Amino acid domains control the circulatory residence time of primate acetylcholinesterases in rhesus macaques (*Macaca mulatta*)

Ofer COHEN, Chanoch KRONMAN, Baruch VELAN and Avigdor SHAFFERMAN¹

Department of Biochemistry and Molecular Genetics, Israel Institute for Biological Research, Ness-Ziona 74100, Israel

An array of 13 biochemically well defined molecular forms of bovine, human and newly cloned rhesus macaque (*Macaca mulatta*) AChEs (acetylcholinesterases) differing in glycosylation and subunit assembly status were subjected to comparative pharmacokinetic studies in mice and rhesus macaques. The circulatory lifetimes of recombinant bovine, macaque and human AChEs in mice were governed by previously determined hierarchical rules; the longest circulatory residence time was obtained when AChE was fully sialylated and tetramerized [Kronman, Chitlaru, Elhanany, Velan and Shafferman (2000) J. Biol. Chem. 275, 29488–29502; Chitlaru, Kronman, Velan and Shafferman (2001) Biochem. J. 354, 613–625]. In rhesus macaques, bovine molecular forms still obeyed the same hierarchical rules, whereas primate AChEs showed significant deviation from this behaviour. Residence times of human and rhesus AChEs were effectively extended by extensive sialylation, but subunit tetramerization and N-glycan addition had a marginal effect on their circulatory longevity in macaques. It appears that the major factor responsible for the differential pharmacokinetics

of bovine and primate AChEs in macaques is related to differences in primary structure, suggesting the existence of a specific mechanism for the circulatory clearance of primate AChEs in rhesus macaques. The 35 amino acids that differ between bovine and primate AChEs are clustered within three defined domains, all located at the enzyme surface, and may therefore mediate the facilitated removal of primate cholinesterases specifically from the circulation of monkeys. These surface domains can be effectively masked by poly(ethylene glycol) appendage, resulting in the generation of chemically modified human and macaque AChEs that reside in the circulation for extraordinarily long periods of time (mean residence time of 10 000 min). This extended residence time is similar to that displayed by native macaque butyrylcholinesterase (9950 min), which is the prevalent cholinesterase form in the circulation of adult macaques.

Key words: acetylcholinesterase, N-glycan, pharmacokinetics, poly(ethylene glycol), protein oligomerization, sialic acid.

INTRODUCTION

AChE (acetylcholinesterase; EC 3.1.1.7) plays a pivotal role in the cholinergic system, where it functions in the rapid termination of nerve impulse transmission. AChE, unlike the related BChE (butyrylcholinesterase) which is abundant in the serum of adult primates, is found preferentially in insoluble, cell-associated forms [1]. Previous studies suggest that exogenous cholinesterase can serve as an effective therapeutic agent in the prophylaxis and treatment of organophosphate poisoning. Indeed, the successful exploitation of the scavenging potential of administered cholinesterase has been demonstrated in rodents and in non-human primates [2–5]. The use of HuAChE (human AChE) as an efficient therapeutic bioscavenger has been advanced by the development of recombinant production systems [6,7] and the introduction of catalytically favourable mutations [7–11].

In addition to their high reactivity towards toxic organophosphate agents, native serum-derived cholinesterases (serum BChE and AChE from fetal bovine serum) were found to be retained in the circulation of experimental animals for extended periods of time, exhibiting MRTs (mean residence times) of more than 1000 min [12–15]. In contrast, rHuAChE (recombinant HuAChE) or rBoAChE (recombinant bovine AChE) produced in stably

transfected cell lines derived from the HEK-293 (human embryonic kidney-293) cell line were eliminated from the bloodstream of experimental animals within much shorter periods of time (MRT = 60–100 min).

In order to understand the structural basis of the circulatory residence of AChE, we examined [16,17] the post-translational differences between serum-derived native BoAChE and its recombinant HEK-293-expressed counterpart by extensive MALDI-TOF (matrix-assisted laser-desorption/ionization—time-of-flight) MS analyses of the appended N-glycans and sucrose-gradient analyses of enzyme subunit assembly status. These studies, together with extensive pharmacokinetic profiling of various AChE forms in mice, allowed us to demonstrate that sialic acid occupancy and enzyme oligomerization determine the circulatory residence of BoAChE in an hierarchical manner. This was manifested by the findings that: (a) desialylated enzymes varying in their degree of subunit assembly were eliminated from the circulation of mice in an equally rapid manner, (b) conversion of enzyme forms into uniformly assembled tetramers conferred only a limited increase in the circulatory retention of suboptimally sialylated AChEs, and (c) the full effect of tetramerization on circulatory longevity could be appreciated only in fully sialylated enzyme forms [16]. Further studies showed that the circulatory

Abbreviations used: AChE, acetylcholinesterase; rAChE, recombinant acetylcholinesterase; (r)BoAChE, (recombinant) bovine AChE; (r)HuAChE, (recombinant) human AChE; (r)RhAChE, (recombinant) rhesus AChE; BChE, butyrylcholinesterase; DFP, di-isopropyl fluorophosphate; HEK, human embryonic kidney; IGF, insulin-like growth factor; iso-OMPA, tetraisopropylpyrophosphoramide; MALDI-TOF, matrix-assisted laser-desorption/ionization—time-of-flight; MRT, mean residence time; MRT_{Rh}/MRT_m, ratios of the MRT in rhesus macaques to the MRT in mice; PEG, poly(ethylene glycol); PRAD, proline-rich association domain; t-PA, tissue plasminogen activator.

¹ To whom correspondence should be addressed (e-mail avigdor@iibr.gov.il).

The nucleotide sequence data reported (exons 2–6 of the RhAChE gene) will appear in DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession numbers AY372522–AY372526.

longevity of the human form of AChE in mice is regulated by the same set and pattern of rules [17].

Increasing the number of N-glycans appended to HuAChE was also shown to contribute to the circulatory performance of the enzyme [18]. Extensive pharmacokinetic analyses of an array of enzyme species differing in their state of sialylation, subunit assembly and number of appended N-glycans demonstrated that the effect of N-glycan addition on the pharmacokinetics of AChE is fully manifested only in the case of efficiently sialylated tetrameric forms of the enzyme. Thus N-glycan loading operates as a third component, together with sialylation and tetramerization, within an hierarchical set of rules governing the circulatory longevity of AChE.

Using the previously defined sets of rHuAChE and rBoAChE molecular arrays [16–18], as well as a similar set of newly cloned and expressed AChEs from rhesus macaque (*Macaca mulatta*), we now reveal that specific amino acid domains promote the elimination of soluble primate AChEs from the circulation of rhesus monkeys, but not that of mice, thereby mitigating the positive contribution of post-translational processing to circulatory retention. This specific process for the removal of their own and related AChE molecules by macaques may, however, be countermanded by the effective masking of these domains, for example by chemical conjugation of PEG [poly(ethylene glycol)] moieties, resulting in the generation of primate AChEs exhibiting extraordinarily long-term circulatory residence in monkeys.

EXPERIMENTAL

Cell culture techniques, enzyme production and purification of rAChEs

The generation of HEK-293 cell lines stably expressing high levels of rHuAChE and rBoAChE was described previously [16,17]. The generation of a HEK-293 cell line stably expressing high levels of rRhAChE (recombinant rhesus AChE) was performed in the same manner, by transfecting the cells with the pRhAChE-nc vector (see below), followed by G418 selection to form rRhAChE stable producer cells. The generation of *in vivo* highly sialylated rHuAChE (wild type and tetraglycosylated mutant), rBoAChE and rRhAChE was achieved by stably expressing the human (wild type or D61N mutant), BoAChE and RhAChE genes in the genetically modified 293ST-2D6 cells that express high levels of heterologous α 2,6-sialyltransferase, followed by G418 selection to form rAChE stable producer cells, as described previously [19]. The method for purification of the secreted rHuAChE and rBoAChE enzymes was described previously [12], and served also for the purification of secreted rRhAChE.

Sequencing of the RhAChE gene

The coding sequence of the gene encoding RhAChE was determined through PCR amplification of rhesus genomic adult female lymphocyte DNA fragments. Five amplified overlapping DNA fragments were generated, using primers based on the HuAChE sequence (see Figure 2A). Sequence determination (ABI Prism Rhodamine kit using the ABI310 Genetic Analyzer; Applied Biosystems) was based on two independent clone isolates for each amplified DNA fragment. The sequences of exons 2–6 of the RhAChE gene were deposited in the GenBank database under accession numbers AY372522–AY372526.

Generation of rRhAChE cDNA

To generate the rRhAChE gene, the HuAChE gene cloned in the pBluescript vector (Stratagene) was subjected to site-directed mutagenesis to replace human-specific codons with those coding for amino acids of the rhesus monkey protein. Using the QuikChange site-directed mutagenesis kit (Stratagene), each of the codons ATG, CAC and GCA, corresponding to amino acids Met⁴², His²⁸⁴ and Ala⁴⁶⁷ respectively of the HuAChE gene, was replaced individually with the codons ACA, AAT and ACG respectively, to code for Thr⁴², Asn²⁸⁴ and Thr⁴⁶⁷. The fragments *EcoRV*–*BstEII* containing the rhesus codon coding for Thr⁴², *BstEII*–*SbfI* containing the rhesus codon coding for Asn²⁸⁴, and *SbfI*–*NotI* containing the rhesus codon coding for Thr⁴⁶⁷ were excised from the corresponding mutated pBluescript vectors and used to replace the parallel fragments in the pAChE-nc expression vector [6], to generate the pRhAChE-nc vector. The AChE coding region within pRhAChE-nc was verified by DNA sequencing as described above.

Enzyme activity

The AChE activities of purified AChE preparations were measured according to Ellman et al. [20]. Assays were performed in the presence of 0.5 mM acetylthiocholine, 50 mM sodium phosphate buffer, pH 8.0, 0.1 mg/ml BSA and 0.3 mM 5,5'-dithiobis-(2-nitrobenzoic acid). The assay was carried out at 27 °C and monitored using a Thermomax microplate reader (Molecular Devices).

To allow the measurement of residual AChE activity in rhesus macaque serum samples, endogenous BChE background activity was inhibited by pretreatment of the samples with the BChE-specific inhibitor, iso-OMPA (tetraisopropylpyrophosphoramide). This was achieved by preincubating the serum samples in the presence of 50 mM sodium phosphate buffer, pH 8.0, 0.1 mg/ml BSA, 0.3 mM 5,5'-dithiobis-(2-nitrobenzoic acid) and 40 μ M iso-OMPA for 10 min at room temperature before adding the substrate, acetylthiocholine (final concn 0.5 mM). Under these conditions, > 98 % of the endogenous BChE activity was inhibited without affecting AChE activity.

Removal of sialic acid from rAChEs

rHuAChE, rBoAChE or rRhAChE (15–30 nmol of enzyme) in PBS was incubated for 16 h with 0.2–0.4 unit of agarose-bound sialidase at room temperature. Sialidase was removed by centrifugation in an Eppendorf microfuge. The desialylated enzyme was dialysed against PBS to remove free sialic acid.

In vitro tetramerization of rAChEs

Synthesis and quality control of the human ColQ PRAD (proline-rich association domain) peptide CLLTTPPPPLFPFFFRG were described previously [16]. Preparative tetramerization for the generation of milligram amounts of tetrameric rHuAChE (wild type or D61N mutant), rBoAChE and rRhAChE for pharmacokinetic studies was performed by incubating 70 nmol of highly sialylated rAChE with 140 nmol PRAD synthetic peptide for 12–16 h at room temperature, in the presence of 5 mM phosphate buffer, pH 8.0, in a final volume of 2 ml. Prior to administration to monkeys, the various *in vitro* tetramerized rAChEs were dialysed extensively against PBS. Complete conversion of the enzymes into tetrameric forms was verified by sucrose-gradient density analysis, as described previously [16].

MALDI-TOF analysis of glycans

N-glycans of rAChEs (~100 µg of protein) were purified, labelled with 2-aminobenzamide [21] and converted into their neutral methylated forms by methyl iodide esterification, as described previously [16]. Mass spectra of 2-aminobenzamide-labelled esterified rAChE glycans were acquired on a Micromass ToFSpec 2E reflectron TOF mass spectrometer. Portions of 1 µl of glycan samples diluted 1:10 (v/v) in water were mixed with an equal volume of freshly prepared 2,5-dihydroxybenzoic acid (10 mg/ml in 70 % acetonitrile) and loaded on to the mass spectrometer target. Dried spots were recrystallized by adding 0.5 µl of ethanol and allowed to dry again. Glycans were observed as $[M + Na]^+$ ions. A 1 µl portion of peptide mixture [renin substrate, corticotropin ('ACTH') fragment 18–39 and angiotensin; 10 pmol/µl; all from Sigma], which served as a three-point external calibrant for mass assignment of the ions, was mixed with freshly prepared α -cyano-4-hydroxycinnamic acid (10 mg/ml in 49.5 % acetonitrile/49.5 % ethanol/0.001 % trifluoroacetic acid), loaded on the mass spectrometer target and allowed to dry. All oligosaccharides were analysed at 20 kV with a single-stage reflectron in the positive-ion mode. Between 100 and 200 scans were averaged for each of the spectra shown.

Conjugation of PEG to AChE

Attachment of PEG chains to primary amines in rHuAChE was performed using succinimidyl propionate-activated methoxy-PEG (SPA-PEG; Shearwater Polymers, Inc.), as described previously [22]. Briefly, purified rHuAChE or rRhAChE (5 µM) was incubated with PEG-20 000 at a ratio of 50:1 (mol/mol) $[PEG]_0/[AChE \text{ primary amines}]_0$ in 50 mM phosphate buffer, pH 8.0, for 2 h at room temperature. The chemically modified products were dialysed extensively against PBS and analysed on 6 % SDS/PAGE gels.

Animal procedures

Female rhesus macaque monkeys (2.7–3.0 kg) were obtained from Covance (Princeton, NJ, U.S.A.). Animals were quarantined upon arrival and screened for evidence of disease. The animals were housed individually in stainless-steel cages in animal rooms that were maintained at 20–22 °C and a relative humidity of 50 ± 10 % on a 12-h light/dark cycle. The monkeys were fed commercial certified Primate Chow (Koffolk Inc., Tel Aviv, Israel) and provided with tap water *ad libitum*. Male outbred ICR mice (Charles River Laboratories) were maintained at 20–22 °C and a relative humidity of 50 ± 10 % on a 12-h light/dark cycle, fed with commercial rodent chow (Koffolk Inc.) and provided with tap water *ad libitum*. Treatment of animals was in accordance with regulations outlined in the USDA Animal Welfare Act and the conditions specified in *Guide for Care and Use of Laboratory Animals* (National Institute of Health, 1996). All pharmacokinetic studies (see below) in monkeys and mice were approved by the local ethical committee on animal experiments.

Clearance experiments and analysis of pharmacokinetic profiles in mice and rhesus macaques

Following extensive dialysis against PBS (pH 7.4), aliquots of 1000 units of the various rAChEs (165, 330 and 165 µg for the human, bovine and rhesus forms respectively) were injected

intravenously into three rhesus macaques per group (injection volumes < 1 ml/kg). Samples (0.25 ml) of blood were collected after various periods of time into Microtainer tubes (Becton Dickinson), centrifuged for 1 min at 11 000 g in an Eppendorf microfuge and stored at –20 °C until AChE activity in the serum samples was determined. Enzymic activity was determined following iso-OMPA-mediated BChE inhibition, as described above. AChE activity values in samples removed 2 min after injection were referred to as 'input activities' and were used for the calculation of residual activity throughout the experiment. AChE values were corrected for background activity determined in blood samples withdrawn 1 h before performing the experiment. Exogenously administered AChE activity was at least 20-fold higher than background endogenous iso-OMPA-resistant cholinesterase activity. To avoid the possible generation of anti-cholinesterase antibodies, which might affect the circulatory retention times of the injected proteins, in all cases each of the monkeys was injected with AChE only once.

Clearance experiments in mice (three to six mice per enzyme sample) were carried out essentially as described previously [12]. Mice were injected intravenously with the various rRhAChE preparations (100 units/mouse in 0.2 ml of PBS). Residual AChE activity in blood samples was measured and all values were corrected for background hydrolytic activity in the blood (using samples withdrawn 1 h before performing the experiment). AChE activity values in samples removed 1 min after injection were assigned a value of 100 % and used for calculation of residual activity. Background cholinesterase levels in the blood of mice before administration of AChE were less than 2 units/ml.

The clearance patterns of the various enzyme preparations were usually biphasic and fitted to a bi-exponential elimination pharmacokinetic model ($C_t = Ae^{-k_{el}t} + Be^{-k_{\beta}t}$), as described previously [16,17]. This model enables determination of the parameters A and B , which represent the fractions of the material removed from the circulation in the first (fast) and second (slow) elimination phases respectively, and $t_{1/2\alpha}$ and $t_{1/2\beta}$, which represent the circulatory half-life values of the enzyme in the fast and slow phases respectively. The pharmacokinetic parameter MRT (which reflects the average length of time that the administered molecules are retained in the organism) was obtained independently by analysing the clearance data according to a non-compartmental pharmacokinetic model using the WinNonlin computer program [23].

Clearance of native and radiolabelled rhesus BChE in rhesus macaques

Serum BChE collected from rhesus macaques was purified on procainamide columns as described previously [12]. Purified native BChE was radiolabelled by incubating the enzyme (7.2 nmol) with a tritiated organophosphorus compound, [3H]DFP ([3H]di-isopropyl fluorophosphate; 60 µCi, 8.4 Ci/mmol; Amersham Biosciences). Excess radioactive material was removed by passing the labelling mixture through a G-50 column. The labelled enzyme was administered to three rhesus macaques, blood samples were removed at various time points and residual labelled BChE in blood samples was measured by counting the radioactivity in trichloroacetic acid precipitates in a liquid scintillation counter. Radioactivity values in samples removed 2 min after injection were assigned a value of 100 % and used for residual enzyme calculation.

To determine the pharmacokinetic profile of non-labelled rhesus serum BChE, ~10 000 units of the enzyme was administered to a single monkey. Residual BChE enzymic activity in blood

Table 1 Pharmacokinetic parameters of rBoAChE and rHuAChE in rhesus macaques

A and B are the fractions of AChE removed from the circulation in the first (fast) and second (slow) elimination phases respectively, and $t_{1/2\alpha}$ and $t_{1/2\beta}$ are the circulatory half-life values of the enzyme in the fast and slow phases respectively

AChE source	AChE type	A (%)	B (%)	$t_{1/2\alpha}$ (min)	$t_{1/2\beta}$ (min)	MRT (min)
rBoAChE	Sialidase treated	100	—	3.5 ± 0.5	—	3.9 ± 0.6
	Partially sialylated	85 ± 6	20 ± 5	5 ± 0.5	57 ± 4	66 ± 2
	Fully sialylated	56 ± 6	46 ± 5	17 ± 4	163 ± 25	205 ± 7
	Fully sialylated, tetramerized	34 ± 2	68 ± 2	14 ± 2	1080 ± 30	1510 ± 45
rHuAChE	Sialidase treated	100	—	2 ± 0.5	—	2.7 ± 0.4
	Partially sialylated	85 ± 5	17 ± 4	6 ± 1	54 ± 6	59 ± 1
	Fully sialylated	37 ± 4	65 ± 6	6 ± 2	73 ± 4	99 ± 3
	Fully sialylated, tetramerized	31 ± 4	72 ± 5	5 ± 1	74 ± 4	96 ± 5
	Tetraglycosylated, fully sialylated, tetramerized	40 ± 3	64 ± 4	6 ± 1.5	73 ± 5	97 ± 6

samples removed after various periods of time was determined as described above, using butyrylthiocholine as the substrate.

RESULTS AND DISCUSSION

Pharmacokinetics of rBoAChE forms obey the same hierarchical rules in rhesus macaques and mice

The effects of post-translational processing on the circulatory retention of heterologous AChE in non-human primates were addressed by monitoring the pharmacokinetic profiles of an array of differently processed rBoAChEs in rhesus macaques. The contribution of sialic acid content to the circulatory retention of the recombinant enzyme forms was evaluated by comparing the clearance profiles of enzymically desialylated enzyme, partially sialylated enzyme produced by HEK-293 producer cells and fully sialylated enzyme produced by the modified 293ST-2D6 cell line, which co-expresses high levels of recombinant α 2,6-sialyl-transferase [19]. The effect of enzyme subunit assembly on the circulatory residence of the rAChE was evaluated by examining the pharmacokinetic behaviour of fully sialylated enzyme following its complete conversion into tetramers in the presence of the synthetic ColQ PRAD peptide [16,24].

Pharmacokinetic analyses of the various rBoAChE forms in rhesus macaques demonstrated that each of the differently processed forms was characterized by a unique circulatory retention pattern (Figure 1A, Table 1). rBoAChE totally devoid of sialic acid residues displayed a monophasic elimination curve and was cleared rapidly from the circulation within a very short period of time ($MRT = 3.9 \pm 0.6$ min). In contrast, all rBoAChE forms containing sialic acid residues displayed biphasic elimination curves, with different circulatory residence values in accordance with the level of sialic acid occupancy and enzyme subunit assembly. Thus rBoAChE preparations consisting of partially sialylated, fully sialylated and fully sialylated/tetramerized forms of the enzyme exhibited increasing circulatory retention, as demonstrated by the corresponding MRT values of 66 ± 2 , 205 ± 7 and 1510 ± 45 min respectively. These results establish that both sialic acid levels and enzyme subunit assembly strongly influence the circulatory retention of BoAChE in non-human primates, and that enzyme sialylation and oligomerization operate in a synergistic manner, resulting in the generation of an enzyme form which resides in the circulation of rhesus macaques for very long periods of time.

The MRT values of the various rBoAChE forms in monkeys are very similar to those obtained previously in mice ([16]; see

Figure 1 Comparison of the circulatory elimination profiles of various molecular forms of rBoAChE and rHuAChE in rhesus macaques

Purified preparations of various molecular forms of rBoAChE (A) or rHuAChE (B), differing in their post-translational processing (glycan sialylation level, subunit assembly state, N-glycan loading), were administered to three rhesus macaques per group, and residual AChE activity in the circulation was assayed at the indicated time points. Circulatory removal curves were determined for all the AChE forms and the derived pharmacokinetic parameters are presented in Table 1. Note the different time scales in (A) and (B).

also Figure 5). Thus the ratios of the MRT in rhesus macaques to the MRT in mice (MRT_{Rh}/MRT_m) for the asialylated, partially sialylated, fully sialylated and fully sialylated/tetramerized forms of rBoAChE were 1.0 ± 0.05 , 1.2 ± 0.09 , 1.0 ± 0.06 and 1.1 ± 0.08 respectively. The finding that the MRT_{Rh}/MRT_m values for all of the differently processed forms of rBoAChE are

approx. 1 demonstrates that the pharmacokinetics of rBoAChE are determined in the rhesus animal system by the same set of principles revealed in the past for mice [16], reflecting the hierarchical rules of fully sialylated/tetramerized > fully sialylated > partially sialylated > asialylated.

The circulatory elimination patterns of rHuAChEs in rhesus macaques appear to deviate from the classical hierarchical rules established in mice

The generation of a therapeutically effective bioscavenger of organophosphate compounds based on exogenously administered cholinesterase would ideally require the use of an enzyme form from the same or a closely related species. We therefore addressed the question of whether the set of rules governing BoAChE pharmacokinetics in monkeys apply to enzyme forms from primates as well. To this end, we monitored the circulatory performance of HuAChE in rhesus macaques. Pharmacokinetic analyses of differently processed forms of human rAChE in monkeys revealed a unique pattern of elimination profiles (Figure 1B, Table 1). Human rAChE entirely devoid of sialic acid residues was eliminated rapidly from the circulation ($MRT = 2.7 \pm 0.4$ min), and partial sialylation of the rHuAChE resulted in improved circulatory residence ($MRT = 59 \pm 1$ min), yet full sialylation of the human enzyme resulted in only an additional 1.7-fold increase in circulatory retention ($MRT = 99 \pm 3$ min), while tetramerization of the fully sialylated human enzyme did not contribute further to the circulatory retention of the human enzyme ($MRT = 96 \pm 5$ min). Thus, while full sialylation of the bovine enzyme and its subsequent conversion into tetramers resulted in 2.8-fold and 7-fold increases respectively in circulatory residence time, full sialylation of the human enzyme form resulted in only a modest increase in MRT, and its subsequent tetramerization did not affect its circulatory retention at all. Notably, when examined in mice ([17]; see also Figure 5B), the circulatory residence of the fully sialylated human enzyme could be significantly extended by tetramerization (fully sialylated, $MRT = 195 \pm 9$ min; fully sialylated/tetrameric, $MRT = 740 \pm 30$ min). These results indicate that the inability of tetramerization to significantly extend the circulatory lifetime of rHuAChE in rhesus macaques does not reflect an intrinsic property of the human enzyme *per se*, but rather some property of the rhesus macaque animal system with regard to the enzyme form of human source.

rHuAChE differs from the bovine enzyme in its number of appended N-glycans. The human enzyme contains three N-glycans at amino acid positions analogous to those of three N-glycans of the bovine enzyme, while the latter carries an additional fourth N-glycan at amino acid 61 [14,25]. Previous findings showed that a mutated form of fully sialylated, tetramerized rHuAChE that contains, like the bovine enzyme, a fourth N-glycan at amino acid 61 resided in the circulation of mice for longer periods of time than its triglycosylated counterpart ([18]; see also Figure 5B). To examine whether increasing the number of N-glycans may increase the retention of rHuAChE in the rhesus macaque background as well, the pharmacokinetic behaviour of the tetra-glycosylated D61N enzyme in its fully sialylated/tetramerized configuration was monitored in monkeys. Pharmacokinetic profiling of this enzyme form demonstrated that its residence was virtually identical to that of its triglycosylated counterpart (MRT values = 97 ± 6 and 96 ± 5 min respectively; Table 1; see also Figure 5B). Thus, unlike the mouse system, where both enzyme tetramerization and the addition of N-glycans increased the circulatory retention of HuAChE, in the rhesus animal system neither tetramerization nor addition of N-glycans contributed in

a measurable manner to the circulatory residence of the fully sialylated enzyme.

These findings suggest that the pharmacokinetics of rHuAChE in the rhesus animal system deviate from the classical hierarchical rules found for the same enzyme in mice due to the operation of a process that specifically promotes elimination of the human enzyme from the circulation of monkeys, while mitigating the positive effect of post-translational processes (i.e. enzyme assembly and glycan loading) on circulatory retention. This inability to augment the circulatory retention of AChE in monkeys cannot be attributed to quantitative or qualitative differences in glycan processing of the two enzymes, since: (a) the tetra-glycosylated mutant form of rHuAChE possesses the same number of appended N-glycans at the same amino acid positions as the bovine enzyme [14], and (b) extensive structural analyses carried out in the past ([16,17]; see also Figure 3B) have clearly demonstrated that the bovine and human versions of rAChE generated in the HEK-293 cell line contain almost identical N-glycan forms in similar abundances. Thus the differential pharmacokinetic hierarchical patterns exhibited by HuAChE and BoAChE in the rhesus animal system cannot be attributed to points of variance in their post-translational modifications, but most probably stem from differences in their primary amino acid sequences.

Cloning, expression and N-glycan analysis of various post-translationally modified rRhAChE forms

The presence of BChE and not AChE in the circulation of primates (e.g. rhesus monkey and human) at the adult stage [26] may suggest that AChE is actively removed from the bloodstream. Such a mechanism could provide an explanation for the unique elimination of HuAChE from the circulation of monkeys, since, unlike BoAChE, this species of the enzyme may be sufficiently similar to the 'self' AChE of the rhesus macaques and therefore be subject to active removal. To examine whether the pharmacokinetic patterns of HuAChE enzyme forms may indeed be attributed to the action of 'self' AChE circulatory elimination mechanisms, we set out to examine the pharmacokinetics of RhAChE in its homologous animal system. Determination of the genomic sequences of rhesus macaque AChE corresponding to exons 2–6 (Figure 2A) revealed that, at the DNA level, RhAChE and HuAChE differ at 32 out of 1971 nucleotides. Only three of these divergences result in changes in amino acids within the coding region of the mature enzyme: $Met^{42}_{human} \rightarrow Thr^{42}_{rhesus}$, $His^{284}_{human} \rightarrow Asn^{284}_{rhesus}$ and $Ala^{467}_{human} \rightarrow Thr^{467}_{rhesus}$ (Figure 2B). The rRhAChE coding sequences were introduced into HEK-293 cells or the modified 293ST-2D6 cell line expressing high levels of recombinant $\alpha 2,6$ -sialyltransferase [19], to generate authentic rRhAChEs which are partially or fully sialylated respectively.

As expected, the levels of production of the partially and fully sialylated rRhAChEs were similar to those of the human enzyme expressed in the same cell systems (approx. 50 units/24 h per ml [19]). The rRhAChE products exhibited a migration pattern on SDS/PAGE (Figure 3A, inset) indistinguishable from that of their human counterparts [19]. As shown previously for the human enzyme, RhAChE derived from the modified 293ST-2D6 cell line displayed lower mobility, reflecting the efficient sialylation of the enzyme product in these $\alpha 2,6$ -sialyltransferase expresser cells. This was verified further by the pharmacokinetic analysis of the enzyme product in mice, as described below.

Examination of the sucrose-gradient profile of partially or fully sialylated rRhAChE demonstrated that these enzyme preparations

Figure 2 Determination of the RhAChE gene sequence

(A) Map of the *Macaca mulatta* AChE gene and DNA genomic fragments generated by PCR amplification for sequence determination. Ex, exon; Int, intron. (B) Amino acid sequence of RhAChE. The deduced sequence of the mature T subunit, signal peptide and the C-terminus of the H subunit (exon 5) of RhAChE, and the corresponding regions of HuAChE and BoAChE, are shown. The conserved N-glycosylation consensus sequences are underlined. The amino acids that differ between rhesus/bovine and rhesus/human are highlighted in bold and shaded respectively.

consist of a mixture of differently assembled forms, most of which are dimers. Incubation of these rRhAChE preparations with the synthetic human ColQ PRAD peptide [16] resulted in the quantitative conversion of the enzymes into assembled tetramers (Figure 3A), in manner similar to that reported previously for HuAChE [17].

To determine whether the basic structures of the N-glycans appended to rRhAChE generated in HEK-293 cells are similar to those of rBoAChE and rHuAChE derived from the same cells, the glycan structures of rRhAChE (partially and fully sialylated) were analysed by MALDI-TOF MS (Figure 3B). Inspection of the MALDI-TOF MS profiles of desialylated glycans of rRhAChE produced by HEK-293 cells and sialyltransferase-

modified 293ST-2D6 cells revealed that both glycan pools consisted of a similar array of varied structures, displaying an overall pattern that was similar to that of the N-glycans associated with partially sialylated and fully sialylated rHuAChE and rBoAChE (Figure 3B). Thus the major glycan species (41–46% of total glycans) corresponded to complex-type biantennary forms carrying a fucose moiety, while triantennary glycans (22–30% of the total glycans) were represented to a greater extent by the non-fucosylated form. The single significant difference between the glycan structures of rRhAChE and those of rHuAChE and rBoAChE was in the level of tetraglycosylated forms, which was higher for rRhAChE. Taken together, characterization of rRhAChE demonstrates that this enzyme displays a strikingly high degree of similarity to the human enzyme both in its primary sequence and in its post-translational processing.

Pharmacokinetic profiles of rRhAChE in rhesus macaques and mice

Examination of the pharmacokinetic behaviour of the various forms of rRhAChE in mice (Figure 4A; Table 2) demonstrated that rRhAChE is retained in the circulation in a manner similar to that displayed by rHuAChE [16]. This was manifested both by the increase in circulatory residence time observed for enzyme forms displaying increasing amounts of appended sialic acid residues (MRT values for asialylated, partially sialylated and fully sialylated rRhAChE of 3.2 ± 0.6 , 92 ± 4 and 171 ± 8 min respectively) and by the significant combined effect of full sialylation and enzyme tetramerization (MRT of fully sialylated tetrameric rRhAChE = 805 ± 25 min). Thus the pharmacokinetics of rRhAChE in mice are governed, like those of rHuAChE, by post-translational processes that operate in an hierarchical manner leading to the extension of circulatory residence by efficient sialylation and tetramerization. The strikingly similar pharmacokinetic behaviour of HuAChE and RhAChE in the mouse system, and the fact that they are influenced equally by the same set of post-translational processes, is in accordance with the high degree of similarity between the two primate AChE enzyme forms.

Pharmacokinetic analyses of the various rRhAChE forms in monkeys demonstrated (Figure 4B) that the pharmacokinetics of RhAChE abide by the classical rules of hierarchy to a lesser extent in this animal system. This was manifested by the finding that conversion of rRhAChE into its fully tetramerized state resulted in only a 2-fold increase (MRT fully sialylated, 143 ± 7 min; MRT fully sialylated/tetramerized, 290 ± 25 min) in circulatory retention, as opposed to a 4.7-fold increase in mice (MRT fully sialylated, 171 ± 8 min; MRT fully sialylated/tetramerized, 805 ± 25 min). Thus, while the MRT_{Rh}/MRT_m values of asialylated, partially sialylated and fully sialylated rRhAChE were 1.0 ± 0.06 , 0.93 ± 0.04 and 0.84 ± 0.04 respectively, the MRT_{Rh}/MRT_m value of the fully sialylated/tetramerized form of rRhAChE declined sharply to 0.36 ± 0.02 . Thus unlike BoAChE, which displayed similar pharmacokinetic behaviour in both mice and monkeys, as exemplified by MRT_{Rh}/MRT_m values of approx. 1 for all the differently processed forms of rBoAChE, the rhesus enzyme resembles its human counterpart in its differential pharmacokinetic behaviour in the mouse and monkey animal systems; circulatory retention of both primate enzymes was clearly less susceptible to post-translational modifications in monkeys.

Taken together, these findings demonstrate that the circulatory residence of bovine and primate AChEs in rhesus macaques is governed differently. Increasing the sialic acid levels of the different enzyme species resulted in extension of their circulatory lifetime in rhesus macaques (Figure 5A), emphasizing the pivotal role of glycan sialylation in determining the circulatory

Figure 3 Characterization of rRhAChE

(A) Sucrose-gradient sedimentation profiles of partially sialylated (■, ●) and fully sialylated (□, ○) rRhAChE, before (■, □) and after (●, ○) *in vitro* tetramerization. G1, G2 and G4 indicate the monomeric, dimeric and tetrameric RhAChE forms respectively. Arrows denote the elution positions of the sedimentation markers alkaline phosphatase (6.1 S), catalase (11.3 S) and β -galactosidase (16 S) included in all samples. Inset: SDS/PAGE analysis of rRhAChE produced in sialyltransferase-expressor 293ST-2D6 cells (lane 1) or in non-modified HEK-293 cells (lane 2). (B) Basic structures of the glycans of rAChEs (produced by HEK-293 cells) and rAChE-sialyltransferase (ST) (produced by 293ST-2D6 cells). Glycans are classified according to their branching and fucosylation. Desialylated glycan structures were deduced from the mass spectral data obtained by MALDI-TOF as described in the Experimental section. The relative abundance (% of total glycans) of each glycan species is shown and compared with the abundances of the corresponding glycan structures in rHuAChE and rBoAChE determined previously [16,17]. □, GlcNAc; ○, mannose; ●, β -galactose; ■, GalNAc; elongated diamond, fucose.

residence of AChEs in these animals. Yet, one can notice that the effect of full sialylation on AChE pharmacokinetics in rhesus macaques was less prominent in the case of the primate enzymes. The differential pharmacokinetic performance of bovine and primate AChEs in rhesus macaques is fully manifested when examining the effect of enzyme tetramerization on circulatory residence (Figure 5B). While assembly of the bovine enzyme into tetramers resulted in a striking increase in its retention, tetramerization affected the circulatory duration of the primate AChEs to a much lower extent, or not at all. The deviation from the classical hierarchical rules established for the different enzyme species in the mouse system is further manifested by the inability to extend the circulatory residence of the human enzyme by increasing its glycan loading (Figure 5C). Thus, unlike the bovine enzyme, AChEs from primate sources appear to be actively eliminated from the circulation of monkeys in a manner that counteracts the positive effect of post-translational processing on circulatory longevity. The somewhat surprising observation that HuAChE is even more prone than RhAChE to elimination from the circulation of rhesus macaques suggests that the differential pharmacokinetics of bovine and primate AChEs

in monkeys cannot be fully explained in terms of self-specific enzyme removal. Specific structural features that are common to the primate enzymes under examination, and not to the bovine enzyme, seem to facilitate the active removal of AChEs in rhesus macaques, while the observed differences in the clearance patterns of the human and rhesus enzymes in rhesus macaques may be attributed to subtle differences in the structures of the two primate AChE species.

Facilitated elimination of cholinesterases from the circulation of rhesus macaques is restricted to primate AChE

The presence of significant levels of circulating BChE in rhesus macaques [27] seems to suggest that active removal of primate cholinesterase in this animal system is restricted to AChE. However, primate BChE may also be actively and effectively eliminated from the circulation at rates similar to those of AChE, while its apparent prevalence in the circulation may be a reflection of its high turnover rate. To examine this issue, BChE from rhesus monkey serum was purified on procainamide columns

Table 2 Pharmacokinetic parameters of rRhAChE in rhesus macaques and mice

A and B are the fractions of AChE removed from the circulation in the first (fast) and second (slow) elimination phases respectively, and $t_{1/2\alpha}$ and $t_{1/2\beta}$ are the circulatory half-life values of the enzyme in the fast and slow phases respectively

Animal system	AChE type	A (%)	B (%)	$t_{1/2\alpha}$ (min)	$t_{1/2\beta}$ (min)	MRT (min)
Rhesus macaque	Sialidase treated	100	—	3.6 ± 0.6	—	3.4 ± 0.4
	Partially sialylated	75 ± 5	32 ± 5	7 ± 1	72 ± 10	86 ± 6
	Fully sialylated	46 ± 4	57 ± 5	16 ± 2	113 ± 8	143 ± 7
	Fully sialylated, tetramerized	57 ± 3	46 ± 3	26 ± 3	255 ± 24	290 ± 25
Mouse	Sialidase treated	100	—	2.9 ± 0.6	—	3.2 ± 0.6
	Partially sialylated	56 ± 8	34 ± 9	8 ± 3	71 ± 16	92 ± 4
	Fully sialylated	51 ± 7	49 ± 7	11 ± 3	118 ± 12	171 ± 8
	Fully sialylated, tetramerized	46 ± 3	52 ± 3	45 ± 10	666 ± 38	805 ± 25

Figure 4 Circulatory elimination profiles of various molecular forms of rRhAChE in mice and in rhesus macaques

Purified preparations of various molecular forms of rRhAChE, differing in their post-translational processing (glycan sialylation level, subunit assembly state), were administered to mice (A) or rhesus macaques (B), and residual AChE activity in the circulation was assayed at the indicated time points. Circulatory removal curves were determined for all AChE forms and the derived pharmacokinetic parameters are presented in Table 2. Note the different time scales in (A) and (B).

and subjected to pharmacokinetic profiling in rhesus macaques. To enable monitoring of the administered enzyme over the high background of endogenous BChE (~ 10 units/ml), purified rhesus serum BChE was radiolabelled by incubating the enzyme with a tritiated organophosphorus compound, [3 H]DFP. The inactivated enzyme was then administered to rhesus macaques, and its circulatory residence was followed by determining radioactivity in blood samples removed after various periods of time (Figure 6). This quantification, which enables detection of relatively small

amounts of administered enzyme, was repeated in three monkeys, and allowed us to determine that rhesus BChE resides in the circulation of rhesus macaques for long periods of time, displaying an MRT value of 9950 ± 1300 min. However, to rule out the possibility that the coupling of the organophosphorous DFP compound to the enzyme affected its circulatory retention significantly, the experiment was performed with native non-labelled rhesus serum BChE, using large enough amounts of enzyme to allow significant quantification of the administered enzyme over background, by monitoring BChE enzymic activity. As with radiolabelled BChE, the native non-labelled form of rhesus BChE resided in the circulation for extended periods of time and displayed a similar MRT value of 10600 ± 1200 min (Figure 6, inset). These values, which testify to the fact that rhesus BChE resides in the circulation of monkeys for very long periods of time, are in good agreement with those reported recently [28], and are similar to those determined in the past for human BChE in humans [29,30]. The occurrence of BChE in the circulation of rhesus macaques therefore reflects the long-term circulatory retention of this enzyme form, rather than the presence at steady state of high levels of short-lived species of BChE. These findings show that removal of RhAChE cannot be attributed to a general mechanism responsible for the elimination of all forms of primate cholinesterase, but rather depends on the operation of an elaborate clearance system which recognizes and removes the soluble self AChE, RhAChE, and the very closely related HuAChE in a unique manner.

Possible role for amino acid domains in the differential pharmacokinetic behaviour of bovine and primate AChEs in rhesus macaques

As reported above, the glycan pool of rRhAChE consists of an array of varied structures whose basic structures display an overall pattern similar to that of the N-glycans associated with rBoAChE and rHuAChE (Figure 3C), the only notable difference being the relatively high level of tetra-antennary glycan forms that is observed for RhAChE. Differences in the basic structures of appended N-glycans therefore cannot provide an explanation for the differential pharmacokinetic performances of bovine compared with primate AChEs. Likewise, the dissimilar pharmacokinetic patterns of bovine and primate AChEs in monkeys cannot be attributed to differences in terminal sialylation, since the various AChEs are all sialylated efficiently in a similar manner when expressed in the genetically modified 293ST-2D6 cells that express high levels of heterologous $\alpha 2,6$ -sialyltransferase (Figure 3A, inset; see also [19]). Examination of the amino

Figure 5 Effects of sialylation, oligomerization and glycan loading on the circulatory residence of BoAChE, HuAChE and RhAChE in mice and rhesus macaques

Bars represent the MRT values in mice and monkeys of the different molecular forms of BoAChE, HuAChE and RhAChE as calculated from the circulatory elimination data presented in Figures 1 and 4 and in previous studies [16–18]. (A) Effect of sialylation of AChEs: solid bars, sialidase treated; hatched bars, partially sialylated; shaded bars, fully sialylated. (B) Effect of oligomerization of sialylated AChEs: shaded bars, dimeric; cross-hatched bars, tetrameric. (C) Effect of number of glycans in fully sialylated tetrameric HuAChEs: cross-hatched bars, wild-type triglycosylated; shaded bars, D61N tetraglycosylated.

acid divergences between HuAChE and RhAChE (Figure 2) demonstrates that these are not involved in the generation or abolition of N-glycosylation consensus sequences, while SDS/PAGE analysis (Figure 3A, inset; [19]) suggests that the human and rhesus enzymes carry similar amounts of glycans. Thus both primate AChEs carry three N-glycans per enzyme subunit, while

Figure 6 Circulatory elimination profiles of labelled and non-labelled rhesus BChE in rhesus macaques

Native rhesus BChE labelled with [^3H]DFP was administered to three rhesus macaques and residual circulatory radiolabelled BChE was assayed at the indicated time points post-administration. Inset: comparison of the MRT values of the radioactively labelled rhesus BChE and enzymically active non-labelled BChE (see the Experimental section).

BoAChE contains four N-glycans per enzyme subunit. However, this quantitative difference in N-glycan loading of BoAChE and primate AChEs is also apparently not responsible for the less efficient retention of the latter in the circulation of monkeys, since addition of a fourth N-glycan to the human enzyme at residue 61, the position analogous to that of the fourth N-glycan of the bovine enzyme, did not alter HuAChE circulatory residence in monkeys (Figure 5B). Furthermore, incubation of both primate and bovine forms of AChE with the synthetic ColQ PRAD peptide resulted in the quantitative conversion of all of the various enzyme forms into tetramers in a similarly highly efficient manner, attesting to the fact that their disparate pharmacokinetics in monkeys are not related to differences in subunit organization.

Taken together, all of these findings suggest that the dissimilarity between the circulatory residence of bovine and primate AChEs does not stem from differences in glycosylation or assembly state, but rather from points of divergence in their primary structure that differentially affect circulatory residence. The divergent amino acid domains may in themselves constitute ligands for receptor-directed clearance, or may lead to the appendage of as yet undefined structures involved in clearance. Such species-specific amino acid domains that facilitate the elimination of primate AChEs from the circulation of monkeys apparently operate via a mechanism of considerable potency, as judged by their ability to counteract the positive effect of post-translational modifications on the circulatory residence of primate AChEs, and function as clearance epitopes in an enzyme-assembly-independent manner, as demonstrated by their ability to abolish tetramerization-induced circulatory lifetime extension (Figure 5B).

Alterations in the plasma clearance of proteins due to amino acid replacements that affect interactions of the protein with its clearance receptor in a direct or indirect manner have been documented previously in other systems. In the case of IGF-I (insulin-like growth factor-I), the single and double amino acid mutants F49A and E3A/F49A were cleared more rapidly from the circulation due to their diminished ability to form high-molecular-mass soluble complexes with IGF-binding proteins, without changing the affinity of the mutant forms towards the IGF type I receptor [31]. On the other hand, the plasma clearance of apolipoprotein E is severely compromised by a single Arg¹⁵⁸ → Cys mutation, which prevents efficient binding of apolipoprotein E to the low-density lipoprotein receptor [32]. Amino acid substitutions were also demonstrated to affect t-PA

Figure 7 Mapping of amino acids that differ between BoAChE and RhAChE/HuAChE on to the three-dimensional model of HuAChE

The protein backbone is depicted as a line ribbon and the divergent residues are shown as space-fill spheres. Note that all divergent residues map to the enzyme surface. (A) Mapping of amino acid residues that differ between BoAChE and RhAChE within three enzyme surface-located domains. The diverging amino acid residues are shaded according to their location: black, domain A; dark grey, domain B; light grey, domain C. Arrows point to the three residues (Met⁴², His²⁸⁴ and Ala⁴⁶⁷) that differ between RhAChE and HuAChE. (B) Mapping of the fourth glycosylation site (Asn⁶¹) of BoAChE to the centre of domain A. (C) Mapping of the BoAChE/RhAChE divergent domains within the tetrameric structure of HuAChE.

(tissue plasminogen activator) clearance from the circulation, but in this case the altered pharmacokinetic properties were due to changes in the N-glycan content of the protein, rather than to the presence of altered amino acids. Thus the 9-fold reduction in circulatory clearance of the Thr¹¹⁰ → Asn t-PA mutant is probably due to the addition of a complex-type N-glycan structure [33], while the 6.5-fold reduction in plasma clearance of the Asn¹¹⁷ → Gln t-PA mutant was attributed to the prevention of high-mannose glycosylation at this site [34]. As noted above, in the case of AChE the differential circulatory behaviour of primate and bovine enzymes cannot be attributed to glycosylation-related factors.

Comparison of the sequences of BoAChE and RhAChE (Figure 2B) demonstrates that the divergent amino acids are dispersed throughout the primary enzyme structure, yet examination of the three-dimensional structure of AChE reveals that all of these diverging amino acids are clustered within three domains, A, B and C (Figure 7). These divergent patches are located at the surface of the enzyme in both its monomeric (Figure 7A) and tetrameric (Figure 7C) states of assembly, with only part of domain B being obscured by tetramerization (Figure 7C). BoAChE as well as the tetraglycosylated form of the human enzyme mentioned above carry an additional N-glycan at amino acid 61, which maps to a central position within domain A (Figure 7B). Interestingly, HuAChE carrying this additional N-glycan displayed enhanced circulatory retention in mice [18]. We note that the three amino acids that differ between HuAChE and RhAChE (positions 42, 284 and 467), which are apparently responsible for the somewhat different pharmacokinetic patterns of these two enzymes in the rhesus system, indeed map within domains B (42 and 284) and C (467).

Effect of masking primate-specific amino acid domains by PEGylation on the circulatory lifetime of AChE in rhesus macaques

Previous studies carried out in mice [22] demonstrated that the conjugation of PEG chains to lysine residues of AChE resulted in the generation of a molecule that resides in the circulation for exceedingly long periods of time. Most importantly, PEG conjugation prevented plasma clearance of undersialylated

Figure 8 Pharmacokinetic profiles of PEGylated rRhAChE and rHuAChE in rhesus macaques

Purified rRhAChE (○, □) or rHuAChE (●, ■) generated in HEK-293 cells (partially sialylated, dimeric forms) was administered to three rhesus macaques before (○, ●) or after (□, ■) chemical conjugation to PEG. The residual AChE activity in the circulation was assayed at the indicated time points, as described in the Experimental section.

rHuAChE as well, suggesting that the appendage of PEG chains obstructs accessibility of domains at the surface of the enzyme to hepatic asialoglycoprotein receptors. It was therefore interesting to examine whether PEGylation can prevent the putative species-specific amino acid domain removal process as well. To address this issue, partially sialylated rHuAChE and rRhAChE produced in HEK-293 cells and, consisting mostly of dimeric forms, were subjected to PEG appendage. To allow efficient PEG conjugation of the two primate AChEs without compromising AChE bioactivity, the enzymes were reacted with succinimidyl propionate-activated PEG at molar ratios and under conditions determined previously [22]. Under these conditions, the PEGylated product carries an average of four PEG chains per enzyme subunit and retains full enzymic activity.

PEGylated rHuAChE and rRhAChE were administered to rhesus macaques and the pharmacokinetic profiles of the chemically modified enzymes were monitored (Figure 8). The PEGylated enzymes were retained in the circulation for exceedingly long periods of time, exhibiting MRT values of 10360 ± 280 min and 9900 ± 390 min for PEGylated rHuAChE and

rRhAChE respectively. Thus the chemical conjugation of PEG to the primate enzymes resulted in an extraordinarily large increase (>100-fold) in the residence of the human and rhesus enzymes in the circulation of monkeys, demonstrating that PEGylation of AChE indeed leads to the efficient masking of the various determinants that promote enzyme clearance in monkeys. The finding that the PEGylated forms of the primate enzymes exhibited MRT values much greater than that of rBoAChE even in its fully processed form (1510 ± 45 min; see Table 1) demonstrates that the appendage of PEG chains not only masks the elements that cause the minor inequality in the pharmacokinetics of HuAChE and RhAChE, but also overcomes the contribution of the primate-specific amino acid sequences to the inferior pharmacokinetic performance in monkeys of both rHuAChE and rRhAChE as compared with BoAChE. In fact, the MRT values of the PEGylated primate AChEs are similar to those exhibited by native long-lived rhesus BChE (Figure 6), indicating that PEG appendage results in the generation of enzyme forms that are no longer accessible to circulatory removal systems that recognize and interact with the self primate AChE-specific domains.

In conclusion, the present study defines amino acid-related domains as an important element which, together with post-translational factors such as oligosaccharide sialylation, enzyme assembly and glycan loading, determine the circulatory lifetime of AChE in non-human primates. In fact, in the rhesus macaque system, amino acid-related elimination of AChE is effective to the extent that it prevents full manifestation of the positive effect of efficient sialylation on circulatory retention of the enzyme. Even so, chemical conjugation of PEG to AChE species containing amino acid epitopes that promote circulatory elimination effectively protected these enzymes from amino acid-related removal.

The amino acid configurations that promote enzyme clearance in rhesus macaques seem to be restricted to those presented by self or self-like AChE species. Unlike in rhesus macaques, differences in amino acid sequences had only a minor effect on the differential clearance of BoAChE, HuAChE and RhAChE in the mouse system, yet this apparent insensitivity to amino acid alterations may stem from the fact that the three AChE species examined are equally 'foreign' and therefore do not present amino acid epitopes that promote enzyme removal in mice. Indeed, primates are characterized by a paucity of soluble AChE in the circulation at the adult stage [26]. The predominance of BChE in the circulation of adults, and the finding that self AChE is eliminated from the circulation, may suggest that organisms have evolved elaborate mechanisms to support the circulatory residence of BChE in particular. Although the biological significance of the active elimination of soluble AChEs from the bloodstream remains to be elucidated, it may be speculated that this process has to do with one or more of the non-catalytic functions attributed to this enzyme, which include cell differentiation, haematopoietic and thrombopoietic activities and involvement in cell adhesion [35–37].

We thank Ms Shirly Lazar, Nehama Seliger and Tamar Sery for excellent technical assistance, Dr Trevor Weiner, Dr Orit Cohen and Mr Yosef Shlomovitch for assistance with animal experiments, and Dr Theodor Chitlaru for fruitful discussions and critical comments in preparation of the manuscript. This work was supported in part by the U.S. Army Research and Development Command, contract DAMD17-00-C-0021 (to A.S.).

REFERENCES

- Massoulie, J., Anselmet, A., Bon, S., Krecji, E., Legay, C., Morel, N. and Simon S. (1999) The polymorphism of acetylcholinesterase: post-translational processing, quaternary associations and localization. *Chem.-Biol. Interact.* **119–120**, 29–42
- Wolfe, A. D., Rush, R. S., Doctor, B. P., Koplovitz, I. and Jones, D. (1987) Acetylcholinesterase prophylaxis against organophosphate toxicity. *Fund. Appl. Toxicol.* **9**, 266–270
- Broomfield, C. A., Maxwell, D. M., Solana, R. P., Castro, C. A., Finger, A. V. and Lenz, D. E. (1991) Protection by butyrylcholinesterase against organophosphorus poisoning in nonhuman primates. *J. Pharmacol. Exp. Ther.* **259**, 683–698
- Maxwell, D. M., Castro, C. A., De La Hoz, D. M., Gentry, M. K., Gold, M. B., Solana, R. P., Wolfe, A. D. and Doctor, B. P. (1992) Protection of rhesus monkeys against soman and prevention of performance decrement from submicrogram amounts of glycoproteins. *Toxicol. Appl. Pharmacol.* **115**, 44–49
- Raveh, L., Grunwald, J., Marcus, D., Papier, Y., Cohen, E. and Ashani, Y. (1993) Human butyrylcholinesterase as a general prophylactic antidote for nerve agent toxicity. *Biochem. Pharmacol.* **45**, 37–41
- Kronman, C., Velan, B., Gozes, Y., Leitner, M., Flashner, Y., Lazar, A., Marcus, D., Sery, T., Grosfeld, H., Cohen, S. and Shafferman, A. (1992) Production and secretion of high levels of recombinant human acetylcholinesterase in cultured cell lines: microheterogeneity of the catalytic subunit. *Gene* **121**, 295–304
- Lazar, A., Reuveny, S., Kronman, C., Velan, B. and Shafferman, A. (1993) Evaluation of anchorage-dependent cell propagation systems for production of human acetylcholinesterase by recombinant 293 cells. *Cytotechnology* **13**, 115–123
- Millard, C. B., Lockridge, O. and Broomfield, C. A. (1995) Design and expression of organophosphorus acid anhydride hydrolase activity in human butyrylcholinesterase. *Biochemistry* **34**, 15925–15933
- Ordentlich, A., Barak, D., Kronman, C., Ariel, N., Segall, Y., Velan, B. and Shafferman, A. (1996) The architecture of human acetylcholinesterase active center probed by interactions with selected organophosphate inhibitors. *J. Biol. Chem.* **271**, 11953–11962
- Shafferman, A., Ordentlich, A., Barak, D., Stein, D., Ariel, N. and Velan, B. (1996) Aging of phosphorylated human acetylcholinesterase: catalytic processes mediated by aromatic and polar residues of the active centre. *Biochem. J.* **318**, 833–840
- Hosea, N. A., Radic, Z., Tsigelny, I., Berman H. A., Quinn D. M. and Taylor, P. (1996) Aspartate 74 as a primary determinant in acetylcholinesterase governing specificity to cationic organophosphonates. *Biochemistry* **35**, 10995–11004
- Kronman, C., Velan, B., Marcus, D., Ordentlich, A., Reuveny, S. and Shafferman, A. (1995) Involvement of oligomerization, N-glycosylation and sialylation in the clearance of cholinesterases from the circulation. *Biochem. J.* **311**, 959–967
- Saxena, A., Raveh, L., Ashani, Y. and Doctor, B. P. (1997) Structure of glycan moieties responsible for extended circulatory life time of fetal bovine serum acetylcholinesterase and equine serum acetylcholinesterase. *Biochemistry* **36**, 7481–7489
- Mendelson, I., Kronman, C., Ariel, N., Shafferman, A. and Velan, B. (1998) Bovine acetylcholinesterase: cloning, expression and characterization. *Biochem. J.* **334**, 251–259
- Saxena, A., Ashani, Y., Raveh, L., Stevenson, D., Patel, T. and Doctor, B. P. (1998) Role of oligosaccharides in the pharmacokinetics of tissue-derived and genetically engineered cholinesterases. *Mol. Pharmacol.* **53**, 112–122
- Kronman, C., Chitlaru, T., Elhanany, E., Velan, B. and Shafferman, A. (2000) Hierarchy of post-translation modifications involved in the circulatory longevity of glycoproteins: demonstration of concerted contributions of glycan sialylation and subunit assembly to the pharmacokinetic behavior of bovine acetylcholinesterase. *J. Biol. Chem.* **275**, 29488–29502
- Chitlaru, T., Kronman, C., Velan, B. and Shafferman, A. (2001) Effect of human acetylcholinesterase subunit assembly on its circulatory residence. *Biochem. J.* **354**, 613–625
- Chitlaru, T., Kronman, C., Velan, B. and Shafferman, A. (2002) Overloading and removal of N-glycosylation targets on human acetylcholinesterase: effects on glycan composition and circulatory residence time. *Biochem. J.* **363**, 619–631
- Chitlaru, T., Kronman, C., Zeevi, M., Kam, M., Harel, A., Ordentlich, A., Velan, B. and Shafferman, A. (1998) Modulation of circulatory residence of recombinant acetylcholinesterase through biochemical or genetic manipulation of sialylation levels. *Biochem. J.* **336**, 647–658
- Ellman, G. L., Courtney, K. D., Andres, V. and Featherstone, R. M. (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.* **7**, 88–95
- Bigge, J. C., Patel, T. P., Bruce, J. A., Goulding, P. N., Charles, S. M. and Parekh, R. B. (1995) Nonselective and efficient fluorescent labeling using 2-amino-benzamide and anthranilic acid. *Anal. Biochem.* **230**, 229–238
- Cohen, O., Kronman, C., Chitlaru, T., Ordentlich, A., Velan, B. and Shafferman, A. (2001) Chemical modifications of recombinant human acetylcholinesterase by polyethylene glycol generates an enzyme with exceptional circulatory longevity. *Biochem. J.* **357**, 795–802

- 23 Laub, P. B. and Gallo, J. M. (1996) NCOMP - a Windows-based computer program for noncompartmental analysis of pharmacokinetic data. *J. Pharm. Sci.* **85**, 393–395
- 24 Simon, S., Krejci, E. and Massoulie, J. (1998) A four-to-one association between peptide motifs: four C-terminal domains from cholinesterase assemble with one proline-rich attachment domain (PRAD) in the secretory pathway. *EMBO J.* **17**, 6178–6187
- 25 Velan, B., Kronman, C., Ordentlich, A., Flashner, Y., Leitner, M., Cohen, S. and Shafferman, A. (1993) N-glycosylation of human acetylcholinesterase: effects on activity, stability and biosynthesis. *Biochem. J.* **296**, 649–656
- 26 Li, B., Stribley, J. A., Ticu, A., Xie, W., Schopfer, L. M., Hammond, P., Brimijoin, S., Hinrichs, S. H. and Lockridge, O. (2000) Abundant tissue butyrylcholinesterase and its possible function in the acetylcholinesterase knockout mouse. *J. Neurochem.* **75**, 1320–1331
- 27 Carmona, G. N., Baum, I., Schindler, C. W., Goldberg, S. R., Jufer, R., Cone, E., Slaughter, C. E., Belendiuk, G. W. and Gorelick, D. A. (1996) Plasma butyrylcholinesterase activity and cocaine half-life differ significantly in rhesus and squirrel monkeys. *Life Sci.* **59**, 939–943
- 28 Rosenberg, Y., Luo, C., Ashani, Y., Doctor, B. P., Fischer, R., Wolfe, G. and Saxena, A. (2002) Pharmacokinetics and immunologic consequences of exposing macaques to purified homologous butyrylcholinesterase. *Life Sci.* **72**, 125–134
- 29 Cascio, C., Comite, C., Ghiara, M., Lanza, G. and Ponchione, A. (1988) Use of serum cholinesterase in severe organophosphorus poisoning. Our experience. *Minerva Anesthesiology* **54**, 337–338
- 30 Jenkins, T., Balinsky, D. and Patient, D. W. (1967) Cholinesterase in plasma: first reported absence in Bantu; half-life determination. *Science* **156**, 1748–1750
- 31 Dubaquié, Y., Mortensen, D. L., Intintoli, A., Hogue, D. A., Nakamura, G., Rancatore, P., Lester, P., Sadick, M. D., Filvaroff, E., Fiedler, P. J. and Lowman, H. B. (2001) Binding protein-3-selective insulin-like growth factor I variants: engineering, biodistributions, and clearance. *Endocrinology* **142**, 165–173
- 32 van Vlijmen, B. J., van Dijk, K. W., vant Hof, H. B., van Gorp, P. J. J., van der Zee, A., van der Boom, H., Breuer, M. L., Hofker, M. H. and Havekes, L. M. (1996) In the absence of endogenous mouse apolipoprotein E, apolipoprotein E*2(arg-158 → cys) transgenic mice develop more severe hyperlipoproteinemia than apolipoprotein E*3-Leiden transgenic mice. *J. Biol. Chem.* **271**, 30595–30602
- 33 Keyt, B. A., Paoni, N. F., Refino, C. J., Berleau, L., Nguyen, H., Chow, A., Lai, J., Pena, L., Pater, C., Ogez, J. et al. (1994) A faster-acting and more potent form of tissue plasminogen activator. *Proc. Natl. Acad. Sci. U.S.A.* **91**, 3670–3674
- 34 Aoki, S., Kitagawa, M. and Okumura, K. (2001) Pharmacokinetic studies of Gln117 tissue-type plasminogen activators in rats. *J. Pharm. Biomed.* **26**, 453–462
- 35 Balasubramanian, A. S. and Bhanumathy, C. D. (1993) Noncholinergic functions of cholinesterases. *FASEB J.* **7**, 1354–1358
- 36 Grisaru, D., Sternfeld, M., Eldor, A., Glick, D. and Soreq, H. (1999) Structural roles of acetylcholinesterase variants in biology and pathology. *Eur. J. Biochem.* **264**, 672–686
- 37 Soreq, H. and Seidman, S. (2001) Acetylcholinesterase - New roles for an old actor. *Nat. Rev. Neurosci.* **2**, 294–302

Received 26 August 2003/9 October 2003; accepted 23 October 2003

Published as BJ Immediate Publication 23 October 2003, DOI 10.1042/BJ20031305