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Aluminum access to the brain: A role for transferrin and its receptor

(metal neurotoxicity/iron transport)

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ABSTRACT **The toxicity of aluminum in plant and animal cell biology is well established, although poorly understood. Several recent studies have identified aluminum as a potential, although highly controversial, contributory factor in the pathology of Alzheimer disease, amyotrophic lateral sclerosis, and dialysis dementia. For example, aluminum has been found in high concentrations in senile plaques and neurofibrillary tangles, which occur in the brains of subjects with Alzheimer disease. However, a mechanism for the entry of aluminum (Al^{3+}) into the cells of the central nervous system (CNS) has yet to be found. Here we describe a possible route of entry for aluminum into the cells of the CNS via the same high-affinity receptor–ligand system that has been postulated for iron (Fe^{3+}) delivery to neurons and glial cells. These results suggest that aluminum is able to gain access to the central nervous system under normal physiological conditions. Furthermore, these data suggest that the interaction between transferrin and its receptor may function as a general metal ion regulatory system in the CNS, extending beyond its postulated role in iron regulation.**

The major aluminum (Al^{3+}) binding fraction of plasma has been shown to be transferrin (Tf), the chief iron transport protein in vertebrates (1, 2). Tf has recently been shown to specifically bind Al^{3+} ions with a high affinity, approaching its affinity for iron (Fe^{3+}) (3–5). Tf also specifically binds a variety of other metal ions, such as gallium, indium, manganese, and zinc (6–8). Accordingly, we first investigated the binding kinetics for transferrin–iron (Tf–Fe^{3+}) in the central nervous system (CNS) to examine how cells in the brain may accumulate iron and regulate its content. Second, we examined the relationship between Tf–Fe^{3+} and Tf–Al^{3+} regulation in the brain at the level of the Tf receptor (TfR). Tf–Fe^{3+} normally enters tissues throughout the body by receptor-mediated endocytosis of the TfR–$(Tf)_2$ complex (1, 2, 9, 10). Tf receptors have been identified on the endothelial cells of brain vasculature (11–13) and a bidirectional transport of iron and Tf into and out of the brain has also been demonstrated (14). Histochemical, immunohistochemical, and autoradiographic studies have outlined a differential distribution of iron, Tf, and its receptor throughout the CNS (15–18).

Initially in this study, we characterize the CNS TfR of the adult rat by using ^{125}I-labeled Tf–Fe^{3+} (^{125}I-Tf–Fe^{3+}). Furthermore, Al^{3+} has been demonstrated to complex to specific binding sites on human Tf at physiological pH, and this association is ligand concentration dependent and reversible (3, 4, 19). We have extended these results to include rat Tf (see Fig. 1). Second, we iodinated rat Tf–Al^{3+} and used it in a variety of saturation binding techniques to demonstrate that Al^{3+} is capable of gaining access to the cells in the CNS via this Tf–TfR interaction under normal physiological conditions.

Previous studies on the putative brain TfR for Tf–Fe^{3+} have employed a variety of approaches, including *in vitro* autoradiography on frozen sections (15), cultured oligodendrocytes (18), and neuroblastoma cell lines (19). However, this study examined the kinetics of the brain TfR, which is found on membranes of a mixed CNS cell population. All binding experiments were performed with iodinated rat Tf on the rat tissue, in contrast to previous studies in which only human Tf was used in their binding experiments. Our initial competition experiments indicated that there may be a slight species difference between the interaction of rat and human Tf with this TfR.

MATERIALS AND METHODS

Ion Loading of Tf. Rat ApoTf (Sigma) is first rendered ion-free by reconstitution in an EDTA buffer at pH 5.0, dialysis for 6–8 hr in a Centricon-10 microconcentrator, and washing and resuspension in a 0.1 M Tris·HCl/10 mM $NaHCO_3$/100 mM NaCl buffer, pH 7.2. All plasticware used was first washed in EDTA buffer to minimize the exposure to environmental ions that could contaminate the preparation. In the loading procedure, $AlKSO_4$ was used as the source of Al^{3+} ions and $FeNH_3$ citrate was used as the source for Fe^{3+} ions. Ion loading, using a 5-fold molar excess of metal ion, was performed in a stepwise (pH 5–7.4) fashion. Unbound metal ions were then removed by dialysis against citric phosphate buffer. The Tf–Fe^{3+} and Tf–Al^{3+} complexes were iodinated by using a modified chloramine-T procedure (20) and remonitored spectrophotometrically to confirm that no dissociation of ions had occurred as a result of iodination of the Tf–metal complex (as tyrosine residues are involved in the active binding of ions to Tf). Fig. 1 shows the typical difference spectrum obtained when Al^{3+} is loaded onto Tf, and maxima at 238 and 285 nm are observed.

Receptor Binding Procedure. All conditions were defined empirically by time, temperature, tissue concentration, and ligand concentration and were held constant for both Tf–Fe^{3+} and Tf–Al^{3+} experiments. Briefly, whole rat brain (including pons and cerebellum) (1:5, wt/vol) was homogenized in 100 mM Hepes/250 mM sucrose/150 mM NaCl, pH 7.4, and centrifuged at 1000 × *g* for 10 min. The supernatant was centrifuged for 15 min at 12,000 × *g* and resuspended in an equal volume of oxygenated Krebs–Ringer buffer for a repeat centrifugation; the resuspended pellet was then analyzed for protein content. The incubation conditions were determined empirically as follows: 100 μg of membrane protein (verified by electron microscopy) was incubated for 1 hr at room temperature (22°C) in a 200-μl total incubation volume. All

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Abbreviations: Tf, transferrin; TfR, Tf receptor; CNS, central nervous system.

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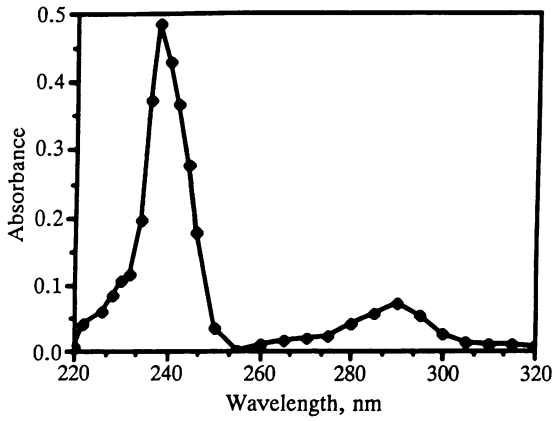

FIG. 1. Saturation of Tf with either Al^{3+} or Fe^{3+} is monitored by difference spectroscopy (against ApoTf, ion-free Tf) at 465 nM (Tf–Fe^{3+}) and 238 nM (Tf–Al^{3+}). This spectrum (320–220 nm) indicates peaks at 238 and 285 nm, indicative of Tf–Al^{3+} binding (peak at 238 nm is not seen with Tf–Fe^{3+}). Preparation of Tf–metal ion complexes (Tf–Al^{3+} and Tf–Fe^{3+}) is according to established methods (3, 4, 8).

binding experiments utilized a range of concentrations of 0.1–20 nM ^{125}I-Tf and 150–200,000 cpm per incubation. A 1000-fold excess of unlabeled Tf was used to determine nonspecific binding. All buffers contained 10 μM leupeptin and 1 mM phenylmethylsulfonyl fluoride. Binding reactions were terminated by the addition of a 10 times vol excess of ice-cold 50 mM Hepes. After incubation for 15 min at 4°C the samples were centrifuged at 15,000 × g for 15 min at 4°C. Pellets were then excised and assayed in a gamma counter (Micromedic Assay CompuCenter) and analyzed for fmol of ^{125}I-Tf bound per mg of membrane protein. All saturation data were directly plotted and then analyzed by the method of Scatchard and appeared to fit a classic one-site binding model (21). The ^{125}I-Tf bound when these procedures were used could not be displaced by addition of a 1000-fold excess of bovine serum albumin or bovine immunoglobulin, but it could be displaced by a 1000-fold excess of either rat or human Tf. The data presented are the means of data from all animals used.

Dissociation of Binding. Competitive displacement assays utilized a 1-hr incubation with saturating levels of ^{125}I-Tf–Fe^{3+} or ^{125}I-Tf–Al^{3+} followed by the addition of 10 μM unlabeled Tf for a period ranging from 2 min to 1 hr before termination of the binding reaction.

RESULTS AND DISCUSSION

The saturation data for Tf–Fe^{3+} (n = 10 animals; means of all data) indicate that the TfR of the adult rat brain has a K_d of 5.7 nM and a B_{max} of 59 fmol per mg of membrane protein when ^{125}I-Tf–Fe^{3+} was used as a ligand (Fig. 2a). Saturation experiments with Tf–Al^{3+} (n = 8 animals; means of all data) indicate that the K_d of the TfR increases to 13.1 nM with a B_{max} of 49 fmol per mg of membrane protein when ^{125}I-Tf–Al^{3+} was used as a ligand (Fig. 2b).

The relatively lower affinity of Tf–Al^{3+} (compared to Tf–Fe^{3+}) for the Tf receptor is still, however, higher than the affinity of Tf–Fe^{3+} for the receptor in a wide range of other tissues (e.g., hepatocytes, K_d 80 nM; myotubes, K_d 37 nM; lymphocytes, K_d 15 nM) (2, 10). The affinities for Tf–Al^{3+} and Tf–Fe^{3+} are lower but within the range of those reported earlier for the brain TfR (1.0 nM) (15). These different affinities probably reflect a difference in technical approach (including the use of rat instead of human Tf) but are in agreement that the brain TfR has a very high affinity for both Tf–Fe^{3+} and Tf–Al^{3+}.

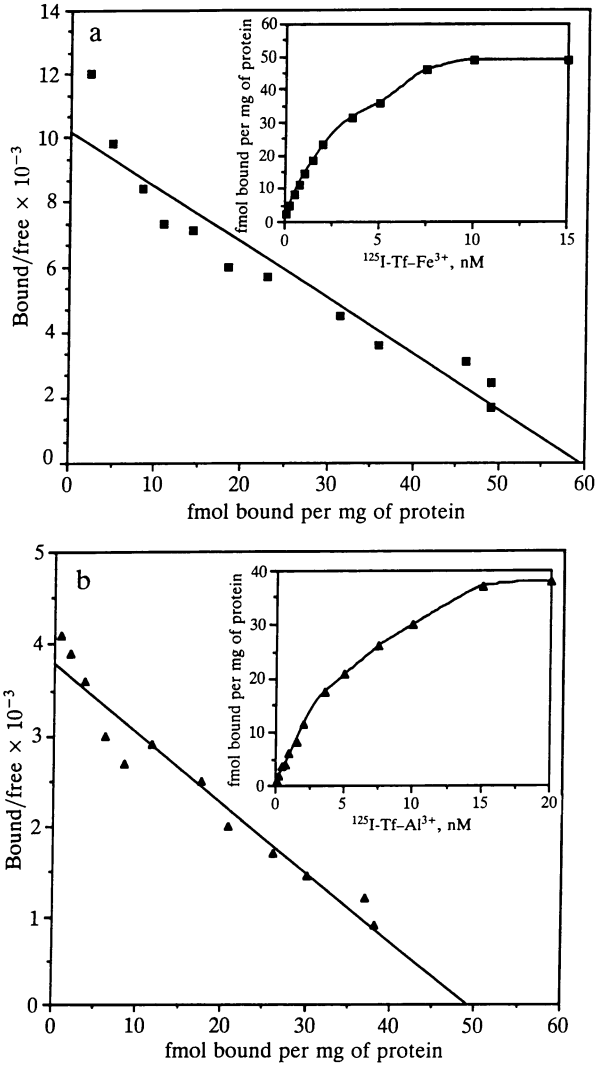

FIG. 2. All binding experiments were performed on a P2 fraction of rat brain. Each point represents the mean of all experiments (a, n = 10; b, n = 8). The average variations (percent standard error) for each mean were 25% (a) and 33% (b). (a) Scatchard plot and saturation plot (*Inset*) for TfR–Tf–Fe^{3+} interaction reveal a K_d of 5.7 nM with a B_{max} of 59 fmol per mg of protein (r = −0.94). (b) Scatchard plot and saturation plot (*Inset*) for TfR–Tf–Al^{3+}. The K_d for the TfR–Tf–Al^{3+} interaction was found to be 13.1 nM with a B_{max} of 49 fmol per mg of protein (r = −0.97).

Because of reports (8) that some Tf complexes—e.g., gallium–Tf (Ga–Tf)—could disrupt normal iron regulatory processes by binding in a relatively nondisplaceable manner with the TfR, we chose to examine whether this could also be the case for Tf–Al^{3+}. This approach would enable us to determine true equilibrium kinetics between the two ligands (Tf–Al^{3+} and Tf–Fe^{3+}) and the brain TfR. The first part of this study demonstrated a differential affinity of the brain TfR for Tf–Fe^{3+} and Tf–Al^{3+}; the second part was to determine that these ligands were, indeed, interacting with the same receptor. A series of displacement experiments were performed, and a time course was plotted for the dissociation of the labeled Tf–Fe^{3+} or Tf–Al^{3+} from the receptor (Fig. 3). Controls in which $KAl(SO_4)_2$ or ferrous ammonium citrate was used alone (not coupled to Tf) showed no displacement of labeled Tf from the receptor, indicating that the ion source alone did not interfere with the Tf–TfR interaction. The results indicate that the interactions of both Tf–Al^{3+} and Tf–Fe^{3+} with the receptor are completely reversible over the time periods indicated. (Incomplete reversibility was noted

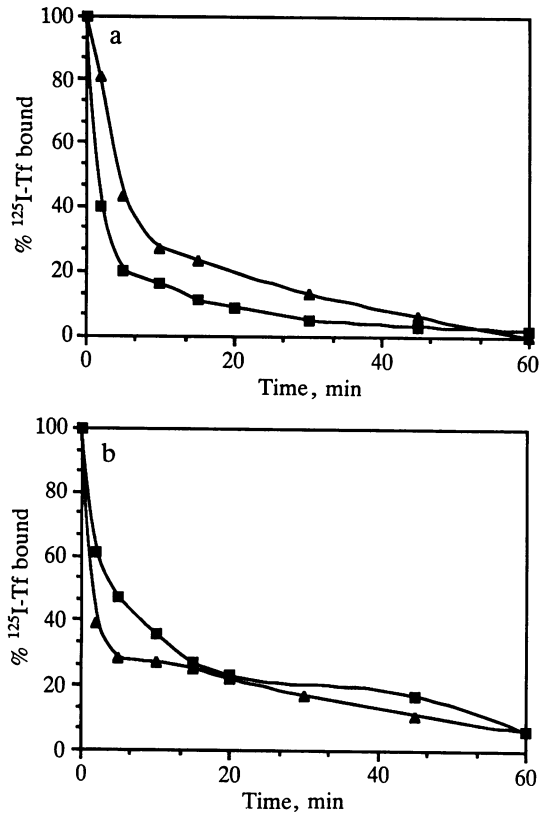

FIG. 3. Each point represents the mean of all experiments (n = 6 in both groups), and the standard error was generally 8% of the mean (both groups). (a) Displacement of ^{125}I-Tf–Al^{3+} (▲) and ^{125}I-Tf–Fe^{3+} (■) following the addition of a 1000-fold excess of unlabeled Tf–Fe^{3+}. (b) Displacement of ^{125}I-Tf–Al^{3+} (▲) and ^{125}I-Tf–Fe^{3+} (■) following the addition of a 1000-fold excess of unlabeled Tf–Al^{3+}. Both unlabeled Tf–Fe^{3+} and unlabeled Tf–Al^{3+} proved equally effective at displacing the iodinated ligands completely within 60 min.

when unlabeled ApoTf was used to displace either labeled Tf–Al^{3+} or Tf–Fe^{3+}.) The identical nature of the displacement rate for both Tf–Al^{3+} and Tf–Fe^{3+} demonstrates that they are acting interchangeably with the same receptor.

Collectively, these results demonstrate that cells in the brain possess a specific high-affinity receptor for Tf that is independent of the metal being transported. This Tf–TfR system is postulated to be the route whereby the brain can access iron from the general circulation to meet its high metabolic requirements. However, it is generally accepted that only 30% of the ion binding sites that plasma Tf has available in the circulation are saturated with iron at any time (4), leaving the remaining 70% available to other ions. Analysis of batches of commercially available "iron saturated" Tf by this laboratory and others has also demonstrated that up to 30% of these binding sites are actually occupied by Al^{3+} (3). The results presented in this paper demonstrate that a metal ion other than iron is capable not only of binding to Tf but also of utilizing this interaction to gain access to cells in the brain via the Tf–TfR system. Although our work demonstrates a normal physiological path for Al^{3+} entry into the brain, it does not directly address the controversial role of Al^{3+} in neurodegenerative disorders (22–24). However, it does suggest that Al^{3+} may be capable of interfering with normal cellular iron homeostasis and could disrupt iron-dependent cellular processes (e.g., oxidative phosphorylation) in the CNS. In this regard, it is interesting to note that the chief iron storage protein, ferritin, when isolated from the brains of Alzheimer disease subjects, has a 6-fold higher Al^{3+} content than normal age-matched controls (25). This latter observation further suggests that aluminum is accessing the same cellular regulatory routes as iron. Recent studies on the ^{67}Ga binding activity of the plasma Tf obtained from Alzheimer and Down syndrome patients have also suggested that altered Tf–ion binding activity may play a role in differential metal ion access to the brains of these patients (26).

CONCLUSION

In conclusion, because of the number of recent studies noting the relatively high affinity of Tf for a variety of metal ions, it would appear that Tf may have been defined as an iron transporter protein mainly because of the relatively high abundance of iron in the circulation in relation to these other ions. If this balance were to change because of some environmental, nutritional, or disease factor, Tf could transport these other metal ions and deposit them intracellularly instead of or in addition to iron. Because CNS neurons (in which Al^{3+} accumulation is most marked) are terminally differentiated, the Al^{3+} transported into these cells will accumulate unless specific systems are available to remove them. Other tissues that access circulatory Tf have a set turnover rate, and metal ion accumulation over time will not be so pronounced. The results of this study thus provide the baseline data for a Tf-specific, but not an iron-specific, system by which metal ion regulatory processes in the normal brain may be examined. A major criticism of the aluminum hypothesis in neurodegenerative disorders, regarding the relative inaccessibility of aluminum to the brain, is now seriously questioned.

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