[CANCER RESEARCH 39, 4971-4974, December 1979]
0008-5472/79/0039-0000$02.00

Effect of Hypoxia on ^{67}Ga Incorporation into Human Granulocytes[1]

F. Ostroy,[2] R. B. Johnston,[3] and R. A. Gams

Comprehensive Cancer Center, University of Alabama in Birmingham, Birmingham, Alabama 35294

ABSTRACT

Human granulocytes accumulate ^{67}Ga when incubated under anoxic conditions and exclude the isotope when oxygenated. When incorporated, ^{67}Ga is associated with the lysosomal fraction of the granulocytes. The ability of granulocytes to exclude the isotope except under hypoxic conditions may explain ^{67}Ga localization in abscesses and some tumor masses.

INTRODUCTION

Scintiscanning with ^{67}Ga citrate has become a standard technique for the detection and staging of a broad group of neoplastic disorders (4, 6, 11, 14, 16). A number of clinical and experimental studies have demonstrated that, in addition to binding to tumor cells of various types and certain normal tissues, including liver, spleen, and bone, ^{67}Ga is localized in inflammatory lesions (see Ref. 7 for additional references). It has been suggested that the localization of ^{67}Ga in these areas may be due to the binding of the isotope by granulocytes (2, 3). *In vitro* studies, however, have shown that very little gallium is taken up by granulocytes and therefore, compared to other isotopes, the technique of reintroducing gallium-labeled leukocytes for localizing inflammatory lesions has been ineffective (18). It is possible, however, that *in vivo* binding to granulocytes in abscesses may be influenced by conditions found in these lesions not reproduced in *in vitro* systems.

We have previously used *in vitro* methods to study the incorporation of ^{67}Ga into L1210 leukemic lymphoblasts (7-9). Here, we describe the application of similar methods to investigate the effect of oxygen on the uptake of ^{67}Ga by granulocytes.

MATERIALS AND METHODS

^{67}Ga Samples. Stock solutions of ^{67}Ga citrate (carrier free) (New England Nuclear, Boston, Mass.) containing 2 mCi/ml at noon of the day of delivery and 2 mg sodium citrate per ml were used within 2 weeks of the delivery date ($t_{1/2}$, 78.1 hr). Dilutions of this isotope were made in 0.9% NaCl solution and counted before use. Radioactivity was determined in a Model 1185 automatic well scintillation counter (Nuclear-Chicago Corp., Des Plaines, Ill.) previously calibrated with ^{67}Ga samples of known activity.

Preparation of Human Granulocytes and Mononuclear Cells. Human granulocytes were prepared by a previously detailed method (15). Briefly, whole blood from normal adult volunteers was defibrinated with glass beads, and granulocytes and erythrocytes were separated from lymphocytes and monocytes by centrifugation through Ficoll and Hypaque. Erythrocytes were removed by dextran sedimentation and osmotic lysis leaving 96 to 99% granulocytes and 1 to 4% lymphocytes. Granulocytes and the mononuclear cell layer were pelleted at 4°, washed 3 times with glucose-Locke's solution (0.9% NaCl, 0.024% KCl, 0.042% $CaCl_2$, 0.02% $NaHCO_3$, and 0.1% glucose, pH 7.5), and suspended to a concentration of 1.3×10^8/ml. Mononuclear cell preparations contained 65 to 80% lymphocytes; the remainder was monocytes (15). Routine examination of preparations prior to and following incubations showed that greater than 90% of cells excluded trypan blue. Similar preparations have been shown to be functionally intact (15).

Cell Binding Studies. Cell suspensions (10 ml) containing granulocytes or mononuclear cells in a concentration of 10^7 cells per ml were added to each of 3 stoppered 100-ml beakers and incubated under a stream of 100% nitrogen, 100% oxygen, or room air for 30 min on a horizontal shaker at 4°. The beakers were then transferred to a 37° water bath without interrupting the gas flow. After an additional 30 min preincubation, ^{67}Ga in 0.9% NaCl solution was added (100 μl/ml) to each of the cell suspensions for a final concentration of 1.05 μCi (1.5×10^6 cpm) per ml. Because of the ability of ^{67}Ga to bind to glass surfaces, total radioactivity was determined by sampling the entire mix. Duplicate 0.5-ml samples were removed from each incubation mixture at intervals, and the reaction was quenched by the addition of 2 ml iced 0.9% NaCl solution to each sample. The cells were pelleted in a Brinkman microcentrifuge and washed 3 times with iced 0.9% NaCl solution. There was no binding of gallium to the centrifuge tubes in the absence of cells.

Cell Fractionation. The cell fractionation procedure used is a modification of the method of Spitznagel *et al.* (22). Granulocytes were suspended in 20 ml glucose-Locke's solution at a concentration of 10^7/ml. To this suspension were added 40 μCi of ^{67}Ga, and the mixture was incubated at 37° for 1 hr under nitrogen. The cells were then washed 3 times, resuspended in 4 ml of 0.34 M sucrose per 0.1 ml of granulocyte pellet, and homogenized in a Dounce glass homogenizer. After centrifugation to remove unbroken nuclei and cells, the supernatant (postnuclear fraction) was layered over a linear sucrose gradient (16 ml of 30 to 53% sucrose) and centrifuged in a SW 27.1 rotor at 70,000 × *g* for 40 min at 4°. The gradient was then displaced from the tubes with 57% sucrose and collected in 0.7-ml fractions. The sucrose concentration of each fraction was determined on a Bausch and Lomb Abbe 3-L refractometer. Lysozyme was assayed by its lytic action on *Micrococcus lysodeikticus* as described by Gorin *et al.* (10) using chicken egg white lysozyme (Worthington Biochemical Corp., Freehold, N. J.) as a standard.

RESULTS

The results typically obtained by incubating human granulo-

[1] This work was supported in part by USPHS Grants CA13148, AI14148, CA16673, CA14148, and CA20709.

[2] To whom requests for reprints should be addressed.

[3] Present address: National Jewish Hospital and Research Center, Denver, Colo. 80206.

Received June 11, 1979; accepted September 12, 1979.

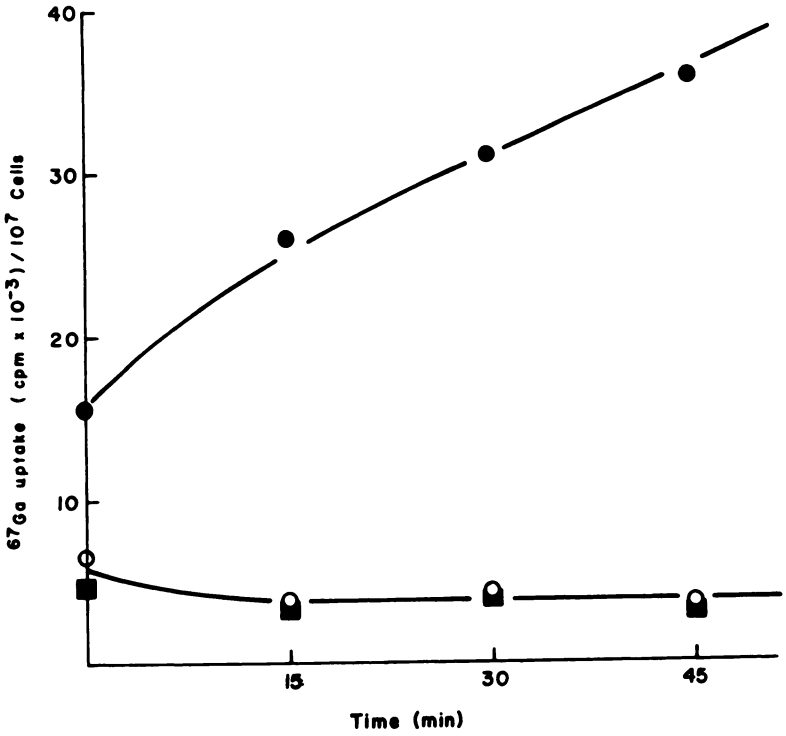

Chart 1. Uptake of ^{67}Ga by human granulocytes. Cells were maintained in either a nitrogen (●) or oxygen (○) atmosphere both before and after ^{67}Ga addition. Incubation conditions are described in the text. Data are the average of duplicate determinations. Also shown are the results of preincubating granulocytes under nitrogen for 30 min followed by uptake in an oxygen atmosphere (■). *Points* taken immediately after the addition of ^{67}Ga to the incubation media are plotted as occurring at 0 time.

cytes with ^{67}Ga are shown in Chart 1. Time-dependent uptake of the isotope occurred in granulocytes only when they were incubated under nitrogen. Although some radioactivity was associated with the cells when incubated in the presence of room air or oxygen, uptake was not time dependent. The rapidity of the initial rate of incorporation under hypoxic conditions and the method of sampling allowed a significant level of uptake at zero time, as is indicated by the higher level of radioactivity at the intercept under those conditions with which incorporation increased with time. Preincubation of granulocytes under nitrogen followed by incubation in an oxygen atmosphere after the addition of ^{67}Ga did not alter either the level or time course of ^{67}Ga uptake obtained by preincubating the cells in an oxygen atmosphere, contrary to what would be expected if uptake were related to cell death caused by anoxia. Normal peripheral human blood lymphocytes showed no time-dependent uptake of ^{67}Ga under either anoxic or fully oxygenated conditions.

Subcellular fractionation of granulocytes incubated with ^{67}Ga in the presence of nitrogen (Chart 2) showed the majority of radioactivity to be associated with those fractions containing lysozyme activity, a marker enzyme for lysosomal granules. Some radioactivity can also be found at the top of the gradient, which contains vesicles and the majority of the cell protein (22).

DISCUSSION

In the present study, we have used *in vitro* techniques to investigate the uptake of ^{67}Ga into human granulocytes and lymphocytes. The data presented show that granulocytes take up ^{67}Ga in a time-dependent manner only when incubated under nitrogen. Gallium appeared to be excluded from the cells when normal or above-normal oxygenation was used. Human lymphocytes showed no time-dependent uptake under either condition. These data suggest that leukocytes normally exclude the isotope. The ability of granulocytes to exclude ^{67}Ga has also been reported by Tsan *et al.* (25), who concluded that uptake is proportional to the number of dead cells present. It is possible that the time course of ^{67}Ga uptake observed in these studies coincides with the rate of a process such as cell death during the incubation period. However, if this were the case, preincubation of the cells in a nitrogen atmosphere would be expected to lead to an increase in the initial or zero time association of ^{67}Ga due to the increased number of "dead" cells. However, our data show that preincubation under nitrogen followed by incubation in oxygen does not increase the level of cell-associated ^{67}Ga over that found by preincubation under oxygen. These observations lead us to suggest that hypoxia may cause the membranes of both L1210 leukemic lymphoblasts and granulocytes to become permeant to gallium whereas, in their normal state, a transferrin-dependent process is the dominant mechanism of gallium accumulation (20).

Although we have previously failed to demonstrate a lysozomal localization of ^{67}Ga in the L1210 lymphoblast cells (8), granulocytes show a marked association of the isotope with fractions rich in lysozyme, an enzyme known to reside only in lysosomal granules of neutrophils. Examination of the subcellular distribution of the isotope in other cells has often demonstrated a marked affinity for lysosomes and lysosome-like granules (1, 12, 23). On the other hand, Merz *et al.* (19) proposed that cultured human lymphocytes bind gallium on their membranes as indicated by a lack of time-dependent uptake and the release of isotope by trypsinization. Studies by Sephton and Kraft (21) have confirmed the inability of cultured human lymphocytes to accumulate ^{67}Ga even when incubated in the presence of transferrin or after phytohemagglutinin stimulation. Likewise, we have also been unable to obtain ^{67}Ga

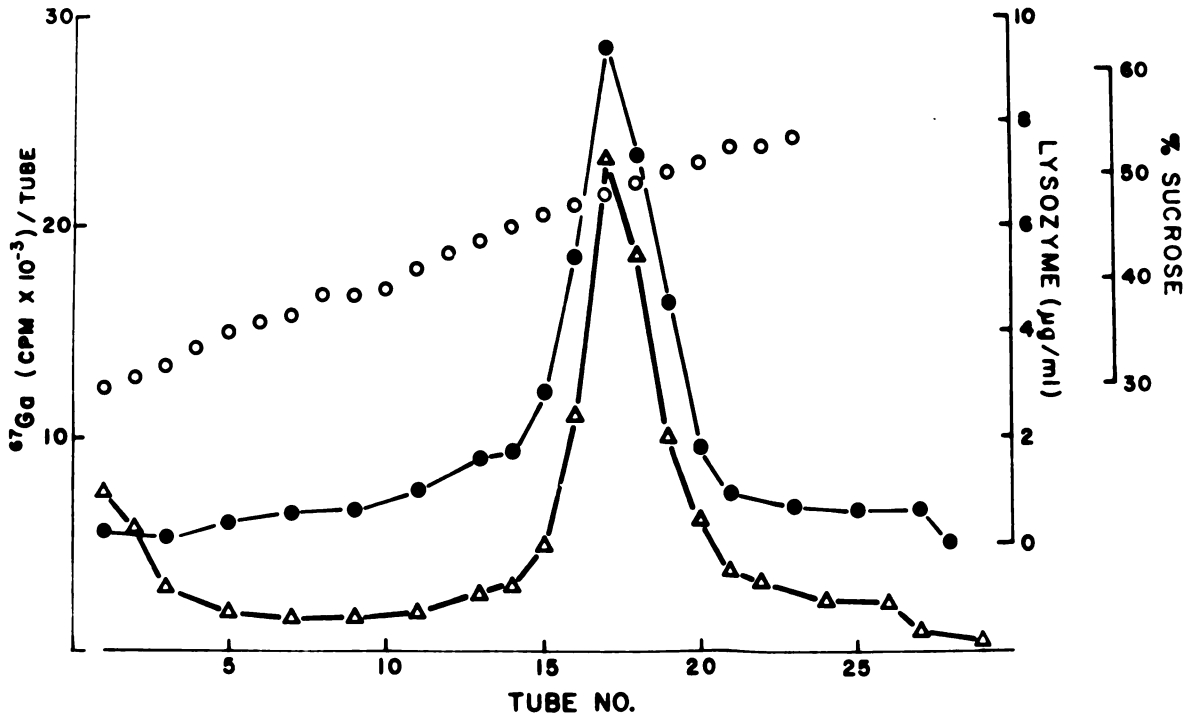

Chart 2. Fractionation of isolated human granulocytes by sucrose gradient centrifugation following incubation with ^{67}Ga for 2 hr under N_2 at 37°. △, total radioactivity in each tube collected after centrifugation; ○, percentage of sucrose; ●, lysozyme content expressed as the concentration of chicken egg white lysozyme that gives the equivalent level of activity.

accumulation with normal human lymphocytes even in a nitrogen atmosphere.

It has recently been noted that ^{67}Ga may accumulate in inflammatory lesions even in the absence of circulating granulocytes (5, 24). Tsan (24) noted gallium accumulation in a sterile inflammatory exudate in an animal that had been rendered agranulocytic by prior chemotherapy. Although these studies suggest that granulocytes may not be required for ^{67}Ga localization in inflammatory lesions, it certainly remains possible that sufficient granulocytes were present to migrate to the site of inflammation, undergo lysis, and discharge their lysosomal contents into the inflammatory exudate. The existence of gallium binders and their source in inflammatory exudates remain to be demonstrated.

In conclusion, it is proposed that granulocytes can incorporate ^{67}Ga under hypoxic conditions. It is therefore possible that the localization of gallium in regions of inflammation and in some tumors may be due to the presence of granulocytes in the localized hypoxic regions present in these lesions. Once within the cell, the largely irreversible binding of gallium by various proteins found in the lysosomal granules is likely to prevent its subsequent release from the cell (19), thereby accounting for an increased cellular level without the need for postulating an active accumulation process under anoxic conditions.

REFERENCES

1. Brown, D. H., Schwartzendruber, D. C., Carlton, J. E., Byrd, B. L., and Hayes, R. L. The isolation and characterization of gallium-binding granules from soft tumors. Cancer Res., *33:* 2063–2067, 1973.
2. Burleson, R., Johnson, M., and Head, H. Scintigraphic demonstration of experimental abscesses with intravenous 67-Ga citrate and 67-Ga labeled blood leukocytes. Ann. Surg., *128:* 446–450, 1973.
3. Burleson, R. L., Holman, B. L., and Tow, D. E. Scintigraphic demonstration of abscesses with radioactive gallium labeled leukocytes. Surg. Gynecol. Obst., *141:* 379–382, 1975.
4. De Land, F. H., Saverbrunn, B. J. L., Boyd, C., Wilkinson, R. H., Friedman, B. I., Moinuodin, M., Preston, D. F., and Kniseley, R. M. ^{67}Ga-citrate imaging in untreated primary lung cancer: preliminary report of cooperative group. J. Nucl. Med., *15:* 408–411, 1974.
5. Dhawan, V. M., Sziklas, J. J., and Spencer, R. P. Localization of Ga-67 in inflammations in the absence of circulating polymorphonuclear leukocytes. J. Nucl. Med., *19:* 292–294, 1978.
6. Edwards, C. L., and Hayes, R. L. Scanning malignant neoplasms with gallium-67. J. Am. Med. Assoc., *212:* 1182–1191, 1970.
7. Gams, R. A., Webb, J., and Glickson, J. D. Serum inhibition of *in vitro* ^{67}Ga binding by L1210 leukemic cells. Cancer Res., *35:* 1422–1426, 1975.
8. Glickson, J. D., Ryel, R. B., Bordenca, M. D., Kim, K. H., and Gams, R. A. *In vitro* binding of Ga-67 to L1210 cells. Cancer Res., *33:* 2701–2713, 1973.
9. Glickson, J. D., Webb, J., and Gams, R. A. Effects of buffers and pH on *in vitro* binding of 67-Ga by L1210 leukemic cells. Cancer Res., *34:* 2957–2960, 1974.
10. Gorin, D., Wang, S. F., and Papapalou, L. Assay of lysozyme by its lytic action on *M. lysodeikticus* cells. Anal. Biochem., *39:* 113–127, 1971.
11. Greenlaw, R. H., Weinstein, M. G., Brill, A. A., McBain, J. K., Murphy, L., and Kniseley, R. M. 67Ga-citrate imaging in untreated malignant lymphoma: preliminary report of cooperative group. J. Nucl. Med., *15:* 403–407, 1974.
12. Halbert, E., and Hambold, U. Isolation of the 67gallium accumulating fraction in normal rat liver. J. Nucl. Med., *13:* 72–84, 1974.
13. Harris, A. W., and Sephton, R. G. Transferrin promotion of 67Ga and 59Fe uptake by cultured mouse myeloma cells. Cancer Res., *37:* 3634–3638, 1977.
14. Johnston, G., Bencea, R. S., Teates, C. D., Edwards, C. L., and Kniseley, R. M. 67Ga-citrate imaging in untreated Hodgkin's disease: preliminary report of cooperative group. J. Nucl. Med., *15:* 399–403, 1974.
15. Johnston, R. B., Jr., Lehmeyer, J. E., and Guthrie, L. A. Generation of superoxide anion and chemiluminescence by human monocytes during phagocytosis and on contact with surface-bound immunoglobulin G. J. Exp. Med., *143:* 1551–1556, 1976.
16. Kay, D. N., and McCready, V. R. Clinical scanning using 67Ga-citrate in the management of Hodgkin's disease. Br. J. Radiol., *45:* 437–443, 1972.
17. Kumar, B., Alderson, P. O., and Geisse, G. The role of 67-Ga citrate imaging and diagnostic ultrasound in patients with suspected abdominal abscesses. J. Nucl. Med., *18:* 534–537, 1977.
18. McAfee, J. A., and Thacker, M. L. Survey of radioactive agents for *in vitro* labeling of phagocytic leukocytes. 1. Soluble agents. J. Nucl. Med., *17:* 480–487, 1976.
19. Merz, T., Malmud, L., McKusick, K., and Wagner, H. N. The mechanism of 67Ga association with lymphocytes. Cancer Res., *34:* 2495–2499, 1974.

20. Sephton, R. G., and Harris, A. W. Gallium 67 citrate uptake by cultured tumor cells stimulated by serum transferrin. J. Natl. Cancer Inst., *54:* 1263–1266, 1975.
21. Sephton, R. G., and Kraft, N. 67Ga and 59Fe uptakes by cultured human lymphoblasts and lymphocytes. Cancer Res., *38:* 1213–1216, 1978.
22. Spitznagel, J. K., Dalldorf, F. G., Leffell, M. S., Folds, J. D., Welsh, R. H., Cooney, M. H., and Martin, L. E. Character of azurophil and specific granules purified from human polymorphonuclear leukocytes. Lab. Invest., *30:* 774–785, 1974.
23. Swartzendruber, D. C., Nelson, B., and Hayes, R. L. Gallium 67 localization in lysozyme-like granules of leukemic and non-leukemic murine tissues. J. Natl. Cancer Inst., *46:* 941–952, 1971.
24. Tsan, M. F. Studies on gallium accumulation in inflammatory lesions: III. Roles of polymorphonuclear leukocytes and bacteria. J. Nucl. Med., *19:* 492–495, 1978.
25. Tsan, M. F., Chen, W. Y., Scheffel, V., and Wagner, H. N., Jr. Studies on gallium accumulation in inflammatory lesions. I. Gallium uptake by polymorphonuclear leukocytes. J. Nucl. Med., *19:* 36–43, 1978.