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Lysosomal Accumulation of ^{67}Ga–Transferrin in Malignant Tumors in Relation to Their Growth Rate

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Abstract—*The binding of ^{67}Gallium to serum and transferrin was investigated by Sephadex G50 gel filtration and cellulose acetate electrophoresis combined with autoradiography.*

The uptake of ^{67}Ga-labelled transferrin in the Morris hepatoma 5123 C, the Novikoff hepatoma and the Yoshida hepatoma AH 130 was studied. A direct correlation between ^{67}Ga-transferrin incorporation and growth rate of the tumors was evident. Increased ^{67}Ga-transferrin accumulation in the tumors of higher proliferation rate was demonstrated qualitatively by tumor scintigraphy as well as quantitatively by measuring the ^{67}Ga-transferrin activity per mg of wet wt tissue.

The intracellular localization of the incorporated ^{67}Ga-transferrin was investigated by isolation of subcellular fractions using differential centrifugation. Fractions were identified enzymatically. The maximum ^{67}Ga-transferrin activity was found within the lysosomes of the Morris hepatoma 5123 C, the Novikoff hepatoma, and the Yoshida hepatoma AH 130 very similar to the accumulation within the lysosomes of the hepatic cell.

Disappearance of ^{67}Ga-labelled transferrin from the blood was investigated in tumor bearing rats. The blood disappearance rate of ^{67}Ga-transferrin showed a direct correlation to the proliferation rates of the tumors. Furthermore, a close correlation between blood disappearance of ^{67}Ga-transferrin and the mass of tumor cells was evident.

The findings indicate the uptake of transferrin into the tumor cell by a process of endocytosis very similar to that found in the normal liver cell. The endocytotic incorporation of transferrin correlates to the proliferation rate of the tumors.

INTRODUCTION

THE PROGRESS of finding substances which can be used for an effective and more selective chemotherapy of malignant tumors requires a better understanding of the mechanisms of cellular uptake of tumoraffine agents and of the intracellular site of their action. Among several radiopharmaceuticals used in the detection of malignant tumors, carrier-free ^{67}Gallium citrate has attracted the most attention [1–3]. The uptake of this radiocompound in different malignant tumors has been described in numerous publications in the last few years [4–8]. However, it is still unclear what mechanisms are responsible for the higher relative uptake of ^{67}Ga by tumor tissues.

In previous reports, a differing intensity of ^{67}Ga accumulation in different types of malignant tumors was evident [9]. Furthermore, microscopic autoradiograms indicated that ^{67}Ga is mainly accumulated in viable tumor cells, and to a greater concentration at the periphery of the lesions than at the necrotic center [10]. Moreover, tumors treated by chemotherapy or irradiation showed a significant reduction in the accumulation of the isotope [11]. These phenomena and the mechanism of the interaction between radiogallium and the tumor cell was nevertheless poorly understood.

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Recent observations showed that ^{67}Ga [12–16]—very similar to scandium [17–19] and indium [20–23]—is bound to transferrin in the blood. There is also evidence that transferrin acts as a carrier in transporting the isotope into the cell. By electron microscopy and electron microscopic autoradiography, transferrin could be demonstrated within endocytotic vesicles of reticulocytes, which had been incubated with ^{125}iodine labelled transferrin [24–27]. In addition, reticulocyte fractionation studies have localized transferrin within the cell as well as on the surface membrane [28, 29]. It is thus concluded that transferrin binds itself to a surface membrane receptor of reticulocytes, and is subsequently internalized by a process of endocytosis. In previous fractionation studies of the normal rat liver, ^{67}Ga was found to be taken up by the hepatic cell through endocytosis and accumulated in the lysosomes [30, 31]. By immunoelectrophoresis combined with autoradiography, the intralysosomal ^{67}Ga binding protein could be identified as transferrin. The results suggest the presence of a transport mechanism for ^{67}Ga–transferrin uptake in the liver cell similar to that shown in reticulocytes [32].

The observation that serum transferrin concentrations are significantly lower in patients with different types of malignant tumors [33], raise the question that transferrin may be consumed by the malignant cells. The present study was undertaken to investigate the behaviour of ^{67}Ga-labelled transferrin in the blood of tumor bearing rats, and the uptake of ^{67}Ga-labelled transferrin in tumors of a different growth rate.

MATERIALS AND METHODS

Experimental tumors

The experiments were performed with control ACI rats, ACI rats with s.c. grown Morris hepatoma 5123C [34], Holtzman rats with s.c. grown Novikoff hepatoma [35], and Wistar–Nagoya rats with s.c. grown solid Yoshida hepatoma AH 130 [36, 37]. All the tumors being obtained from Hoechst AG (Frankfurt/Main). The average body wt of the rats was 180–220 g. Under aseptic conditions, the tumor cells were transplanted s.c by respectively using an aliquot of gently homogenized Morris and Novikoff hepatomas and 1 ml of Yoshida ascites cells. The Morris hepatoma grows very slowly to become a large tumor taking up 40–50% of the body wt. The animals usually die after 5–6 weeks. The Novikoff hepatoma grows moderately fast and the animals usually die after 3–4 weeks. Due to visible necrotic lesions in the center of the tumor, we used parts of the viable periphery for the experiments. After i.p. injection, the Yoshida hepatoma grows in ascites form. In contrast, after s.c. injection of the Yoshida ascites cells, a solid tumor grows very fast and aggressive and the animals die after about 10 days.

Sephadex G 50 column chromatography

Purified human transferrin (Behring Werke, Marburg/Lahn, Germany) was labelled with carrier free ^{67}Ga citrate (NEN, Boston, Massachusetts). The binding of ^{67}Ga to transferrin was checked by Sephadex G 50 gel filtration (Pharmacia, Uppsala, Sweden) and cellulose acetate electrophoresis (Sartorius, Göttingen, Germany). Half a ml (5 mg) of either ^{67}Ga labelled transferrin or ^{67}Ga labelled serum was applied to a column of Sephadex G 50 (18 cm length, 1 cm dia) equilibrated with isotonic saline of pH 7.2 and eluted with the same solvent in fractions of 8 drops. The protein and non-protein eluates were assayed for radioactivity and for protein concentration (extinction at 280 nm).

Cellulose acetate electrophoresis

Electrophoresis of ^{67}Ga labelled human transferrin and ^{67}Ga labelled human serum, respectively, was carried out in a horizontal tray using a 0.075 M sodium barbital buffer, pH 8.6. A current of approximately 20 mA was passed for about 30 min. The distribution of radioactivity in each sample after electrophoresis was determined by autoradiography of the unstained electrophoretic strip using an industrial X-ray film Kodak X-Omat S. Thereafter, electrophoretic strips were stained for protein with Ponceau-S red (Merck, Darmstadt, Germany).

Blood disappearance rate of ^{67}Ga–transferrin complex

Approximately 100 μCi of ^{67}Ga-transferrin was injected by cardiac puncture under light ether anesthesia. Blood samples (10 μl) were taken at 1, 3, 6, 12, 24 and 30 hr after injection, and radioactivity was measured in a Packard Autowell γ-Spectrometer.

Tissue distribution of ^{67}Ga–transferrin in tumor-bearing rats

Deposition of ^{67}Ga-transferrin in normal

and malignant tissues was assayed in untreated tumor-bearing rats and in rats pretreated by i.v. administration of 2 mg of iron by which a complete saturation of the circulating transferrin is attained. ^{67}Ga–transferrin was injected 30 min after iron pretreatment. ^{67}Ga–transferrin activity per mg of wet wt tissue was expressed as per cent of administered dose.

Tumor scintigraphy

Whole body scans were performed under light ether anesthesia 30 hr after the injection of ^{67}Ga labelled transferrin using a γ-camera (Nuclear-Chicago Pho/γHP).

Preparation of subcellular fractions

Thirty hr after injection of the isotope, rats were killed by decapitation and exsanguinated. A part of the liver and tumor, respectively, was removed and weighed. The preparation of liver and tumor tissue was performed under iso-osmotic conditions at 4°C according to previous reports [30]. The following subcellular fractions were isolated: crude nuclear fraction (0–700 ***g*** sediment), mitochondria (1500–6500 ***g*** sediment), lysosomes (6500–9000 ***g*** sediment), microsomes (10,000–40,000 ***g*** sediment), and cytoplasm (105,000 ***g*** supernatant) (Fig. 2). Aliquots of each fraction were used for enzymatic determinations and for the measuring of radioactivity.

Chemical analysis

Acid phosphatase (E.C. 3.1.3.2.) was measured as a marker enzyme for lysosomes [38, 39] according to Andersch *et al.* [40] and Fishman *et al.* [41]. Non-tartrate-inhibited acid phosphatase was determined in the presence of 15 mM sodium tartrate. Glucose-6-phosphatase (E.C.3.1.3.9.) was used as a marker enzyme for microsomes and endoplasmatic reticulum according to Swanson [42] and Hübscher and West [43].

Protein was determined by the method of Lowry *et al.* [44] with crystalline bovine albumin as a standard (Behring Werke, Marburg/Lahn, Germany). Specific activity (SA) is expressed as nanomoles of substrate consumed, or product formed per min per mg protein. Relative specific activity (RSA) is defined as the ratio of specific activity of the fraction/specific activity of the homogenate.

RESULTS

In vitro labelling of purified transferrin was performed with ^{67}Ga citrate. The binding of the tracer to transferrin was shown by Sephadex G 50 gel filtration. Figure 1 contains the results of Sephadex G 50 filtration of serum, transferrin, and saline containing ^{67}Ga (as the citrate). When ^{67}Ga in normal saline was filtered, ^{67}Ga radioactivity was eluted only in the later fractions (Nos. 18–28). When serum containing ^{67}Ga was filtered, the elution pattern of ^{67}Ga radioactivity paralleled that of purified tranferrin containing ^{67}Ga. In this case maximum radioactivity was eluted together with the 280 nm absorbing material in the early fractions (Nos. 10–12) where protein with mol. wt greater than 30,000 normally occurs. The binding of ^{67}Ga by transferrin was confirmed by cellulose acetate electrophoresis which revealed a migration of ^{67}Ga radioactivity within the β-globulin fraction of human and rat serum. This data agrees with the concept of a specific binding

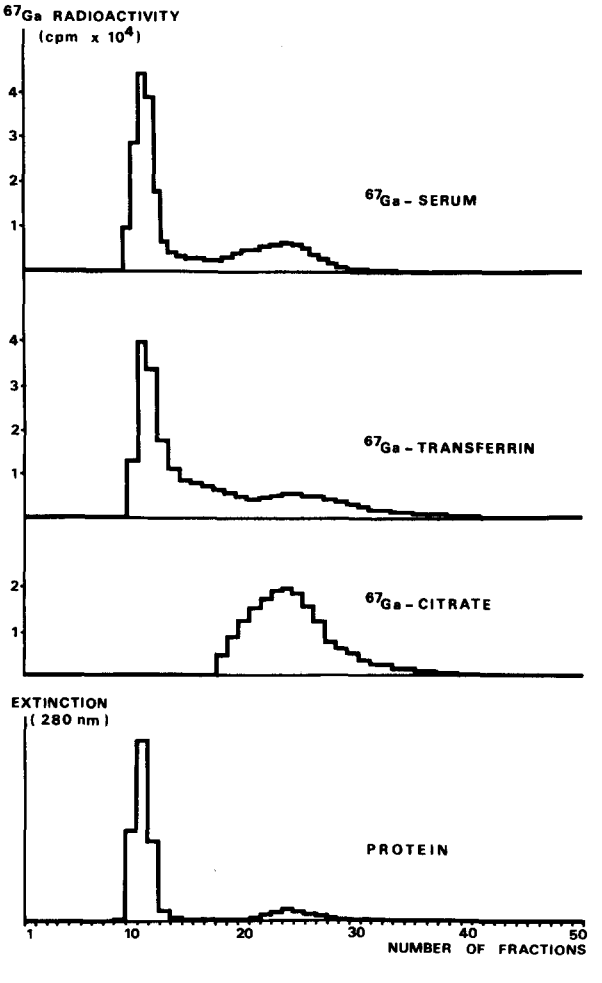

Fig. 1. *Sephadex G50 gel filtration of* ^{67}Ga *citrate and* ^{67}Ga *labelled human serum and purified transferrin.*

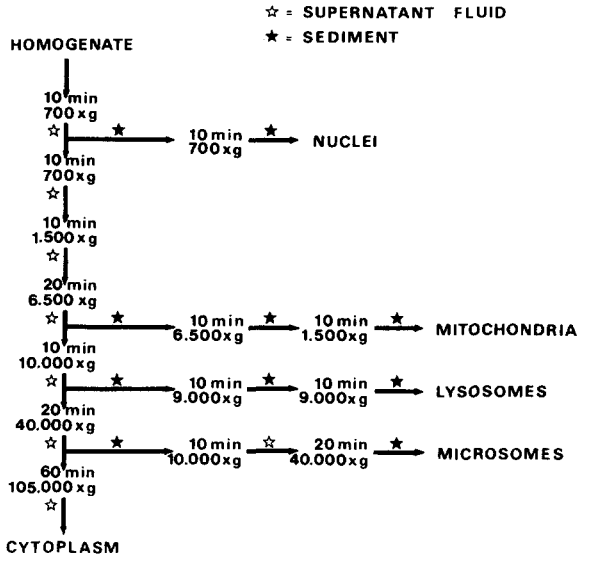

Fig. 2. Preparation of subcellular fractions by differential centrifugation.

of ^{67}Ga by transferrin *in vivo* and *in vitro* [12–16, 45]. Moreover the specific binding of ^{67}Ga to transferrin explains the identical results we obtained in whole body scintigraphy of tumor-bearing rats by using either ^{67}Ga citrate or *in vitro* labelled ^{67}Ga–transferrin.

In a first series of experiments, the intracellular site of the accumulated ^{67}Ga–transferrin within the tumor cell was investigated. Isolation of subcellular fractions was performed by differential centrifugation 30 hr after i.v. injection of ^{67}Ga–transferrin. Nuclei, mitochondria, lysosomes, microsomes and cytoplasm were isolated and identified enzymatically (Fig. 2). Lysosomes were identified by the determination of tartrate-sensitive acid phosphatase in the fractions. Contamination by microsomes and endoplasmatic reticulum was examined by controlling the glucose-6-phosphatase. The subcellular distribution pattern of ^{67}Ga–transferrin and tartrate-sensitive acid phosphate is shown in Fig. 3. The distribution pattern in the normal rat liver is demonstrated by the white colums. The subcellular distribution of ^{67}Ga–transferrin activity parallels that of tartrate-sensitive acid phosphatase. The highest specific activity of both ^{67}Ga–transferrin and tartrate-sensitive acid phosphatase was found in the lysosomes. Enrichment in this fraction was 10-fold and 12-fold, respectively. The subcellular distribution found in the Morris hepatoma 5123 C, Novikoff hepatoma and Yoshida hepatoma AH 130 resembled the distribution in the Normal liver. The maximum ^{67}Ga–transferrin activity as well as the maximum acid phosphatase activity was found within the lysosomes of the tumors. These results indicate that ^{67}Ga–transferrin is accumulated within tumor lysosomes very similar to the accumulation within the lysosomes of the hepatic cell. The identical intracellular site of ^{67}Ga–transferrin accumulation suggests the presence of a very similar mechanism of cellular ^{67}Ga–transferrin incorporation in the tumor as in the hepatic cell.

In a further series of experiments, we investigated whether the uptake of ^{67}Ga–transferrin by tumor cells results in a faster disappearance of the tracer from the blood. The disappearance of ^{67}Ga–transferrin from the blood was measured up to 30 hr after i.v. injection. The data shown in Fig. 4 are expressed as per cent of 0-time. The disappearance of the tracer from the blood shows a direct correlation to the rapidity of the tumor growth. In the normal rat, the biological half-life of ^{67}Ga–transferrin in the blood was found to be 3.9 hr. A significant more rapid disappearance was found in tumor bearing rats. In rats with a relatively slowly growing Morris hepatoma 5123 C, the biological half-life of ^{67}Ga–transferrin was 2.5 hr, and in rats with a moderately growing Novikoff hepatoma, 1.7 hr. The shortest disappearance half-time was found in rats with the rapidly growing Yoshida hepatoma AH 130 (0.9 hr). In these experiments, the tumor mass was approximately 15% of body wt. In a further series of experiments, ^{67}Ga–transferrin disappearance from the blood was measured in relation to the tumor mass. In Fig. 5, blood disappearance curves of ^{67}Ga–transferrin in rats with Morris hepatomas of different tumor sizes are shown. The correlation between blood disappearance of the isotope and the mass of tumor cells is evident. With the increasing tumor mass, a more rapid disappearance of ^{67}Ga-labelled transferrin from the blood takes place. A similar correlation was found in the Novikoff and Yoshida hepatomas (Fig. 6). The figure summarizes the dependence of blood half-life from both, the rapidity of tumor growth, and the mass of tumor cells. In our experiments, as can be seen in the figure, the largest tumor mass of the Morris hepatoma was 37% of body wt. In this animal, the half-life of ^{67}Ga–transferrin in blood was 1.2 hr. A comparable half-life time was found in rats with Novikoff hepatomas with a tumor mass of only 23% of body wt and in rats with Yoshida hepatomas with a tumor mass of only 15% of body wt.

Not only the disappearance of ^{67}Ga–transferrin from the blood but also the accu-

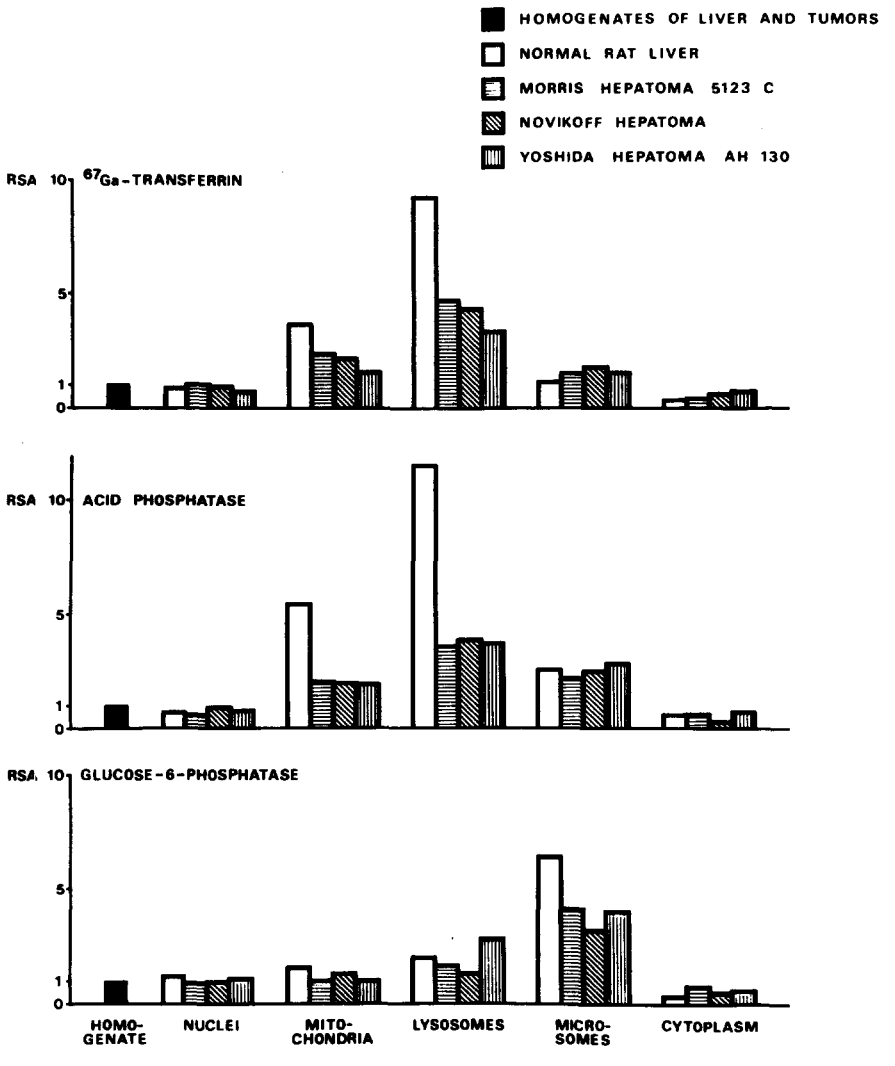

Fig. 3. Subcellular distribution of ^{67}Ga–*transferrin, acid phosphatase and glucose-6-phosphatase.*

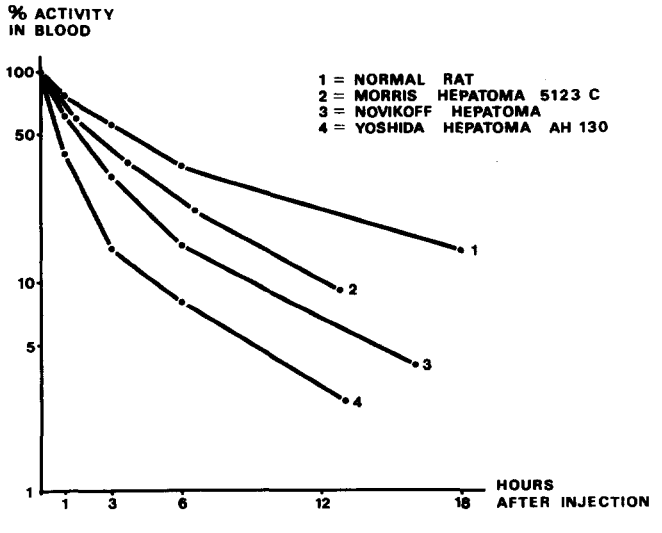

Fig. 4. Disappearance of ^{67}Ga–*transferrin from the blood in tumors of different malignancy (tumor mass* = 15% *of body wt).*

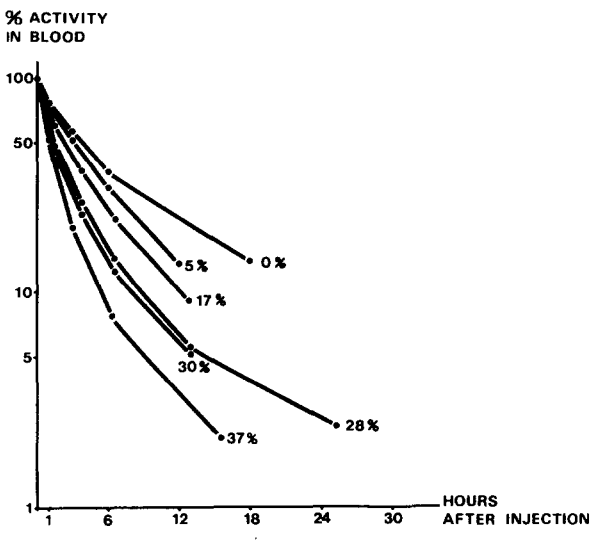

Fig 5. Disappearance of ^{67}Ga–transferrin from the blood in Morris hepatoma 5123 C related to tumor mass (% of body wt).

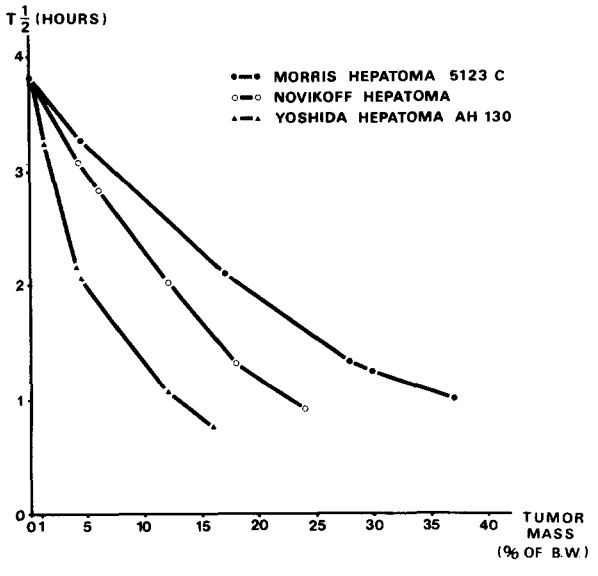

Fig. 6. Half-life ($T_{\frac{1}{2}}$) of ^{67}Ga-transferrin in blood related to tumor mass and malignancy of tumor.

mulation of ^{67}Ga–transferrin in the tumor tissue can be demonstrated qualitatively and quantitatively. Figure 7 shows ^{67}Ga–transferrin whole body scans of normal rat compared with rats with Morris hepatoma 5123 C, Novikoff hepatoma and Yoshida hepatoma AH 130. The whole body scan of the control rat shows the maximum activity of ^{67}Ga–transferrin in the liver. The second scan demonstrates a rat with a large Morris hepatoma. Besides the light radioactivity in the tumor, the pattern of the liver can still be marked off distinctly in the scan. The third scan demonstrates a rat with a Novikoff hepatoma. In this scan, a comparable accumulation of radioactivity can be seen in the tumor as well as in the liver. As is apparent in the fourth scan, the greatest ^{67}Ga–transferrin accumulation took place in the Yoshida hepatoma.

In order to investigate whether the unsaturated iron binding capacity (UIBC) influences the tissue distribution of i.v. injected ^{67}Ga–transferrin in tumor-bearing rats, deposition of ^{67}Ga–transferrin in normal and malignant tissues was assayed with and without pretreatment by i.v. administration of 2 mg of iron 30 min before injection of ^{67}Ga–transferrin. Figure 8 and Table 1 demonstrate

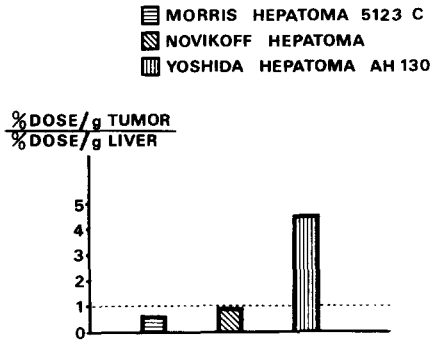

Fig. 8. Accumulation of ^{67}Ga–transferrin in tumor tissue related to accumulation in liver tissue.

the quantitative amount of ^{67}Ga–transferrin accumulation in the blood, in the liver tissue and in tissue of the Morris hepatoma 5123 C, the Novikoff hepatoma and the Yoshida hepatoma AH 130. Radioactivity was expressed as percentage dose per g of wet wt tissue. Since liver tissue accumulates ^{67}Ga–transferrin avidly [30, 31] the tumor to liver ratio of ^{67}Ga–transferrin accumulation was calculated. As seen before in the whole body scan, ^{67}Ga–transferrin accumulation in the Morris hepatoma was 0.67-fold less than the accumulation in liver tissue. In contrast, the accumulation in the Novikoff hepatoma was approximately the same as in liver tissue (0.9-fold). The accumulation in the Yoshida hepatoma exceeded 4-fold the accumulation of the isotope in liver tissue. This data indicates that there is a close positive correlation between lysomal accumulation of ^{67}Ga-labelled transferrin and the proliferation rate of tumor cells. The ^{67}Ga–transferrin concentration in the tumor tissue of rats pretreated with 2 mg of iron 30 min before application of ^{67}Ga–transferrin did not differ from that in rats without iron pretreatment (Table 1).

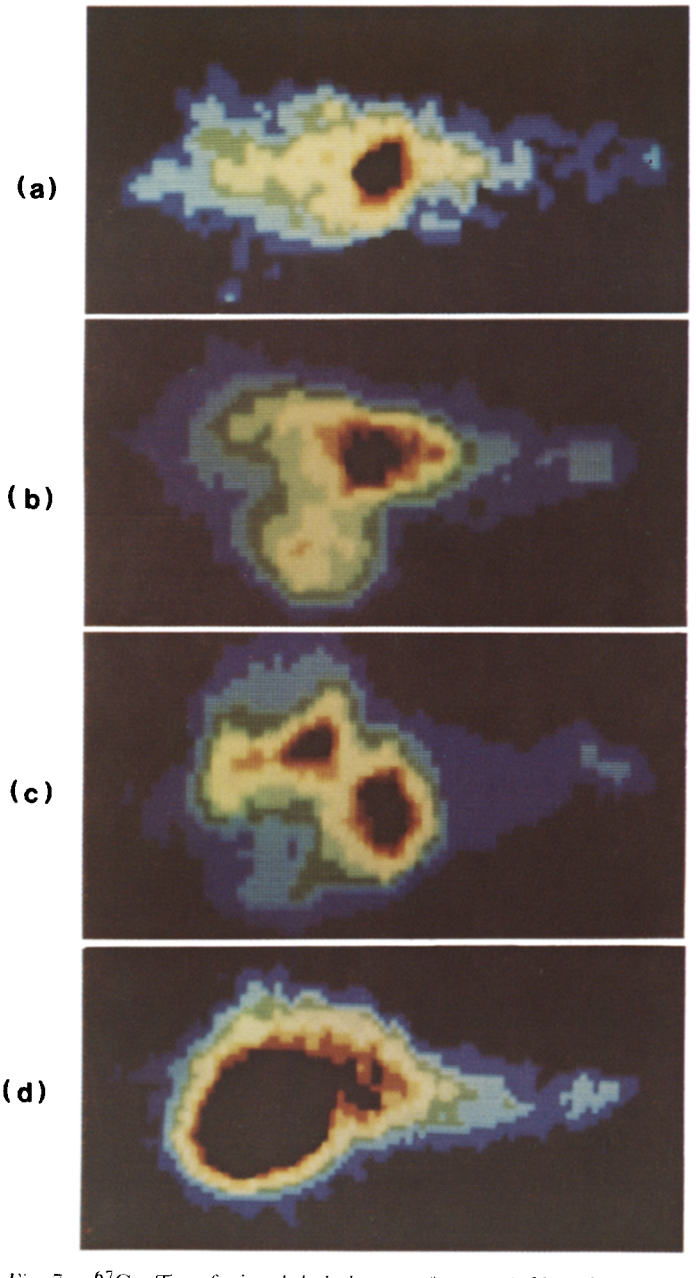

Fig. 7. ^{67}Ga-*Transferrin whole body scan of rats.* (*a*) *Normal rat;* (*b*) *Morris hepatoma* 5123 *C;* (*c*) *Novikoff hepatoma;* (*d*) *Yoshida hepatoma AH* 130.

Table 1. *Concentration of* ^{67}Ga–*transferrin in tissues of tumor-bearing rats* 30 hr *after injection*

	Blood (% dose/g)*	Liver (% dose/g)	Tumor (% dose/g)	Fe-pretreated Tumor (% dose/g)†	Ratio Tumor/liver tissue	Ratio viable/necrotic tumor
Morris hepatoma	0.36±0.08	4.33±0.8	2.92±0.5	2.89±0.7	0.67	1.9‡
Novikoff hepatoma	0.28±0.04	4.11±1.6	3.50±1.5	—	0.85	1.93
Yoshida hepatoma	0.16±0.09	3.50±0.5	13.86±2.8	13.98±2.8	4.00	1.92

*Values depend on tumor mass.
†2.0 mg Fe^{3+} were injected i.v. 30 min prior to ^{67}Ga–transferrin injection.
‡R. L. Hayes and C. L. Edwards, In *Medical Radioisotope Scintigraphy.* (IAEA) Vol. II, p. 531. Vienna (1973).

DISCUSSION

^{67}Gallium shows promise as a clinically useful agent in the detection of a variety of malignant tumors in animals and human beings [4–8]. However, it is unknown how and why malignant tumor cells incorporate ^{67}Ga faster and to a higher concentration than most normal cells do. There are various opinions about the mechanism involved in the uptake and concentration of the tracer in normal and tumor cells. Several studies have raised the possibilty that transferrin acts as carrier in transporting the tracer into the cell.

Binding of ^{67}Ga *to transferrin*

In earlier studies it was shown that ^{67}Ga binds to serum proteins *in vitro* [15]. The ^{67}Ga binding component was identified as transferrin by Gunasekera *et al.* [13] and confirmed in our laboratory [12] under *in vivo* and *in vitro* conditions. The kinetics of the binding of carrier-free ^{67}Ga to human transferrin were recently thoroughly investigated by Larson *et al.* [45]. The binding of ^{67}Ga to serum transferrin as well as to purified transferrin in our system is shown in Fig. 1. In recent investigations of liver parenchymal cells it could be demonstrated that ^{67}Ga is taken up by the cell through endocytosis and accumulated within the lysosomes [31]. Furthermore, it could be shown that the simultaneous administration of scandium at the mg/kg level, greatly decreases the incorporation of radiogallium in the liver cell by competition for ^{67}Ga–transferrin binding sites [17–19]. A similar effect was produced by stable gallium which greatly decreases the deposition of ^{67}Ga in all soft tissues, both normal and malignant [10, 46]. Alteration of ^{67}Ga–transferrin binding in the serum can also be observed by addition of iron. Thus, hyperferremia induced by whole body irradiation [47] or by treatment with *N*-acetylphenylhydrazine [48] produces a significant decrease of ^{67}Ga serum binding and ^{67}Ga uptake in tumor and liver tissue whereas urinary excretion of inorganic ^{67}Ga increased markedly [48, 49]. In the agreement with these observations of the relationship between ^{67}Ga–transferrin binding and ^{67}Ga tissue uptake it was recently reported, that the uptake of ^{67}Ga citrate and ^{59}Fe citrate by cultured mouse tumor cells was greatly enhanced with the addition of transferrin to the cultured medium [50–52]. This data indicates that transferrin probably causes the uptake of the ^{67}Ga by a process of endocytosis. This conception agrees with the fact that an increased endocytosis is a characteristic attribute of malignant cells [53–56]. This might be an explanation for the observation that malignant tumors incorporate ^{67}Ga faster and to a higher concentration than normal cells do. More recently Sephton *et al.* [57] reported data of differences in the tissue distribution of ^{67}Ga and ^{59}Fe. It must be considered that the behaviour of ^{59}Fe in different tissues reflects an overlap of iron metabolism and transferrin metabolism. It is worth noting that the main differences of ^{67}Ga and ^{59}Fe distribution were found in the blood cells and in the spleen. Therefore,—because of its inability to replace iron in specific metabolic pathways such as synthesis of heme and enzymes—^{67}Ga might preferentially reflect the fate of transferrin in the tissue. If these differences in the ^{67}Ga and ^{59}Fe distribution reveal different aspects of the interaction of transferrin with the cell or if they reflect more basic differences between the uptake mechanisms of ^{67}Ga and ^{59}Fe is not yet clear.

Lysosomal accumulation of ^{67}Ga–transferrin

In the normal rat liver cell, ^{67}Ga–transferrin could be seen to be accumulated within the lysosomes after being taken up through endocytosis [30, 31]. In histoautoradiographic studies, ^{67}Ga was found to be localized over electron dense lysosome-like cytoplasmic granules of murine leukemic cells [58, 59]. Our present investigation shows that the incorporated ^{67}Ga–transferrin is accumulated in the lysosomes of the Morris hepatoma 5123 C, the Novikoff hepatoma, and the Yoshida hepatoma, as well as in the lysosomes of liver parenchymal cells. The subcellular distribution pattern of ^{67}Ga–transferrin is very similar to that of tartrate sensitive acid phosphatase. The highest SA of both ^{67}Ga–transferrin and tartrate sensitive acid phosphatase was found in the same fraction. Since tartrate sensitive acid phosphatase is localized in the lysosomes [38, 39], we derive from our results that after cellular uptake, ^{67}Ga–transferrin is predominantly concentrated in the lysosomes (Fig. 3). In contrast, glucose-6-phosphatase activity, which was used as 'marker enzyme' for microsomes and endoplasmatic reticulum, showed maximal enrichment in the microsomal fraction. This indicates that the contamination of the lysosomal fraction by microsomes or endoplasmatic reticulum was negligible. As can be seen in the figure, the activities of ^{67}Ga–transferrin and acid phosphatase were found to be lower in the specific fractions of the three tumors, than in the normal liver. This probably is an artifact. It may be due to the increased permeability of the lysosomal membrane in the tumor cell, which leads to a loss of lysosomal enzymes during the washing procedure [38, 39, 60]. In addition, a main problem of the isolation of subcellular fractions is the homogenization technique. Preparation of the more fibrotic tumor tissue requires increased homogenization forces. Furthermore, tumor lysosomes are known to be more fragile. Thus, differences in particle size of the disrupted subcellular organelles, and consequently a slight shift in the sedimentation pattern, must be tolerated. Moreover, it can be considered that disseminated necrosis could be detected microscopically within the tumor tissue. It is known that the ^{67}Ga concentration and the enzyme content is greater in viable tissues than at the sites of necrosis [10]. In the Morris hepatoma 5123 C the viable-to-necrotic tissue ratio is 1.9 [46]. In the Novikoff hepatoma and in the Yoshida hepatoma AH 130 we found the same ratio of viable-to-necrotic tissue (Table 1). Since ^{67}Ga and enzyme activities within the isolated fractions depend highly on the grade of purification of the sediments, contamination by necrotic material results in impaired specific activities. Therefore, the aim of these subfractionation experiments can only be a qualitative statement of the subcellular localization of accumulated ^{67}Ga–transferrin.

Our data of lysosomal accumulation of ^{67}Ga–transferrin in Morris hepatoma 5123 C, Novikoff hepatoma and Yoshida hepatoma AH 130 agrees with the data reported by other authors who found ^{67}Ga to be localized in the lysosomes of different tumor models [58, 59]. The data correspond with the finding of Hayes [46], who noted that after centrifugation of various tumor homogenates 84–95% of the ^{67}Ga activity was spun down. They found after various membrane disruptive treatments that 65–70% of the sedimented ^{67}Ga activity was lost to the supernatant, but remained in macromolecular form and was not removed by dialysis or ultrafiltration. In contrast, Clausen *et al.* [16] found the major part of ^{67}Ga to be located in the crude nuclei fraction of a Hodgkin's granuloma and an anaplastic carcinoma. This may be caused by the crude isolation procedure in which purified fractions cannot be expected.

The role lysosomes play in malignancy is not yet understood completely. The activity of lysosomal enzymes is often significantly elevated in solid tumors as compared with their tissue of origin [61]. Higher enzyme levels of tumor lysosomes, and the release of lysosomal enzymes, appear to be associated with an increased invasiveness (malignancy) and mitotic index of tumors [62, 63]. The ease in which malignant cells can release their cytoplasmic enzymes appears to be related to differences in the composition and organization of the tumor membrane [61]. These differences could be related to differences in membrane fusion reactions in tumor cells, associated with increased endocytosis and exocytosis. They might also be related to differences in cell growth rate [61].

Disappearance of ^{67}Ga–transferrin from blood

In the last few years, numerous observations of evident differences in positive tumor imaging by ^{67}Ga citrate were reported [4–8]. One of the major differences between several types of malignant tissues is the growth rate. The finding of the lysosomal uptake of the ^{67}Ga–transferrin complex supports the idea

that differences in the accumulation of the tracer within the tumor are dependent on differences in tumor growth rate. However, a relation between the proliferation rate and endocytotic activity of tumor cells has not been described. Important for this question are recent reports that serum transferrin levels in patients with clinically active Hodgkin's disease are lower than in inactive disease, and increase to higher levels correlating with the return to the inactive state of the disease [64]. Corresponding changes of imaging of the Hodgkin's lesions by radiogallium are known [65–68]. From these reports, a correlation must be expected between the disappearance of the transferrin bound isotope from the blood, and the endocytotic activity and proliferation rate of tumor cells. Our studies were undertaken to evaluate whether the ability of cells to concentrate ^{67}Ga-labelled transferrin by endocytosis might be related to their proliferation rate, and whether this accumulation of ^{67}Ga-labelled transferrin within tumor cells results in a more rapid disappearance of the tracer from the blood. In our experiments, the very slow growing Morris hepatoma 5123 C [34], the moderate growing Novikoff hepatoma [35] and the rapid growing solid Yoshida hepatoma AH 130 [36, 37] were used. The time course of radioactivity in the blood was determined up to 30 hr after i.v. injection of the tracer. Disappearance of ^{67}Ga–transferrin from the blood showed a close correlation to the proliferation rate of the tumors (Figs. 4 and 6). As seen in the figure, in the normal rat, 40% of the initial level of the isotope was still in the blood 6 hr after i.v. injection of ^{67}Ga–transferrin. Whereas, at the same time of the initial level of ^{67}Ga–transferrin 21% in the Morris hepatoma, 18% in the Novikoff hepatoma, and only 7% in the Yoshida hepatoma could be measured. In these experiments, the mass of tumors was identical (approximately 15% of body wt). It can be expected that the blood disappearance rate of ^{67}Ga–transferrin is not only dependent on the activity of endocytosis of the tumor cell, but also on the mass of tumor cells. Correspondingly, a close correlation between blood disappearance of the isotope, and the amount of tumor cells could also be demonstrated (Figs. 5 and 6). In the Morris hepatoma, tumor sizes ranging from 5 to 37% of body wt were investigated. A shortening of the half-life of transferrin bound ^{67}Ga in blood was visible in tumors with a mass of at least 5% of body wt, with a more rapid occurance in larger tumor masses. In a tumor taking up 37% of the body wt, the biological half-life was reduced from 3.9 hr, in normal rats, to 1.0 hr. A similar correlation between blood disappearance of the tracer and the amount of tumor cells was seen in the Novikoff and Yoshida hepatomas (Fig. 6).

^{67}Ga–*transferrin accumulation in tumor*

The disappearance of ^{67}Ga–transferrin from the blood is an indirect parameter for the uptake of the tracer in the tumor tissue. In a further study, direct evidence of the increased ^{67}Ga–transferrin incorporation in tumors of higher proliferation rate could be demonstrated scintigraphically. Typical examples for the ^{67}Ga–transferrin whole body scans of rats with the three different tumors are shown in Fig. 7. Increased malignancy of the tumor corresponded with an increase of ^{67}Ga–transferrin accumulation in the tissue. In addition to these scans, which exhibit a qualitative information, the quantitative incorporation of ^{67}Ga–transferrin in the tumor tissue is demonstrated in Fig. 8 and Table 1. The data shows a comparison of the specific activities in blood, liver, and tumor tissue (percentage per g wet tissue). It can be noted that ^{67}Ga–transferrin concentration in the blood depends highly on the tumor mass—as can be derived from our blood clearance studies.

Whereas variations in the serum unsaturated iron binding capacity (UIBC) can lead to alterations of ^{67}Ga–serum-binding and ^{67}Ga–tumor-uptake when the ^{67}Ga is applied as the citrate [47–49], no alterations should be expected to occur when the ^{67}Ga is applied in the form of *in vitro* labelled ^{67}Ga–transferrin. This is because there is no significant redistribution of transferrin bound iron between saturated and unsaturated transferrin molecules [69–72]. Thus, we compared ^{67}Ga–transferrin tissue distribution of untreated tumor-bearing rats with rats pretreated with 2 mg of iron which causes a complete saturation of endogenous transferrin with iron. Since nearly all excess iron (which is not bound to transferrin) disappears from the plasma within a few minutes [73, 74], it can be expected that 30 min after pretreatment with excess iron no unbound ionized iron exists in the circulating blood which could replace ^{67}Ga from its transferrin binding site. Table 1 demonstrates that there was no difference in the ^{67}Ga–transferrin tumor uptake of iron-pretreated or untreated rats. We derive from this finding that the accumulation of *in*

vitro labelled ^{67}Ga–transferrin within the tumor cell is not influenced by the serum unsaturated iron binding capacity (UIBC).

Since the target to non-target ratio is the most important information for determining both the specificity of tumor imaging and the selectivity of tumor therapy and since liver tissue is known to accumulate ^{67}Ga–transferrin very avidly [30, 31], the tumor-to-liver ratio of ^{67}Ga–transferrin accumulation is demonstrated in Fig. 8 and Table 1. The tumor-to-liver ratio increased from 0.67-fold in the case of Morris hepatoma to 4-fold in the Yoshida hepatoma. This data indicates a positive correlation between the cellular incorporation of the ^{67}Ga–transferrin complex and the rapidity of tumor proliferation. This correlation might also be an explanation for the known variety of radiogallium incorporation of different types of tumors [9]. In the case of the Morris hepatoma 5123 C, Hayes *et al.* [46] describe a much higher tumor-to-liver ratio of ^{67}Ga incorporation (i.e., 7.3–16.0) than we found (i.e., 0.67). It is noteworthy that their absolute measurements of ^{67}Ga concentration in the tumor tissue (exhibiting a wide range from 4.2 to 10.0% dose per g) do not differ so much from ours (i.e., 2.9% dose per g). Their higher tumor-to-liver ratio is due to a much lower ^{67}Ga concentration in the liver tissue (0.3–0.6% dose per g) than we found in agreement with Sephton *et al.* [57] i.e., 4.3 and 4.5% dose per g, respectively. These differences might be explained by their use of ^{67}Ga in the citrate form which is dependent on the serum unsaturated iron binding capacity (UIBC) [47–49], or by possible differences in the course of ^{67}Ga–transferrin uptake in the liver and tumor tissue.

Our observation of a correlation between ^{67}Ga–transferrin incorporation and the proliferation rate of the tumor cell are confirmed by the findings that ascites tumor cells, shortly after their inoculation during the period of a high proliferative activity, show a considerably higher rate of radiogallium incorporation than they do after reaching their plateau phase with a decreased growth rate [75]. This data also compares to the recently reported findings in normal tissue that hyperactive bone marrow cells of daily bled mice showed highest radiogallium incorporation, while the depressed proliferation of hypoxia treated bone marrow cells by O_2 exposure resulted in a loss of affinity to ^{67}Ga [75, 76]. In addition, mammary glands of pregnant hamsters showed a 20 times greater ^{67}Ga incorporation than that in resting mammary glands [77].

The biological significance of transferrin in tumor growth and cell division is not clearly understood. Numerous studies, using cultured fibroblasts [78, 79], lymphocytes [80, 81] and spleen cells [82], indicate that the addition of low concentration of serum stimulates cell growth and DNA synthesis. In serum, transferrin could be identified as the factor which promotes cell growth and which stimulates DNA synthesis and cell division. As a comparison, inhibitors of endocytosis such as colchicine and vinblastine are also effective inhibitors of mitogenesis in neuroblastomas, mouse 3T3 cells and lymphocytes [83, 84]. As a result, transferrin seems to be required for the growing and dividing cell. These observations agree with our findings that tumor cells of a higher proliferation rate absorb ^{67}Ga-labelled transferrin very rapidly from the blood, and results in depressed serum transferrin levels as described in patients with different types of malignant tumors [33].

CONCLUSION

We conclude from our results: (1) After cellular uptake, ^{67}Ga-labelled transferrin is accumulated within the lysosomes in the tumor cell as well as in normal liver cell; (2) There is a close positive correlation between lysosomal accumulation of ^{67}Ga-labelled transferrin and rapidity of the tumor growth; (3) The disappearance of ^{67}Ga-labelled transferrin from the circulating blood depends on the rapidity of tumor growth and the mass of tumor cells.

The parallelism of blood disappearance rate and the cellular incorporation rate of the ^{67}Ga–transferrin, and the localization of the accumulated isotope within the lysosomes of tumor and liver cells, suggest a cellular uptake through endocytosis by the tumor cell as described previously in the hepatic cell. Nevertheless, a better understanding of the correlation between the rate of endocytosis of transferrin, and the rapidity of tumor growth demonstrated in our experiments, requires further investigations.

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