloid without iron (<0.22 μm)	140 μl
^{111}In-bleomycin liposomes (<<0.1 μm)	5 μl
^{198}Au-colloid* (7–10 nm) (17)	2 μg
^{131}I-microaggregated albumin (19)	2 mg
^{14}C-chylomicrons† (0.5–1.5 μm) (18)	14 mg

* Abbott Laboratories, North Chicago, Ill.
† ^{14}C-chylomicra—surface adsorption only.

TABLE 2. LABELED PARTICLES BOUND BY NEUTROPHILS

	Dose per 2 ml of cells	Percent bound after 1 hr at 36°C: Fresh supernatant	Percent bound after 1 hr at 36°C: Old supernatant
^{99m}Tc-sulfur colloid (0.1–1 μm)	1.5–100 μl (0.4–30 μg)	42	15
^{99m}Tc-millimicrospheres (0.5–2 μm)	0.2 ml (0.2 mg)	19–30	0.4–4
^{99m}Tc-millimicrospheres, boiled	0.2 ml (0.2 mg)	15–46	65
^{99m}Tc-millimicrospheres, boiled + gelatin	0.2 ml (0.2 mg)	65	44
^{99m}Tc-zymosan, boiled	10 μl (30 μg)	34–56	3
^{99m}Tc-zymosan, boiled + gelatin	10 μl (30 μg)	44–69	44
^{111}In-Fe-colloid (1–5 μm)	0.14 ml	62	14–28
^{23}Pb-RBC, heat-denatured	10–100 μl (5–50 × 10^6 cells)	53	58–70
^{111}In-acetylacetone–RBC, heat-denatured	10–100 μl (5–50 × 10^6 cells)	27	28
^{111}In-acetylacetone–RBC, tannic acid	10–100 μl (5–50 × 10^6 cells)	19	18

increased nonspecific binding to red cells. Therefore, a sizeable but variable fraction of the cell-bound activity with ^{99m}Tc-sulfur colloid appears to be due to nonspecific reversible surface adsorption.

According to Colombetti (*9*), the majority of gelatinized ^{111}In-colloid particles containing carrier iron are 1–5 μm in diameter, with no particles smaller than 0.22 μm. By microscopy, clumping was found to be greater than with ^{99m}Tc-sulfur colloid, but nevertheless, the indium product was more stable to centrifugation. In eight experiments, the percentage of cell-bound activity for fresh supernatant was higher than for ^{99m}Tc-sulfur colloid. On the other hand, the percentage adherence to old supernatant was, as a rule, higher than for ^{99m}Tc-sulfur colloid. No great difference in the degree of binding was noted at different incubation temperatures and the binding did not increase significantly between 15 min and 4 hr. Considerable cell-bound activity was lost by repeated washing of the cell sediment, and no consistent difference was detected in the degree of cell binding when the amount of colloid was varied from 0.05 to 1 ml per 2 ml of cell-rich supernatant. The percentage of cell-bound activity was markedly reduced ($\sim$ 50%) after the addition of excess nonradioactive colloid, again indicating that most of the binding is reversible and not confined to the viable phagocytes. This conclusion was confirmed microscopically in smears stained for iron. The stained particles were easily seen adhering to the surface of the polymorphs, and very few were intracellular.

Both millimicrospheres and boiled zymosan particles, labeled with ^{99m}Tc and suspended in plasma, settled by gravity within a few minutes. Hence, centrifugation could not be used to separate cell-bound from free particles after incubation with cell suspensions. Differential centrifugation with a layer of commercial Ficoll–Hypaque (Lymphoprep, Nyegaard and Co., Norway), or with other density gradients with specific gravities varying from 1.076 to 1.097, was not successful in separating cell-bound from free particles. Consequently, the only valid method of cell separation was the iron-particle magnet procedure. The millimicrospheres and boiled zymosan particles appeared to be similar in their cell-binding behavior, although the degree of binding in fresh supernatant was greater with the latter. The fraction of total activity that became cell bound was generally not as high as with ^{99m}Tc-sulfur colloid. On the other hand, the cell binding to suspensions without viable phagocytes was consistently low (4% or less). Their in vitro behavior was compatible with true phagocytosis. At room temperature the cell binding after 1 hr was approximately half that at 36°C. The cell binding for cell suspensions in HBSS without plasma was only 10%. The addition of ACD solution or excess nonradioactive particles after incubation did not change the cell-bound percentage. An increase in cell binding of about 10% was obtained by a fivefold increase in leukocyte concentration. Boiling millimicrospheres, or gelatinizing millimicrospheres or zymosan particles, produced a higher degree of cell binding but also greatly increased the nonspecific adherence to suspensions without viable phagocytes.

When erythrophagocytosis was attempted with red cells labeled with either ^{203}Pb or ^{111}In-acetylacetone and denatured either by heat or tannic acid, cell binding invariably occurred in fresh supernatant, but the degree of cell binding to old supernatant without viable phagocytes proved to be just as great. The apparent cell binding, therefore, appears to be largely or entirely due to nonspecific attachment. Unmodified labeled red cells were easily removed from cell suspensions by either hypotonic lysis or passage through nylon columns. After denaturation, on the other hand, the labeled cells adhered to the nylon columns and proved more difficult to destroy by hypotonic lysis.

DISCUSSION

The labeled particles which do not bind neutrophils appreciably (Table 1) have one common characteristic. They are "small particle" colloids, compared with the other preparations evaluated which do become bound. These findings agree with the observations of Roberts and Quastel (*16*) that the optimal size for neutrophilic phagocytosis is 0.26–3.0 μm, using carefully sized polystyrene latex spheres. Various particles less than 0.1 μm in diameter, such as colloidal gold (*17*) or chylomicrons (*18*), are poorly phagocytosed by neutrophils, although they are readily engulfed by macrophages. Likewise, microaggregated albumin does not interact with polymorphs (*19*).

Millimicrospheres of human serum albumin and boiled zymosan particles are optimal in size for neutrophilic phagocytosis and are readily labeled with ^{99m}Tc, giving a high yield. About 30% of these particles undergo true phagocytosis in vitro in 1 hr at 36°C. Their nonspecific surface adsorption to nonphagocytic cells appears low. Their rapid settling by gravity or minimal centrifugation is a disadvantage, however, so that separation of the free from the cell-bound particles is difficult. Labeled millimicrospheres incubated with blood phagocytes and reinjected into dogs became concentrated in the reticuloendothelial organs and lungs, presumably due to margination of unclumped leukocytes and embolization of leukocyte clumps. In addition, activity was found in the

kidneys and bladder, probably due to the liberation of free ^{99m}Tc from the labeled microspheres (*8*).

The percentage of cell-bound radioactivity was higher for ^{111}In-Fe-colloid than for other preparations used in the present study. However, a sizeable fraction of this cell-associated activity resulted from nonspecific adherence to nonphagocytic cells. Labeled red cells denatured by heat or tannic acid proved even less satisfactory for neutrophilic phagocytosis because of excessive surface adsorption to all cellular blood elements. True phagocytosis often can be distinguished in vitro from nonspecific surface adsorption because the latter is a reversible process. Surface adsorption tends to be temperature-independent over 5–37°C and instantaneous, whereas true engulfment progressively increases during one to several hours and is optimal at 37°C (*17,18*).

The cell-associated activity obtained with ^{99m}Tc-sulfur colloid in the present study, averaging 42%, is higher than for labeled millimicrospheres. However, 15% of the binding apparently comes from nonspecific adsorption. These figures agree with the values of 40% and 18%, respectively, obtained by English et al (*7,15*) with buffy coat from human or canine blood. In most commercial kit preparations of this colloid, about 80% of the particles are between 0.1 and 1 μm in diameter and only 5% are greater than 1 μm (*20*). Hence the average particle size is too small for optimal phagocytosis. Nonetheless, this colloid offers a practical advantage over the millimicrospheres: the cell-bound activity is better separated from supernatant activity by simple centrifugation. The fraction of cell-associated radioactivity due to surface adsorption may be reduced significantly, after incubation but before cell separation, by the addition of an excess of nonradioactive colloid or ACD solution (which chelates calcium and magnesium ions). The lipid-soluble radioactive agents discussed previously (*14*), however, do not suffer from these technical difficulties, and the unwanted activity remaining in the suspension fluid is easily removed by centrifugation and resuspension of the labeled cells.

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