Radiolabeling of Erythrocytes with Technetium-99m: Role of Band-3 Protein in the Transport of Pertechnetate Across the Cell Membrane

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This study analyzes the transport characteristics of the pertechnetate anion across membranes of human erythrocytes during labeling with technetium-99m (^{99m}Tc). Transport of this anion is inhibited after incubation at low temperature, indicating the involvement of a process with high activation energy. Transport is also decreased by two well-known inhibitors of the band-3 anion transport system and is not affected by inhibition of the Na/K/Cl co-transport system. From these results we conclude that, in the process of labeling red blood cells with ^{99m}Tc, the pertechnetate anion may reach the interior of the erythrocyte through the band-3 anion transport system.

Technetium-99m-labeled autologous red blood cells (^{99m}Tc-RBCs) are routinely used for the noninvasive evaluation of cardiac function (*1–3*), the localization of gastrointestinal bleeding sites (*4,5*), and for the determination of total RBC mass (*6*). Several methods have been employed to label RBCs, including in vivo (*7*), in vitro (*8,9*), and a combination of these methods (*10, 11*). Although the labeling efficiency of these procedures is high, there are circumstances in which labeling efficiency decreases to levels that interfere with the technical and diagnostic quality of each study. Not all the parameters that determine labeling efficiency are known. For binding to occur, it is essential that the pertechnetate anion reaches the internal compartment of the erythrocyte. Although several of the intracellular binding steps (see Fig. 1), such as reduction of technetium by stannous ion and subsequent binding to hemoglobin, have been well characterized (*12,13*), the

actual transport mechanisms by which tin and technetium reach the internal compartment of the erythrocyte have not been elucidated. In this study, we analyzed the transport characteristics of [^{99m}Tc]pertechnetate by human erythrocytes and compared its properties with those of the anion transport system band-3 protein. The results indicate that the pertechnetate anion reaches the interior of the RBC through a transport system with properties similar to those of the band-3 protein, the anion transport system responsible for chloride-bicarbonate exchange (Hamburger shift) in human and other mammalian red cells.

Identification of this system as the transport mechanism for the pertechnetate anion will permit the identification and prevention of potential pharmacologic interactions since many drugs are known to interact with this transport system (*14–18*). In addition, the use of pertechnetate may create a convenient method for assessing the integrity of the band-3 anion transport system under various physiologic and pathologic conditions.

MATERIALS AND METHODS

Erythrocyte Isolation

Red blood cells were obtained from normal individuals or patients scheduled to have a radionuclide angiocardiographic study. A blood sample was obtained either before or 20 min following i.v. injection of a 3-ml solution containing 10 mg sodium pyrophosphate, 30 mg sodium trimetaphosphate, and 1.8 mg stannous chloride (Pyrolite, DuPont Medical Products, N. Billerica, MA). Anticoagulation was accomplished by the addition of 100 units heparin. Packed red cells were prepared by centrifugation at 1500 × g for 10 min in a Beckman centrifuge model TJ-6R (Beckman Instruments Inc., Palo Alto, CA) at 22°C. After centrifugation, the plasma was removed and the cells resuspended and washed in Earle's balanced salt solution (EBSS). This washing procedure was repeated three times. After the final spin, the cells were resuspended in their respective uptake solutions and used the same day.

Solutions

Uptake assays were performed in a modified EBSS of the following composition (in mM): Na^+, 143; K^+, 5.36; Mg^{2+}, 0.8; Ca^{2+}, 1.8; Cl^-, 117; PO_4^{3-}, 1.0; SO_4^{2-}, 1.0, CO_3H^-, 25; and glucose, 10. The solution was equilibrated with a gas mixture of 5% CO_2 and 95% O_2. After equilibration, the pH of the solution was 7.4. Earle's solutions without CO_3H^- and Cl^- were obtained after replacing these anions with HEPES buffer and gluconate, respectively.

Uptake Assays

Immediately before starting the assay in their respective uptake solutions, the packed cells were resuspended to a final hematocrit of 50%. The uptake assays were carried out at room temperature (22°C) or at 4°C in an ice bath. Technetium-99m-pertechnetate was obtained from a commercially produced $^{99}Mo/^{99m}Tc$ radionuclide generator prepared from fission product ^{99}Mo (E.I. DuPont Medical Products, N. Billerica, MA), which had been previously eluted at least once within the previous 24 hr. The mole fraction of the metastable isomer in the eluate was >25%. At time zero, 100 μCi of pertechnetate-99m (^{99m}Tc) was added to the cell suspension and mixed gently. At intervals of from 1 to 60 min following pertechnetate addition, 100 μl samples were collected from the reaction mixture and placed into a solution containing 0.5 mg·ml^{-1} DTPA (diethylenetriaminepentaacetic acid) and 0.017 mg·ml^{-1} stannous chloride (Medi-Physics, Inc., Richmond, CA). This method has been shown previously to immediately quench the labeling reaction, thus, allowing time for separation of the cells (10). A control experiment performed by simultaneously adding the pertechnetate anion and the mixture of DTPA/SnCl$_2$ demonstrated a complete inhibition of the labeling process.

At the completion of sampling, all samples were centrifuged at 1500 × g for 5 min. Cells and supernatants were separated and their radioactivity content determined in a radioisotope dose calibrator model CRC-12R (Capintec Inc., Ramsey, NJ). Corrections for interstitial trapping determinations were made as described before (19) based on the amount of carbon-14 (^{14}C) inulin associated with each sample. The ^{14}C activity of the samples was determined by liquid scintillation counting using Dimilume-30 (Packard Instrument Co.) scintillation fluid in a Beckman liquid scintillation counter model LS 230 (Beckman Instruments Inc., Fullerton, CA). Corrections for interstitial trapping were minimal (<2%) and not considered in the calculation of the labeling efficiency. Labeling efficiency was expressed as the activity in cells divided by the radioactivity in cells plus supernatant times 100%. To determine the influx of pertechnetate into erythrocytes that were not pretreated with stannous chloride, RBCs were obtained from subjects who had not received Sn^{2+} and prepared as above. Cell-bound activity was determined following separation of the RBCs from the incubation medium by rapid centrifugation on a silicone oil cushion. One hundred microliter samples were added to 1.5 ml conical microcentrifuge tubes containing 500 μl of silicone oil (d 1.05 g/ml). The samples were centrifuged at 15,600 × g for 60 sec in a microcentrifuge (Eppendorf, model 5412, Brinkman Instruments, Westbury, NY). After centrifugation, the liquid phase and one-half of the oil phase were separated and the contents of the tube and the liquid phase counted for radioactivity as described above.

HPLC Analysis

Two milliliters of RBC suspension incubated for 15 min in the presence of [^{99m}Tc]pertechnetate in the absence of SnCl$_2$ were packed by centrifugation at 1500 × g for 5 min. The packed cells (1 ml) were hemolyzed in 5 ml sterile distilled water. An aliquot of the hemolysate was removed and filtered through a 0.22-μm filter to remove cell debris prior to high-performance liquid chromatography (HPLC). Technetium-99m-labeled RBCs were obtained from patients and washed two times with EBSS to remove non-cell-bound activity. Packed cells were prepared and hemolyzed as described above. A 20-μl sample of each hemolysate was injected into an HPLC system (LDC Milton Roy, Bloomfield, CT) using a TSK-250 column and an isocratic mobile phase of phosphate/sulphate buffer at pH 6.8. The flow rate was 1 ml/min. Simultaneous recordings of protein and radioactivity were performed with an ultraviolet (UV) detector at 280 nm and a radiation detector placed in series with the column.

Materials

SITS (4-Acetamido-4'-isothiocyanotestilbene-2,2'-disulfonic acid); furosemide (5-[Aminosulfonyl]-4-chloro-2-[(2-furanylmethyl)-amino] benzoic acid; and HEPES (N-2-Hydroxy-ethylpiperazine-N'-2-ethanesulfonic acid) were obtained from Sigma Chemical Co. (St. Louis, MO). Dipyridamole (2,6-bis-(diethanolamino)-4,8-dipiperidinopyrimido-(5,4-dipyrimidine) was obtained from Boehringer Ingelheim Pharmaceuticals, Inc. (Ridgefield, CT). All other chemicals were analytical grade reagents obtained from Sigma or Aldrich Chemical Co. (Milwaukee, WI).

RESULTS

Effect of Temperature on the Labeling Efficiency

Figure 1 shows that at room temperature (22°C) the labeling efficiency of pre-tinned RBCs increases rapidly to reach values close to 100% after 10 min incubation. Greater than 85% of this labeling took place in the first 5 min of incubation. Incubation at 4°C reduced the
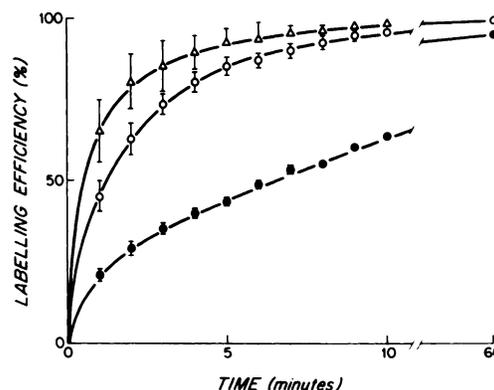

FIGURE 1

Changes in the labeling efficiency during incubation of tinned human erythrocytes incubated in EBSS at 22°C (O) or 4°C (●). Open triangles represent the labeling efficiency of erythrocytes incubated at 22°C but in absence of chloride and bicarbonate. The results are the mean of 6 determinations ± s.e.

labeling efficiency at 5 min to 50% of that obtained at 22°C.

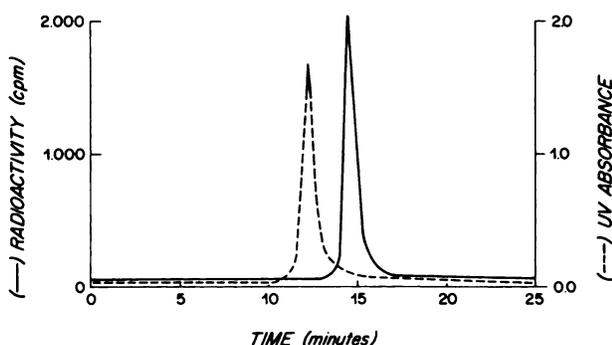## Effect of Stannous Ion on the Efficiency and Reversibility of Labeling

Preincubation of the erythrocytes with stannous chloride is a necessary requirement for a high and permanent labeling to occur. The labeling efficiency of erythrocytes that were not pretreated with stannous chloride was significantly reduced to ~50% even after 60 min incubation. The radioactivity accumulated by these erythrocytes decreased rapidly to very low values when they were incubated in a large volume of EBSS. This rapid washout was not observed with erythrocytes pretreated with stannous chloride.

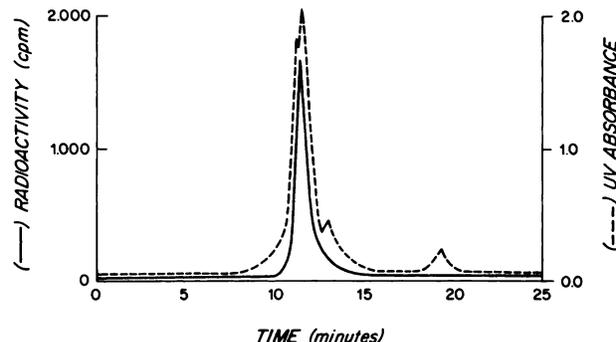## Distribution of Pertechnetate in Non-tinned and Tinned Erythrocytes

To determine the characteristics and distribution of ^{99m}Tc across the RBC membrane, non-tinned and tinned erythrocytes were hemolyzed and the hemolysate analyzed by HPLC. The eluate of the column loaded with the hemolysate from erythrocytes not treated with $SnCl_2$ is shown in Figure 2. A single peak with a retention time of 14.8 min was obtained on the radioactivity tracings. This corresponded to the retention of the pertechnetate ion. A single UV peak corresponding to hemoglobin was observed at 11.5 min. The radioactivity profile of the hemolysate from erythrocytes treated with $SnCl_2$ before labeling (Fig. 3) consisted of two minor peaks with retention times of 13.0 and 18.0 min and two major peaks with very close retention times of 11.3 and 11.8 min. These two peaks have a retention time similar to that (11.5 min) of the single peak observed in the UV tracing.

Effect of Anion Substitution on the Labeling Efficiency

Figure 1 shows the effect of replacing bicarbonate and chloride by HEPES and gluconate anions, respectively, on the labeling efficiency of tinned RBCs. In the

FIGURE 3
HPLC analysis of hemolysate from tinned human erythrocytes incubated for 10 min in presence of $^{99m}TcO_4^-$.

absence of chloride and bicarbonate, there was an increase in the labeling rate of the erythrocytes by pertechnetate. Although the change observed after the anion substitution was not as dramatic as that observed with changes in temperature, the results suggest that these anions compete with the pertechnetate anion when they are present on the same side of the erythrocyte membrane. In addition (see Table 1), the higher retention of ^{99m}Tc by non-tinned cells incubated in buffer without chloride and bicarbonate (40.3% ± 0.9%) when compared to buffer containing both anions (5.1% ± 0.5%) indicates that the presence of these ions outside the erythrocyte increased (trans-stimulated) the efflux of pertechnetate ion across the RBC membrane.

Effect of Sodium and Potassium Substitution on the Labeling Efficiency

Table 2 shows the effect of replacing sodium by choline and potassium by sodium on the labeling efficiency of erythrocytes. No inhibition of the labeling rate of tinned RBCs was observed after substituting sodium and potassium with choline and sodium, respectively.

Effects of SITS on the Labeling Efficiency

Figure 4 shows that SITS at a concentration of 10^{-4} M produced significant inhibition of the labeling rate of RBCs incubated at 22°C. After a 5-min incubation in the presence of the inhibitor, labeling efficiency was 31.0% ± 2.65% compared with 84.0% ± 3.76% ob-

FIGURE 2
HPLC analysis of hemolysate from non-tinned human erythrocytes incubated for 10 min in presence of $^{99m}TcO_4^-$.

TABLE 1
Effect of Chloride and Bicarbonate on the Efflux of Pertechnetate from Non-tinned Human Erythrocytes

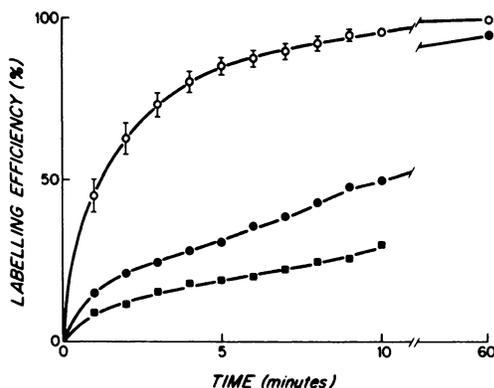| Incubation medium	Labeling efficiency (%)	
	0 min	10 min
EBSS	50.3 ± 0.3	5.1 ± 0.5
Cl^-/HCO_3^- free EBSS	50.8 ± 0.5	40.3 ± 0.9

TABLE 2
Effect of Sodium, Potassium, and Furosemide on the Labeling Efficiency of Tinned Human Erythrocytes with ^{99m}Tc

Incubation condition	Labeling efficiency (%)
Control EBSS	97.8 ± 0.3
Na⁺-free EBSS	99.2 ± 0.3
K⁺-free EBSS	99.1 ± 0.2
Furosemide 10^{-4} M	97.4 ± 1.3
Furosemide 10^{-3} M	93.5 ± 2.1

FIGURE 5
Rate of labeling of tinned human erythrocytes during incubation in the absence (O) or presence (●) of 10^{-4} M dipyridamole. The results are the mean of 4–6 determinations ± s.e. When not shown, the error bars are part of the symbols.
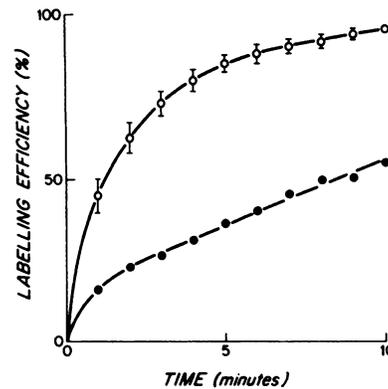
tained in the control group. The inhibition seemed to affect the labeling rate, not total labeling, as shown by an almost identical labeling efficiency obtained in both groups after 60 min incubation. Incubation of the erythrocytes at 4°C in the presence of 10^{-4} M SITS produced an additional inhibition of the labeling efficiency as compared with the inhibition obtained after incubation at 4°C alone or after incubation at 22°C in the presence of SITS. The effect of SITS on the labeling efficiency seemed irreversible since it remained unchanged after several washes of RBCs before the incubation in presence of ^{99m}Tc.

Effect of Dipyridamole on the Labeling Efficiency

The effect of dipyridamole on the labeling efficiency of erythrocytes by pertechnetate is shown in Figure 5. Dipyridamole at a concentration of 10^{-4} M produced a marked inhibition of the labeling rate. This inhibition was of the same magnitude as that observed after incubation at 4°C or after incubation in the presence of 10^{-4} M SITS. Like SITS, the inhibitory effect of dipyridamole remained unchanged after several washes of RBCs in absence of the inhibitor.

FIGURE 4
Rate of labeling of tinned human erythrocytes during incubation in the absence (O) or presence (●) of 10^{-4} M SITS at 22°C. Closed squares represent the rate of labeling of erythrocytes incubated in presence of 10^{-4} M SITS at 4°C. The results are the mean of 4–6 determinations ± s.e. When not shown, the error bars are part of the symbols.

Effect of Furosemide on the Labeling Efficiency

Table 2 shows the effect of furosemide on the labeling efficiency of erythrocytes by pertechnetate. At a concentration of 10^{-4} M, this inhibitor produced no significant inhibition of the labeling rate. Moreover, no significant inhibition was observed after increasing the concentration of the inhibitor to 10^{-3} M.

DISCUSSION

The physiologic function of the anion exchange protein, band-3, in red cells is the chloride-bicarbonate exchange (Hamburger shift) between the erythrocyte and plasma. The actual transport of a particular anion (chloride, for instance) through this transport system takes place in exchange for another anion (bicarbonate, for instance) located on the other side of the erythrocyte membrane. The characteristic properties of the anion exchanger include, for example, a system that is temperature-dependent with a high activation energy. The transport of a particular anion by the system is inhibited by the presence of a similar anion at the same side of the erythrocyte membrane (competition) and is stimulated by the presence of a similar anion at the opposite side of the membrane (trans-stimulation). Finally, the anion transport system, band 3, is specifically inhibited by several agents, including SITS and dipyridamole.

Previous results (12,13) and the data shown above indicate that labeling of erythrocytes by pertechnetate results from the permanent retention of this anion within the intracellular compartment of the RBCs. Although the actual transport mechanisms involved are unknown, these studies indicate that the plasma membrane of the erythrocyte is relatively permeable to the pertechnetate anion. In the present study, we have analyzed the labeling of RBCs by the pertechnetate anion under different experimental conditions and have

compared this process with the chloride-bicarbonate exchange system observed in human erythrocytes.

The inhibition of the labeling efficiency after incubation of the erythrocytes at low temperature is consistent with a process which has a high activation energy, like the chloride-bicarbonate exchange in RBCs. The rate of anion transport in human red cells is highly temperature-dependent with activation energies of 19.6 Kcal·mol^{-1} below a transition point at 16°C; and 11.7 Kcal·mol^{-1} above that transition point (20). The apparent high activation energy for the labeling process indicates the presence of an energy barrier, probably represented by the transport of pertechnetate across the cell membrane rather than the anion binding which has an activation energy less than 4 Kcal·mol^{-1} (21).

The small decrease in the labeling rate of the erythrocytes after incubation in the presence of chloride and bicarbonate is consistent with a competition between these anions and the pertechnetate anion on the outside of the red cell. Since the maximum rate of exchange of intracellular bicarbonate for extracellular chloride in human red cells is higher than that of intracellular chloride for extracellular bicarbonate (22), it appears that most of the inhibition of the labeling process is due to the presence of chloride and not to bicarbonate in the incubation medium. This observation suggests that pertechnetate may reach the interior of the erythrocyte after substituting for chloride in the chloride-bicarbonate exchange. The stimulation of pertechnetate efflux observed when there is chloride in the incubation medium (trans-stimulation) indicates that the pertechnetate anion could replace not only chloride, but also bicarbonate during operation of the anion exchanger.

The stilbene sulphonates, including SITS (23,24), have been shown to be specific inhibitors of the anion transport in RBCs through interaction at the substrate site. Kinetic analysis of the inhibition of anion transport by SITS, for instance, shows negative cooperativity consistent with site-site interactions (25). The large inhibition of the labeling efficiency of human erythrocytes observed in the presence of SITS is consistent with the idea that the pertechnetate anion reaches the interior of the red cells at the anion exchange protein.

Dipyridamole has also been shown to inhibit red cell anion exchange, but in this case, by a mechanism other than straightforward interaction with the substrate site (15,16). This compound is of particular interest because of its frequent use as a therapeutic agent in the secondary prevention of acute myocardial infarction (26) and transient cerebral ischemia (27) or as a vasodilator in studies of myocardial perfusion with thallium-201 (28, 29). The poor image quality of the cardiac radionuclide angiographic studies performed in patients receiving therapeutic or diagnostic doses of dipyridamole suggests that this compound may interfere with the labeling of the red cells by pertechnetate. The reduction of eryth-

rocyte labeling efficiency caused by dipyridamole confirms this possibility and indicates that this inhibition takes place at the level of the anion exchange protein present in the erythrocyte membrane. The inhibition by dipyridamole and SITS seems irreversible, as shown by a persistant and unchanged inhibition after several washes of the RBCs. This observation indicates that the inhibitory effect of these agents is due to their direct interaction with the exchanger and not to the extracellular reduction and chelation of pertechnetate.

Pertechnetate has been shown to be transported in parotid acinar cells (30) by a Na$^+$/K$^+$/Cl$^-$ co-transport system, which is inhibited by furosemide. Two sets of evidence, however, argue against the possibility that the transport of this anion during the labeling of RBCs is similar to that observed in acinar cells. In the first place, the labeling efficiency is not affected by incubating the red cells in the absence of sodium and potassium. Second, maximal doses of furosemide do not inhibit the labeling efficiency by pertechnetate. Although furosemide also inhibits chloride-bicarbonate exchange in red cells (17,31), this inhibition is less specific and occurs at higher concentrations than that observed with stilbene sulphonates. Furosemide has also been shown to inhibit the co-transport of chloride with potassium and sodium or lithium observed in the red cells (32, 33). Two possibilities could explain the observation that although the chloride co-transport system is involved in the transport of pertechnetate by parotid cells it is apparently not involved in the transport of this anion by RBCs. First, although pertechnetate is transported by the chloride co-transport system of acinar parotid cells, this anion is not a substrate for the chloride co-transport system present in RBCs. The second and more likely possibility is that under the present experimental conditions the contribution of the chloride co-transport system to the total transport of pertechnetate by red cells is minimal compared with the contribution of the chloride-bicarbonate exchange. This co-transport system, however, may be responsible for the labeling of neutrophils, lymphocytes, and platelets, since they normally do not have a detectable band-3 protein.

Furosemide is another agent commonly used in the treatment of patients with cardiac failure. Despite inhibition of the chloride-bicarbonate exchange system by furosemide reported in the literature, our results indicate that, at the concentration normally used to treat these patients, no interference with the labeling procedure should be expected.

The transport of pertechnetate by the human erythrocyte exhibits several properties of the band-3 anion transport system. We conclude that in the labeling of RBCs with ^{99m}Tc, the pertechnetate anion reaches the intracellular space in exchange for chloride and/or bicarbonate ions through this transport system (see diagram of the labeling process in Fig. 6). In addition, the
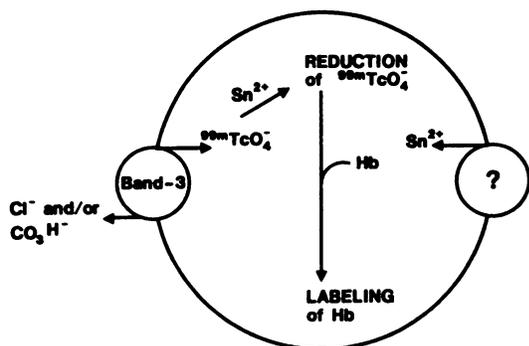
FIGURE 6
Diagram of the different steps involved in the labeling of RBCs with [^{99m}Tc]pertechnetate. The ? indicates the presence of a possible Sn^{2+} transport system involved in the translocation of this cation from the plasma into the RBC.

reducing agent $SnCl_2$ also seems to be transported inside the erythrocyte by a specific transport system (*34*). The identification of these mechanisms will permit the identification and prevention of potential drug interactions and provides a convenient means for assessing these transport systems in vivo under various physiologic and pathologic conditions.

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SELF-STUDY TEST
Skeletal Nuclear Medicine

Questions are taken from the *Nuclear Medicine Self-Study Program I*, published by The Society of Nuclear Medicine

DIRECTIONS

The following items consist of a heading followed by lettered options related to that heading. Select the options that you think are true and those that you think are false. Answers may be found on page 2028.

1. True statements regarding techniques for measurement of bone mineral include which of the following?
A. In quantitative computed tomography (QCT), one generally measures only trabecular bone in the center of the vertebra.
B. The midradius is a sensitive indicator for early bone mineral changes.
C. Dual-photon absorptiometry of the spine and QCT of the spine both result in radiation exposures of between 0.5 and 1.0 rad per examination.
D. An advantage to measuring bone mineral in the calcaneus is its similarity to the spine with respect to the ratio of trabecular to cortical bone.

2. True statements regarding the scintigraphic findings in ischemic necrosis of bone include which of the following?
A. In adult patients undergoing steroid treatment for lupus erythematosus, photon-deficient defects in the femoral diaphysis are common on ^{99m}Tc-colloid images.
B. In patients with sickle cell anemia, photon-deficient defects in the femoral diaphysis are common on ^{99m}Tc-colloid images.
C. Ischemic necrosis of the femoral head in an adult is more reliably detected by imaging with ^{99m}Tc-colloid than with ^{99m}Tc-MDP.
D. Immediately after bone infarction, ^{99m}Tc-MDP scintigraphy will usually show focally increased activity.
E. In a patient with sickle cell anemia, osteomyelitis should be considered much more probable than infarction if a lesion shows uptake of both ^{99m}Tc-MDP and ^{67}Ga of moderate intensity and congruent in distribution.

3. True statements regarding scintigraphy in acute rheumatoid synovitis include which of the following?
A. The increased accumulation of ^{67}Ga is predominantly in periarticular bone.
B. Images obtained with ^{111}In-labeled neutrophils show periarticular photon deficiency because the process is chiefly lymphocyte-mediated.
C. The increased activity seen on images with ^{99m}Tc-pertechnetate is predominantly in the synovium.

D. There is a decreased rate of ^{133}Xe washout after its instillation (dissolved in saline) into the joint space.
E. Delayed images with ^{99m}Tc-MDP show increased activity in the periarticular bone.
F. Acute rheumatoid synovitis of an individual joint is readily distinguished from septic arthritis of that joint by combined bone and gallium scintigraphy.

4. True statements regarding scintigraphy with ^{99m}Tc-MDP for detection of sacroiliitis include which of the following?
A. A low-grade, symmetrical increase in sacroiliac joint activity is difficult to detect by subjective evaluation of the scintigrams.
B. The ratio of activity in the sacroiliac joints to that in the sacrum clearly distinguishes patients with ankylosing spondylitis from those with normal joints.
C. The sacroiliac joint uptakes in ankylosing spondylitis and metabolic bone disease are often similar.
D. Both qualitative and quantitative assessments of sacroiliac joint uptake are usually normal in patients with diffuse idiopathic skeletal hyperostosis.

5. True statements regarding the scintigraphic evaluation of total hip prostheses include which of the following?
A. Increased uptake of ^{99m}Tc-MDP around the stem of the femoral component nearly always indicates loosening.
B. The finding of focally increased uptake of ^{99m}Tc-MDP at the tip of the stem of the femoral component 1 year after implantation nearly always indicates loosening.
C. Diffusely increased uptake of ^{67}Ga in the same distribution as ^{99m}Tc-MDP reliably excludes infection.
D. Uptake of either ^{67}Ga- or ^{111}In-labeled leukocytes in a pattern incongruent with the distribution of ^{99m}Tc-MDP indicates a high probability of infection.
E. Persistent increased uptake of ^{99m}Tc-MDP in heterotopic bone indicates its immaturity.