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Uptake of Ga-67 into rat cerebral hemisphere and cerebellum. Comparison with Fe-59.

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Transferrin and transferrin receptors play an important role in the transport of iron into the brain. To determine whether gallium enters the brain by the same mechanism, uptakes of ^{67}Ga and ^{59}Fe have been compared under controlled conditions. Rates of gallium penetration into brain (K_{in}) were four times slower than those for ^{59}Fe. K_{in} for ^{67}Ga when infused with citrate were 0.88 ± 0.24 and 0.94 ± 0.39 × 10^{-3} ml $g^{-1}h^{-1}$ for cerebral hemisphere and cerebellum, respectively. When infused as the transferrin complex, ^{67}Ga uptake into the brain was not different from that when infused with citrate. The presence of the anti-transferrin receptor antibody OX-26 significantly reduced uptake of ^{59}Fe by 60% and 64% into cerebral hemisphere and cerebellum, respectively. By contrast, pretreatment of rats with OX-26 enhanced the uptake of ^{67}Ga into brain, particularly when infused with citrate; mean increases in uptake of ^{67}Ga were 120% and 144% for cerebral hemisphere and cerebellum, respectively. Purified ^{67}Ga-transferrin was also taken up into both brain regions examined in the presence of OX-26. These results indicate that the transport of non-transferrin bound gallium is an important mechanism for gallium uptake into brain.

Keywords: blood–brain barrier, gallium, iron, transferrin, transferrin receptor

Introduction

Transport of gallium in the body in general, and into brain in particular, is of interest. Because of the lack of a readily available and easily employed radioisotope of aluminium, gallium has been considered (Ganrot 1986) and even used as a marker for transport of aluminium in the body, including the brain (Pullen *et al*. 1990). ^{67}Ga complexed with transferrin has potential as an agent for localising tumours (Chitambar & Živković 1987) and has been appraised as a possible radioactive metal for labelling transferrin in blood plasma and thus estimating capillary permeability and plasma volume (Brunetti *et al*. 1988, Otsuki *et al*. 1989). Gallium has also been employed therapeutically to lower the hypercalcaemia of malignancy (Warrell *et al*. 1986), although the mechanism of this action is unknown.

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In fact, although as the next metal of higher molecular number in Group III of the Periodic Table gallium has a number of physico-chemical similarities to aluminium, it is closer to iron in certain biochemical aspects. Its stability constants for combination with transferrin are almost as high at 20.3 and 19.3 (log *K*) as those for formation of ferric transferrins at 22.6 and 21.7, whereas those for aluminium transferrin are considerably lower at 12.9 and 12.3 (Martin *et al*. 1987); a similar situation exists for gallium and ferric citrates, the stability constants of which are again higher than that for aluminium citrate, but in this case separation of the constant for aluminium citrate from the other two is not so marked (Martell & Smith 1977, 1982, Smith & Martell 1989). The ability of ionic Fe to readily undergo oxido-reduction between Fe(II) and Fe(III) results in a much greater biological activity, unlike Ga or Al which are unable to change oxidative state.

Gallium in plasma, after sufficient exposure, is likely to be largely combined with transferrin, although in contrast to iron there seems to be a

sufficient labile fraction of low molecular weight to allow passage across porous artificial membranes, which retain protein (Raijmakers *et al.* 1992), and across all but the tightest capillaries (Brunetti *et al.* 1988, Otsuki *et al.* 1989). Such biological and physiological background has led us to investigate the uptake into brain of ^{67}Ga-citrate as a tracer, or with additional citrate, or as purified gallium-transferrin, when infused into the bloodstream of the rat, and the effects thereon of an antibody against the transferrin receptor. A comparison between the brain uptakes of ^{67}Ga and ^{59}Fe has also been made.

Materials and methods

Animal preparation

Animals used in this study were female Wistar rats (weight range 200–230 g). All procedures for the care and use of animals were performed within the terms of the appropriate licences from the United Kingdom Home Office. Rats were anaesthetised intraperitoneally (i.p.) with sodium pentobarbitone (30 mg kg^{-1}). Periodic top-up doses of diluted sodium pentobarbitone in saline (6 mg kg^{-1}) were administered to maintain the anaesthesia for up to 5 h. An endotracheal tube was placed into the trachea of each rat to maintain a patent airway, and the right external jugular vein and left femoral artery were cannulated with polyethylene tubing, containing heparinized saline (5 IU ml^{-1}). ^{67}Ga and/or ^{59}Fe were infused into the right external jugular vein for periods up to 5 h and blood samples were collected from the left femoral artery at periodic intervals.

Intravenous infusion and sampling procedure

20 μCi of ^{67}Ga-citrate (Amersham International, Little Chalfont, UK) was infused for periods up to 5 h along with non-radioactive gallium with additional sodium citrate in order to maintain total serum citrate levels of about 0.6 mM. In experiments where direct comparison between ^{67}Ga and ^{59}Fe brain uptakes was made, 20 μCi of ^{67}Ga-citrate (Amersham International) and 10 μCi of $^{59}FeCl_3$ (NEN/Dupont, Hounslow, UK) were simultaneously infused in physiological saline containing only tracer amounts of gallium and iron for periods up to 5 h.

In some experiments ^{67}Ga was infused as gallium-transferrin for 3.5 h. Gallium-transferrin was prepared using a modification of the reported method for labelling transferrin with ^{59}Fe (Cavill 1971). Commercially available apotransferrin (Sigma, Poole, UK) of 98% purity was saturated with gallium containing ^{67}Ga-citrate *in vitro*. Excess non-transferrin bound ^{67}Ga and unlabelled gallium were removed in an IRA-100 iron-exchange column (Sigma, Poole, UK).

All infusions were performed at a diminishing rate using a Harvard syringe pump, in order to maintain an approximately constant serum level of ^{67}Ga and ^{59}Fe. The infusion rate was 13.0 μl min^{-1} over the first 15 min, reduced to 6.5 μl min^{-1} during the next 45 min, and then reduced to 1.0 μl min^{-1} for the rest of the infusion. During each infusion, five to eight arterial blood samples (approximately 40 μl) were periodically collected into capillary tubes.

The uptake of ^{67}Ga and ^{59}Fe into rat brain was also measured in the presence of a monoclonal anti-transferrin receptor antibody OX-26 (Serotec, Kidlington, UK). OX-26 was administered as a single intravenous injection (25 mg per rat) 10 min before the start of the infusion. Previous data have shown that this dose is adequate to inhibit transferrin receptor mediated transport of iron (Ueda *et al.* 1993).

At the end of the infusion period, the vascular system was washed out with 1 mM EDTA-saline, immediately prior to the decapitation to remove any residual blood and any ^{67}Ga or ^{59}Fe that may have adhered to the luminal surface of the tissue capillaries (Bradbury & Deane 1986). Tissue samples were removed and immediately weighed.

^{67}Ga and ^{59}Fe analysis

The activities of ^{67}Ga and ^{59}Fe were measured in a Canberra Packard Cobra II Auto-gamma counter. Counts were corrected for background and isotope decay. In the case of the experiments where both ^{67}Ga and ^{59}Fe were infused together, ^{67}Ga counts were corrected for spillover from the ^{59}Fe window. ^{59}Fe was subsequently counted following the decay of the shorter half-life ^{67}Ga.

Citrate measurements

Citrate concentrations were determined using a Hitachi F-2000 fluorescence spectrophotometer by a standard enzymatic technique (Mollering & Gruber 1966, Warty *et al.* 1984).

Calculation and statistics

The uptake of ^{67}Ga and ^{59}Fe into the brain was expressed as a space (ml 100 g^{-1}) according to equation 1

$$\text{UPTAKE SPACE} = \frac{C_{\text{tis}} \cdot T}{\int_0^T C_{\text{ser}} \cdot \mathrm{d}t} \qquad (1)$$

where C_{ser} is the serum activity of the tracer (cpm ml^{-1}), C_{tis} is the tissue activity (cpm g^{-1}) and T is the total experimental time. The rate of ^{67}Ga and ^{59}Fe uptake into the brain with time was estimated by linear regression analysis where the slope represents the unidirectional transfer coefficient (K_{in} in ml g^{-1} h^{-1}) and the ordinate intercept represents rapidly equilibrating space for ^{67}Ga and ^{59}Fe in brain (in ml 100 g^{-1})

Results are presented as means ± SEM. Differences in the uptake into different tissues were tested for statistical significance by ANOVA, followed by Student's *t*-test (two-tailed) where appropriate. Slopes of the regression lines were tested for statistical significance using the two-tailed *t*-test.

Results

The serum profile of gallium and citrate during 3.5 h infusion of ^{67}Ga-gallium with citrate in two representative rats in the absence and presence of OX-26 is shown in Figure 1. Gallium concentrations were calculated assuming that the specific activity of ^{67}Ga in serum was the same as the known specific activity of ^{67}Ga in the infusion solution. OX-26 reduced the serum level of gallium to 1/10 of the level in the rat which was not treated with the antibody, although the citrate level remained unchanged.

The uptake of ^{67}Ga, in the presence of citrate, into cerebral hemisphere and cerebellum of the rat was linear with time (Figure 2), with K_{in} significantly different from 0 and significant rapidly equilibrating spaces at zero time (Table 1). The slopes of the regression lines for each brain region were not significantly different from each other.

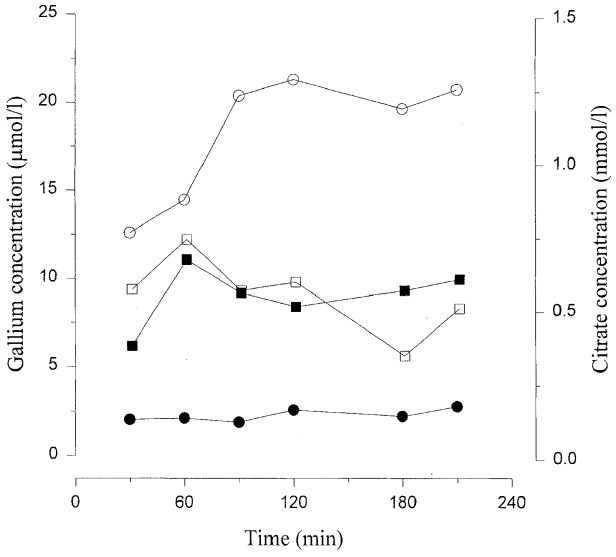

Figure 1. Serum profile of gallium (circles) and citrate (squares) during infusion of ^{67}Ga with citrate in the absence (open symbols) and presence (closed symbols) of anti transferrin receptor antibody OX-26 in two representative rats. Gallium concentrations were calculated on the assumption that the specific activity of ^{67}Ga in serum was the same as the known specific activity of ^{67}Ga in the infusion solution.

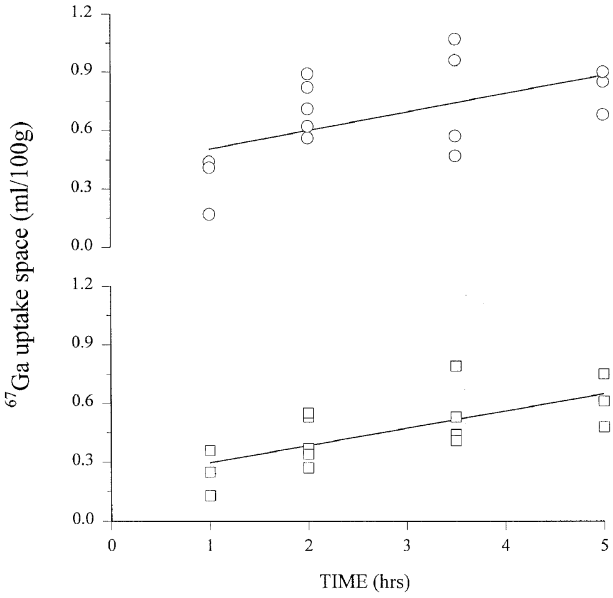

Figure 2. Uptake of ^{67}Ga, with citrate, into cerebral hemisphere (squares) and cerebellum (circles) of the rat with time. Each point represents one animal, $n = 15$.

The brain uptake spaces of ^{67}Ga at 3.5 h after infusion of ^{67}Ga with citrate and gallium-transferrin in the absence and presence of OX-26 are shown in Table 2. The uptake of ^{67}Ga, when infused with citrate, into cerebral hemisphere and cerebellum was significantly enhanced by the presence of OX-26. Purified gallium-transferrin was also taken into both brain regions in the presence of OX-26 and although uptakes of ^{67}Ga were higher in the presence of OX-26, there were not significantly different from purified gallium-transferrin only. The uptake of ^{67}Ga when infused with the citrate was higher in both brain regions than when infused as transferrin complex in both the absence and presence of OX-26. With the antibody, ^{67}Ga uptake spaces after citrate infusion were 143% and 144% greater for cerebral hemisphere and cerebellum, respectively than after infusion of the transferrin complex ($P < 0.025$).

The uptake of ^{67}Ga into rat cerebral hemisphere and cerebellum was compared with that of ^{59}Fe following the intravenous infusion of tracer amounts

Table 1. The rates of uptake (K_{in}; $\times 10^{-3}$ ml g^{-1} h^{-1}, slope ± SEM) and ordinate intercepts ± SEM (ml 100 g^{-1}) of ^{67}Ga-citrate into cerebral hemisphere and cerebellum of the anaesthetised rat ($n = 15$)

	Slope ± SEM	*P*	Intercept ± SEM	*P*
Cerebral hemisphere	0.88 ± 0.24	0.003	0.21 ± 0.08	0.018
Cerebellum	0.94 ± 0.39	0.030	0.41 ± 0.12	0.005

Table 2. Uptake of ^{67}Ga as a space (ml 100 g^{-1}), into rat cerebral hemisphere and cerebellum following 3.5 h intravenous infusion of ^{67}Ga with citrate or as gallium-transferrin, and the effect of pretreatment with the anti-transferrin receptor antibody OX-26. Values shown are means ± SEM. Differences between the control and antibody pretreated groups: $^{a}P < 0.05$; $^{b}P < 0.01$

	^{67}Ga-citrate ($n = 8$)		^{67}Ga-transferrin ($n = 10$)	
	Control	With OX-26	Control	With OX-26
Cerebral hemisphere	0.54 (± 0.08)	1.19 (± 0.11)^{b}	0.44 (± 0.09)	0.49 (± 0.06)
Cerebellum	0.77 (± 0.13)	1.88 (± 0.25)^{a}	0.48 (± 0.08)	0.77 (± 0.18)

of ^{67}Ga and ^{59}Fe in physiological saline (Figures 3 and 4; Tables 3 and 4). The uptake of ^{59}Fe and ^{67}Ga increased linearly with time in the rat cerebral hemisphere (Figure 3) and cerebellum (Figure 4), with K_{in} significantly different from 0 (Table 3). The ordinate intercepts for ^{67}Ga were significant at zero time whereas for ^{59}Fe they were not (Table 3). However, the uptake of ^{59}Fe into brain was significantly faster ($P < 0.01$) than the uptake of ^{67}Ga (Figures 3 and 4).

The effect of OX-26 on the uptake of ^{67}Ga and ^{59}Fe into rat cerebral hemisphere and cerebellum following a 3.5 h intravenous infusion of tracer amounts of ^{67}Ga and ^{59}Fe in isotonic NaCl is shown in Table 4. The uptake of ^{59}Fe was significantly inhibited by the presence of OX-26. Mean inhibitions due to OX-26 were 60% and 64% in cerebral hemisphere and cerebellum, respectively. By contrast with the inhibitory effect of OX-26 on the uptake of ^{59}Fe into brain, there was no significant effect of OX-26 on the uptake of ^{67}Ga into brain.

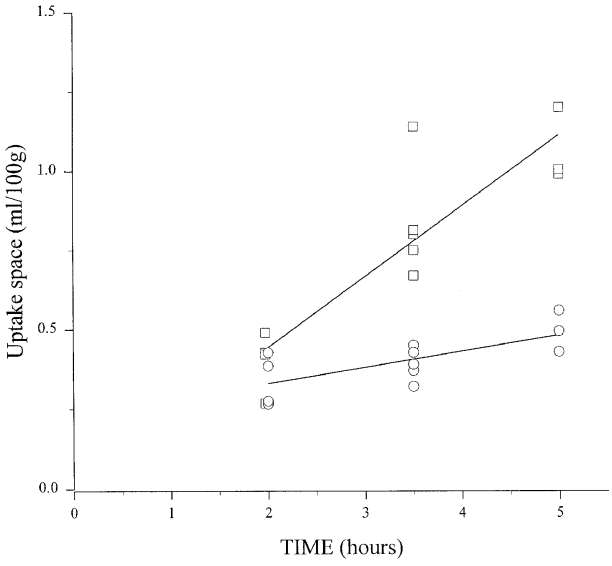

Figure 3. Uptake of ^{67}Ga (circles) and ^{59}Fe (squares) into rat cerebral hemisphere with time. Each point represents one animal, $n = 12$.

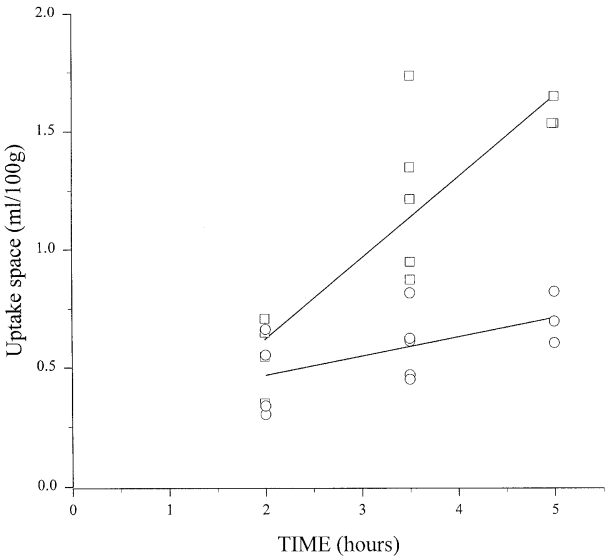

Figure 4. Uptake of ^{67}Ga (circles) and ^{59}Fe (squares) into rat cerebellum with time. Each point represents one animal, $n = 12$.

Discussion

The uptakes of manganese, lead, zinc and iron into brain from the blood are known to be linear with time when an approximately constant level of the trace metal is maintained in the blood by intravenous infusion (Bradbury 1992). However, the individual rates of entry (K_{in}) into brain are quite different for the different metals. For example, iron uptake into brain has a K_{in} of 5.2×10^{-3} ml g^{-1} h^{-1} whereas the K_{in} for $^{54}MnCl_2$ is around 300 times that for iron (Bradbury 1992). Accordingly, if gallium

Table 3. The rates of uptake of (K_{in}; $\times 10^{-3}$ ml 100 g^{-1} h^{-1}, slope ± SEM) and ordinate intercepts ± SEM (ml 100 g^{-1}) of ^{67}Ga and ^{59}Fe into cerebral hemisphere and cerebellum of the anaesthetised rat ($n = 12$).

	^{67}Ga		^{59}Fe	
	Slope ± SEM	Intercept ± SEM	Slope ± SEM	Intercept ± SEM
Cerebral hemisphere	0.51 ± 0.16	0.23 ± 0.06	2.25 ± 0.37	– 0.11 ± 0.13
	$P = 0.009$	$P = 0.003$	$P = 0.001$	$P = 0.934$
Cerebellum	0.81 ± 0.36	0.30 ± 0.13	3.42 ± 0.64	– 0.06 ± 0.23
	$P = 0.048$	$P = 0.038$	$P = 0.001$	$P = 0.787$

Table 4. Uptake of ^{67}Ga and ^{59}Fe, as a space (ml 100 g^{-1}), into rat cerebral hemisphere and cerebellum following 3.5 h intravenous infusion of tracer amounts of ^{67}Ga and ^{59}Fe in isotonic NaCl, and the effect of pretreatment with the anti-transferrin receptor antibody OX-26. Values shown are means ± SEM, $n = 5$. Differences between the control and antibody pretreated groups: [a]$P < 0.05$

	^{67}Ga		^{59}Fe	
	Control	With OX-26	Control	With OX-26
Cerebral hemisphere	0.39 (± 0.02)	0.42 (± 0.03)	0.83 (± 0.08)	0.34 (± 0.01)[a]
Cerebellum	0.59 (± 0.07)	0.67 (± 0.02)	1.22 (± 0.15)	0.44 (± 0.06)[a]

were transported into brain by the same mechanisms as iron, it would be predicted that its rate of entry into brain should be of a similar order of magnitude to that for iron (or less). Although the levels of ^{67}Ga in brain increased with time, its rates of uptake were four times lower than those of ^{59}Fe following infusion of ^{59}Fe-ferric chloride and around six times lower when compared with the K_{in} for ^{59}Fe following infusion of ^{59}Fe/ascorbate (Ueda *et al.* 1993). This lower uptake could reflect the lower affinity of transferrin for gallium compared with iron (see below) or it could indicate that gallium uses a different (or additional) uptake mechanism. Pullen *et al.* (1990) reported a K_{in} value of 1.5×10^{-4} ml g^{-1} h^{-1} for ^{67}Ga entry into rat brain, which is also much slower than the rate of entry for iron (Ueda *et al.* 1993). Iron is taken up into brain largely as a complex with transferrin, mediated by the transferrin receptor, and its uptake is markedly reduced in the presence of antibodies to the transferrin receptor as shown in the present study and by Ueda and associates (1993). It is not known in what form gallium is taken up into brain, or indeed what proportion of the total gallium in blood is associated with each of the putative ligands (transferrin, citrate and albumin). However, the *in vitro* affinity of transferrin for gallium is known to be much greater than the affinity for gallium of any of the other putative ligands in serum (Martin *et al.* 1987). Furthermore, the formation constants for Ga-transferrin are of a similar magnitude to those for Fe-transferrin (Harris & Pecoraro 1983, Martin *et al.* 1987). Thus, it is likely that a significant proportion of the gallium in blood would be complexed with transferrin. Since the trivalent ^{67}Ga ion cannot be reduced to a divalent ion, and readily forms poorly soluble hydroxides, it is likely to be much less mobile within endothelial cells compared with iron. Accordingly, the reduced uptake of gallium into brain may be due to retention of gallium within endothelial cells, such that it only slowly passes beyond the abluminal membrane to enter the brain interstitial fluid. However, ^{67}Ga when combined with transferrin, does appear able to exchange readily with a low molecular weight fraction in blood *in vitro* (Vallabhajosula *et al.* 1980, Raijmakers *et al.* 1992) and *in vivo* (Brunetti *et al.* 1988, Otsuki *et al.* 1989), and it is possible that non-transferrin bound gallium may also enter brain.

When gallium was infused as gallium-citrate, the rates of entry into brain were consistent with previously published K_{in} values for ^{67}Ga (Murphy & Rapoport 1992). However, the brain uptake space for ^{67}Ga infused as the citrate was around 40% higher than for ^{67}Ga-transferrin or tracer levels of ^{67}Ga, although this increase did not reach statistical significance. Greater uptake of ^{67}Ga administered with citrate compared with that of Ga-transferrin was potentially striking and significant in the

presence of the anti-transferrin receptor antibody. It is likely that much of the increased uptake is due to uptake of low molecular weight complexes, such as gallium-citrate, and is not transferrin-mediated. In other tissues where transferrin-mediated transport of gallium appears to dominate, the transport of low molecular weight gallium complexes is an important additional uptake mechanism (Radunović *et al*., in press) and this is likely to be the case in brain tissue as well.

A convenient strategy to highlight the importance of transferrin-mediated uptake of gallium into the brain is to investigate uptake in the presence of antibodies to the transferrin receptor. Administration of anti-transferrin receptor antibodies has been shown to markedly reduce ^{59}Fe transport into mouse and rat brain (Ueda *et al*. 1993). This was confirmed in the present study with OX-26 inhibiting iron uptake into rat brain. By contrast, we found that OX-26 increased the uptake of ^{67}Ga transport into rat brain. This effect on gallium uptake is not a peculiarity of rats and is also observed in mice following the administration of the RI7-208 anti-transferrin receptor antibody (Radunović *et al*. 1997).

Serum gallium concentrations were markedly reduced in the presence of OX-26, suggesting that gallium appears to be cleared more rapidly from the blood into tissues in the presence of the antibody. The uptake of ^{67}Ga into bone and renal cortex, where transferrin-mediated transport is not present or is very low, is increased in the presence of OX-26 (Radunović *et al*., in press) or RI7- 208 (Radunović *et al*. 1997), indicating an enhancement of a non-transferrin-mediated uptake mechanism. Other tissues, like skeletal muscle, may represent a sink for low molecular weight gallium complexes. The uptake of ^{67}Ga into skeletal muscle, where the uptake of ^{59}Fe is low and even lower than into the brain presumably due to few transferrin receptors on the skeletal muscle endothelium, is also substantially greater in the presence of OX-26 and RI7-208 (Radunović, 1994). The greater uptake into tissues in the presence of the antibody was observed both when gallium was infused as transferrin complex, although the reduction in serum gallium levels (1/3) was not as great as for ^{67}Ga-citrate (data not shown). Similarly, ^{67}Ga uptake into brain is also increased in hypotransferrinaemic mice which have very low serum levels of transferrin (Radunović *et al*, 1997). If it is non-transferrin bound ^{67}Ga that constitutes the largest pool that is transported into brain, the small increases in brain uptake of ^{67}Ga, when administered as ^{67}Ga-transferrin or at tracer levels of ^{67}Ga, could be attributed to a relatively low level of non-transferrin bound ^{67}Ga in these animals. The observation that OX-26 inhibits ^{67}Ga-transferrin uptake into rat brain endothelial RBE4 cells, where the composition of the bathing medium can be tightly controlled (Egleton *et al*. 1996), provides further evidence to suggest that the increased uptake of ^{67}Ga that occurs in the presence of the antibody *in vivo* must be due to non-transferrin mediated mechanisms. It is possible that the presence of the antibody to the transferrin receptor prevents the export of iron from the blood and may lead to a greater satura-tion of circulating transferrin with iron. This could reduce the number of sites available to bind other metals or displace other metals from transferrin, leading to a greater pool of non-transferrin bound metals. Following the endocytosis of the antibody–transferrin receptor complex into erythroblasts, the transferrin receptor has been shown to be trapped in the early endosome compartment due to the presence of the antibody (Killisch *et al*. 1992). Thus, the failure of apotransferrin to be recycled back to the plasma membrane in the presence of the antibody could reduce the level of circulating transferrin and effect the equilibrium between transferrin bound and non-transferrin bound metals in the blood.

In conclusion, these experiments indicate that the transport of gallium into rat brain occurs by a different mechanism from that of iron. Transport of non-transferrin bound gallium is likely to be an important mechanism for uptake of gallium into rat brain.

Acknowledgements

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