Marked increases in concentrations of apolipoprotein in the cerebrospinal fluid of poliovirus-infected macaques: relations between apolipoprotein concentrations and severity of brain injury

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Apolipoproteins in cerebrospinal fluid (CSF) might have important functional roles in the pathophysiology of brain and lipid metabolism in the vascular component. The present study examined apolipoprotein A-I (apo-A-I) and apolipoprotein E (apo-E) levels in CSF and serum from poliovirus-infected macaques. Poliovirus-infected macaques developed motor deficits and were classified into three groups: (1) muscle weakness in one or both legs; (2) partial paralysis in one or both legs; (3) complete paralysis in one or both legs. No motor deficits were evident in the control or sham-treated macaques. Apo-A-I concentrations in CSF were markedly elevated in poliovirus-infected macaques with weakness, partial or complete paralysis, in comparison with either control or sham-treated animals, and were proportional to the severity of motor impairment. Apo-E concentrations in CSF

were also significantly elevated in poliovirus-infected macaques with complete paralysis. The magnitude of increase in CSF apo-A-I or apo-E concentrations was also closely associated with the degree of histologic neurological damage and inflammation (lesion scores). However, no changes in serum apo-A-I and apo-E concentrations were observed in the poliovirus-infected macaques compared with control macaques. Furthermore there were no significant correlations apo-A-I or apo-E concentrations between serum and CSF. We hypothesize that the elevation of apo-A-I and apo-E concentrations after poliovirus infection is caused by immune stimulation within the central nervous system (CNS). Measures of CSF apo-A-I and apo-E levels might serve as a useful marker for the severity and/or the range of CNS injury.

INTRODUCTION

Apolipoproteins are constituents of plasma lipoproteins that serve many functions in lipid metabolism [1]. In the central nervous system (CNS) apolipoproteins are also involved in cholesterol homeostasis [2]. The presence of apolipoprotein in human cerebrospinal fluid (CSF) was first described in 1979 by Roheim et al. [3], and five types of apolipoproteins, including apolipoprotein A-I (apo-A-I) and apolipoprotein E (apo-E), have been identified [4]. Little is known, however, about the function or clinical significance of the apolipoproteins in the CNS. Apo-E is produced by astrocytes [5,6] and oligodendrocytes [7] in the CNS. Apo-A-I and apo-E are also thought to play important roles in the degeneration and regeneration of brain tissues, and in the regulation of the immune system [8,9]. Recent studies have demonstrated that nerve regeneration and cholesterol reutilization occur even in the apo-A-I and apo-E double-knockout mice [10]. Therefore other apolipoproteins such as apolipoproteins A-IV, D and J might be substituting for the apo-A-I and apo-E in such animals. However, we still cannot exclude the possibility that apo-A-I and E have a functional role in the degeneration and regeneration of the CNS in genetically normal subjects.

In the present study, CSF apo-A-I and apo-E concentrations were quantified in normal macaques and macaques during an acute viral infection. Animals inoculated with poliovirus into the

lumbar spinal cord showed neurodegeneration and inflammatory lesions within the target areas of the CNS [11]. We determined whether apolipoprotein concentrations change in proportion to the severity of functional deficits and the degree of neurological damage.

MATERIALS AND METHODS

Materials

BSA was obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.), and the remaining compounds were of analytical grade (Wako Pure Chemical Industries, Osaka, Japan). The enhanced chemiluminescence (ECL) Western blotting analysis system and Hyperfilm-ECL were purchased from Amersham Life Science (Little Chalfont, Bucks., U.K.).

Antibody techniques

Monoclonal antibody against apo-A-I or apo-E and polyclonal antibody against apo-E were prepared by established methods. Briefly, apo-A-I and apo-E purified from a fresh human serum pool were used as antigen. Hybridomas that produced specific antibody toward each of apo-A-I or apo-E were cloned three

Abbreviations used: apo-A-I, apolipoprotein A-I; apo-E, apolipoprotein E; CSF, cerebrospinal fluid; CNS, central nervous system; ECL, enhanced chemiluminescence.

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times [12]. Finally, two clones for apo-A-I and one clone for apo-E were chosen for each ELISA. For mass production of antibodies, established hybridomas were injected intraperitoneally into Balb/c mice, and ascites cells were obtained 2–3 weeks after injection. Mouse IgG from ascites cells was partly purified by a combination of $(\text{NH}_4)_2\text{SO}_4$ precipitation (50% saturated solution) and DEAE ion-exchange chromatography [13]. Polyclonal antibodies against apo-E were prepared by immunization of recombinant apo-E3 into goat. The specificity of antibody for apo-E was confirmed by Western blot analysis, with recombinant apo-E3 as a sample. A single band on the nitrocellulose membrane was found at the position corresponding to apo-E, which was revealed by protein staining, by use of polyclonal or monoclonal antibody. Equal reactivity of monoclonal antibody towards apo-E2, E3 and E4 was confirmed by ELISA. In addition, cross-reactivity of monoclonal anti-(human antibody) to apo-A-I or apo-E towards corresponding macaques apolipoproteins was also confirmed by Western blot analysis (see the Results section).

Western blot analysis

For the determination of apo-A-I or apo-E, SDS/PAGE [4–20% (w/v) gel] was performed by established methods. Briefly, human or macaque serum was diluted with loading buffer and then samples were incubated at 100 °C for 4 min before analysis by SDS/PAGE. Samples separated by SDS/PAGE [4–20% (w/v) gel] were electroblotted on Pure Nitrocellulose Membranes (Bio-Rad Laboratories, Hercules, CA, U.S.A.) for immunological detection. Human or macaque apo-A-I or apo-E was detected with antibody generated against human apolipoprotein, with the ECL Western blotting analysis system.

Apolipoprotein determination in CSF by ELISA

Apo-A-I and apo-E in serum and CSF were determined by highly sensitive and accurate ELISA. The assay was two-step sandwich ELISA format. β -Galactosidase-labelled polyclonal antibody and a specific monoclonal antibody as coated antibody were used for the determination of apo-E, and two distinct specific monoclonal antibodies were used for the apo-A-I determination.

Microtest plates (Nunc, Roskilde, Denmark) were coated with 100 μl of primary monoclonal antibodies (apo-A-I, 8.0 $\mu\text{g}/\text{ml}$; apo-E, 3.0 $\mu\text{g}/\text{ml}$; each diluted with PBS, pH 7.2) in each well and incubated overnight at 4 °C. After incubation the plates were washed twice with washing solution [PBS containing 0.05% (v/v) Tween-20 and 0.1% NaN_3 , pH 7.2] and then blocked with 300 μl of blocking reagent [PBS containing 0.005% (v/v) Tween-20 and 0.5% BSA, pH 7.2]. After blocking had proceeded for 5 h, the plates were washed three times with washing solution and were covered until used (filled with 300 μl of washing solution per each well; stability at least 2 months). Appropriate diluted standards and biological samples (50 μl per well) and 100 μl of reaction reagents [PBS containing 0.5% (w/v) BSA, 0.05% (v/v) Tween-20, 10 mM MgCl_2 and 0.1% NaN_3] were incubated at 25 °C for 1 h. After incubation, the plates were washed three times with washing solution and then incubated at 25 °C for 1 h with β -galactosidase-labelled secondary antibodies (expressing the final concentrations of secondary antibodies as the A_{280} , concentrations of 4×10^{-3} and 8×10^{-3} were used for apo-A-I and apo-E assays respectively) in 100 μl of phosphate buffer, pH 7.0, containing 0.1% (v/v) Tween-20, 0.002% BSA, 0.18% NaN_3 , 0.9 M NaCl and 20 mM MgCl_2 . Plates were then washed three times with washing solution and β -galactosidase activity was measured by using 100 μl of *o*-nitrophenol- β -D-

galactopyranoside solution as substrate. Reaction mixtures were incubated for 3 h at 25 °C and the reaction was terminated by the addition of 100 μl of 0.1 M Na_2CO_3 . The plates were analysed on a Behring ELISA processor II at 405 nm, corrected for absorbance at 650 nm. The protein content of purified apo-A-I or apo-E, the primary standard for the assay, was determined by established methods as well as by amino acid analysis. Secondary plasma standard samples were prepared in our laboratory by pooling plasma from different individuals and storing multiple aliquots at –80 °C. The detection limits of apo-A-I and apo-E were 4.0 and 4.5 $\mu\text{g}/\text{l}$ respectively.

Macaques

Samples were collected from colony-bred rhesus macaques (*Macaca mulatta*, 1–2 years old weighing 2–3 kg) that were already being used as part of Congressionally mandated neurovirulence testing and vaccine development before human administration (United States Code of Federal Regulations: 21; Part 632, Sub-parts A-C, 1988). A single vaccine test of 14 macaques defines the safety of enough vaccine for approx. 250 000 human vaccinations. The macaques were housed in accordance with the standards of the American Association for the Accreditation of Laboratory Animal Care, and adhered to the Guide for the Care and Use of Laboratory Animals prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council. Studies were approved by the Food and Drug Administration Animal Care and Use Committee and the National Institute of Mental Health Animal Care and Use Committee.

Intraspinal poliovirus inoculation and sample collection

Monovalent type II and type III poliovirus in 0.1 ml of carrier was administered directly into the lumbar enlargement of the spinal cord of 61 ketamine-anaesthetized (40 mg/kg) macaques by sterile 27-gauge hypodermic needles. Eight sham-treated animals received carrier alone (0.1 ml) and three additional macaques received no injection into the spinal cord. Macaques were inspected daily by experienced laboratory personnel and the degree of motor impairment was carefully recorded. Macaques developed motor deficits by day 4 and were classified on day 17 as: (1) muscle weakness in one or both legs ($n = 38$); (2) partial paralysis in one or both legs ($n = 9$); (3) complete paralysis in one or both legs ($n = 14$). No motor deficits were evident in the control or sham-treated macaques ($n = 11$). Macaques were killed 17 days later by a lethal dose of sodium pentobarbital, and exsanguinated. A sample of serum was isolated by centrifugation. CSF was collected from the cisterna magna and the lumbar sac. Occult blood in CSF was checked by Uropiece II (Fujisawa Pharmaceutical Co., Osaka, Japan) and positive samples were eliminated from this study. A 3 cm segment of the lumbar enlargement around the inoculation site was immersed in 10% (v/v) formalin solution for the quantification of lesion scores.

Quantification of lesion scores

The degree of neuronal damage and inflammation (lesion scores) were quantified in 15 μm sections of poliovirus target areas that were stained with galloxyanin by experienced laboratory personnel, without prior knowledge of the degree of paralysis of the macaques or the biochemical data [11]. Briefly, 18 sections of the lumbar cord enlargement, 18 sections of the cervical enlargement, three sections each of the left and right brain stem and one section of the precentral gyrus (areas 1, 2 and 3; motor cortex)

were examined. The following scores were assigned to each section: grade 0, no vaccine-related lesions; grade 1, a single vascular lesion consisting of perivascular lymphocytic accumulations, or a single accumulation of glial or mononuclear cells or a focal neuronal lesion (one or more cells showing pyknosis); grade 2, a combination of two or three of the above lesions; grade 3, extensive vascular lesions, cellular infiltration and fewer than two foci of neuronal lesions but with at least one normal neuron remaining; grade 4, gross destruction of neurons with no normal neurons left, and extensive areas of cellular infiltration and perivascular lesions extending throughout the hemisection. The average lesion score is the sum of the scores divided by the number of sections.

Statistical analyses

Results were analysed by computerized statistical packages [14,15]. Group mean values were compared by one-way analysis of variance and Dunnett's *t* test for multiple comparisons as the *post hoc* test. There were no significant differences in any parameter measured between control and sham-treated macaques. Therefore the values were pooled for the purposes of statistical comparison to the poliovirus-infected macaques. Correlation coefficients were calculated by the method of least squares. Values presented are means $\pm$ S.E.M. unless otherwise stated. A *P* value of less than 0.05 was considered statistically significant.

RESULTS

Antibody specificity

The specificities of the monoclonal mouse anti-(human apo-A-I and apo-E) antibodies were confirmed by Western blot analysis (Figure 1). Anti-apo-A-I and anti-apo-E antibodies each recognized a single band with an apparent molecular mass of 28 and 34 kDa respectively. The results demonstrate that each antibody preparation is highly specific for apo-A-I or apo-E. Furthermore these also clearly cross-reacted with macaque apolipoproteins.

Apolipoprotein responses in CSF to poliovirus infection

Apo-A-I concentrations in CSF were significantly elevated in poliovirus-infected macaques with weakness, partial or complete paralysis in comparison with control animals. The magnitudes of the increases in CSF apo-A-I concentrations were closely associated with the severity of motor impairment (Table 1). Apo-E

Table 1 Apo-A-I and apo-E in CSF and serum in response to poliovirus infection

Motor deficits were recorded and samples were collected on day 17 after inoculation. Results are means $\pm$ S.E.M. **P* < 0.05, ***P* < 0.01, significantly different from control values, as determined by analysis of variance followed by Fisher's least significant difference multiple comparison matrix.

	Concentration (mg/l)			
	Control	Poliovirus-infected macaques		
		Weakness	Partial paralysis	Complete paralysis
CSF				
Apo-A-I	1.30 $\pm$ 0.18	5.60 $\pm$ 0.72**	7.16 $\pm$ 1.93**	9.50 $\pm$ 1.22**
Apo-E	0.62 $\pm$ 0.05	0.72 $\pm$ 0.04	0.74 $\pm$ 0.07	0.88 $\pm$ 0.05*
Serum				
Apo-A-I	1350 $\pm$ 190	1500 $\pm$ 250	1430 $\pm$ 230	1390 $\pm$ 150
Apo-E	21.6 $\pm$ 5.6	17.0 $\pm$ 5.8	15.5 $\pm$ 1.8	20.0 $\pm$ 7.7

Figure 2 Relation of lesion scores to severity of motor deficits after poliovirus infection

Each point represents an individual lesion score from poliovirus-infected macaques.

Figure 1 Western blot analysis of human and macaque apolipoproteins with monoclonal mouse anti-human antibody

Each sample lane contained 0.05 μ l of human (H) or macaque (M) serum for apo-A-I, or 2.0 μ l of apo-E serum. Abbreviation: kD, kDa.

concentrations in CSF were also significantly elevated in poliovirus-infected macaques with complete paralysis and tended to increase with weakness and partial paralysis (Table 1). However, serum apo-A-I and apo-E concentrations in the poliovirus-infected macaques with any categories of motor impairment were not significantly different from those of control macaques (Table 1).

The degree of neurological damage (lesion score) was determined in each individual macaque (Figure 2). Average lesion scores were: control, 0 (*n* = 11); weakness, 0.87 ± 0.11 (*n* = 38); partial paralysis, 1.49 ± 0.23 (*n* = 9); complete paralysis, 2.20 ± 0.15 (*n* = 14). There were significant positive correlations between lesion score and CSF apo-A-I (*r* = 0.629, *P* < 0.01) or apo-E (*r* = 0.533, *P* < 0.01) concentrations from all polio-infected animals, but the strong correlation was more evident at lesion scores above 2.0 in apo-A-I (*r* = 0.835, *P* < 0.01 for apo-A-I, and *r* = 0.461, *P* < 0.01 for apo-E). There were no correlations between lesion score and serum concentrations of

apo-A-I or apo-E. The ratio of serum to CSF apo-A-I and apo-E varied between 198 and 1038 and between 19.0 and 35.4 respectively, and depended on the severity of motor impairment. However, there were no significant correlations in apo-A-I ($r = 0.06$) or apo-E ($r = 0.03$) concentrations between serum and CSF.

DISCUSSION

Marked increases in apo-A-I and small increases in apo-E concentrations in CSF were observed in poliovirus-infected macaques, whereas no changes in serum apo-A-I and apo-E concentrations were observed. This is the first study to show quantitative correlations between CSF apolipoprotein levels and the severity of functional deficits. Our results are of particular interest because the magnitude of increases in CSF apo-A-I concentrations were closely associated with the severity of motor impairment (Table 1) and the degree of neurological damage (lesion scores; Figure 3A) in the data set as a whole. By inspection of Figure 3(A), apo-A-I concentrations varied widely in animals with a lesion score of more than 2.0. The reason for this is unclear, but could indicate that the maximal capacity to synthesize apo-A-I had been reached. Another possibility is that although apo-A-I production is accelerated in response to the CNS injury, probably as a response for its repairment, its synthesis might instead be impaired in severely injured lesions.

The mean value of the serum-to-CSF apo-A-I and apo-E ratios in control macaques were 1040 and 35 respectively, which are slightly higher than results from human control subjects [16]. There was also no linear correlation between CSF and serum of those apolipoprotein concentrations. These results indicate that CSF apo-A-I and apo-E are synthesized locally within the CNS. Indeed, several studies have demonstrated that apo-E can be

synthesized by astrocytes and oligodendrocytes as well as monocytes/macrophages after nerve injury [5–8,17]. The infiltration of the CNS by macrophages and other lymphocytic cells is not specific for poliovirus infection, but also occurs within the CNS of a large number of other inflammatory conditions and various neurological diseases [11]. In fact clinical investigations have demonstrated that CSF apo-E concentrations are increased in patients with various inflammatory neurological diseases, but not with non-inflammatory diseases [16]. In contrast, little is known about the origin of apo-A-I in the CNS, whereas there are some reports demonstrating the existence of apo-A-I in CSF [3,4,18]. More recently, Weiler-Güttler et al. [19] demonstrated that apo-A-I is synthesized in pig brain microvascular endothelial cells, which might be a candidate for its origin in the CNS. It is also possible that the elevation of CSF apo-A-I concentrations in poliovirus-infected macaques might reflect the CNS responses including neurons and glial cells as well as infiltrated cells, although we still cannot exclude the possibility of apo-A-I infiltration from the blood stream. Both apo-A-I and apo-E concentrations in CSF might also be regulated by the clearance and binding capacity as well as apolipoprotein synthesis in the CNS.

The magnitude of elevation of CSF apo-A-I concentrations is five times higher than that of apo-E after poliovirus infection. This might be because these apolipoproteins play different roles in the repair of neural injury, probably to lipid transport, at least that caused by viral infection. However, because both apo-A-I and apo-E concentrations in the CSF might also be regulated by clearance and binding capacity as well as by apolipoprotein synthesis in the CNS, this should be clarified in a further study.

In summary, apo-A-I concentrations in CSF were markedly elevated, in comparison with control animals, in poliovirus-infected macaques with weakness, partial or complete paralysis. Apo-E concentrations in CSF were also significantly elevated in poliovirus-infected macaques with complete paralysis. The magnitude of the increases in CSF apo-A-I or apo-E concentrations was closely associated with the degree of neurological damage (lesion scores). Taken together, these apolipoprotein levels in CSF might serve as a useful marker of the degree of CNS injury under certain conditions. We hypothesize that the increased apo-A-I and apo-E concentrations after poliovirus infection reflect the immune stimulation within the CNS. Further studies on the role of apolipoproteins in neurological damage, inflammation and recovery are warranted.

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Figure 3 Correlation between lesion scores and apolipoprotein concentrations in CSF

Each point represents an individual rhesus macaque from the results on poliovirus infection presented in Table 1.

REFERENCES

- Mahley, R. W., Innerarity, T. L., Rall, S. C. and Weisgraber, Jr., K. H. (1984) *J. Lipid Res.* **25**, 1277–1294
- Weisgraber, K. H., Roses, A. D. and Strittmatter, W. J. (1994) *Curr. Opin. Lipidol.* **5**, 110–116
- Roheim, P. S., Carey, M., Forte, T. and Vega, G. L. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4646–4649
- Borghini, I., Barja, F., Pometta, D. and James, R. W. (1995) *Biochim. Biophys. Acta* **1255**, 192–200
- Krul, E. S. and Ttong, J. (1992) *J. Neurosci. Res.* **32**, 227–238
- Pitas, R. E., Boyle, J. K., Lee, S. H., Foss, D. and Mahley, R. W. (1987) *Biochim. Biophys. Acta* **917**, 148–161
- Stoll, G., Muller, H. W., Trapp, B. D. and Griffin, J. W. (1989) *Glia* **2**, 170–176
- Boyles, J. K., Pitas, R. E., Wilson, E., Mahley, R. W. and Taylor, J. M. (1985) *J. Clin. Invest.* **76**, 1501–1513
- Ignatius, M. J., Gebicke-Harter, P. J., Skene, J. H., Schilling, J. W., Weisgraber, K. H., Mahley, R. W. and Shooter, E. M. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 1125–1129
- Goodrum, J. F., Boulton, T. W., Zhang, S. H., Maeda, N. and Popko, B. (1995) *J. Neurochem.* **64**, 408–416

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- 11 Heyes, M. P., Saito, K., Jacobowitz, D., Takikawa, O., Markey, S. P. and Vickers, J. (1992) *FASEB J.* **6**, 2977–2989
 - 12 Kohler, G. and Milstein, C. (1975) *Nature (London)* **256**, 495–497
 - 13 Steinbuch, M., Audran, R. and Pejaudier, L. (1970) *C. R. Seances Soc. Biol. Ses Fil.* **164**, 296–301
 - 14 Feldman, D. S., Gagnon, J., Hofmann, R. and Simpson, J. (1987) *Statview II*, Abacus Concepts, Berkeley, CA
 - 15 Gagnon, J., Roth, J. M., Carroll, M., Haycock, K. A., Plamondon, J., Feldman, D. S. and Simpson, J. (1989) *SuperANOVA*, Abacus Concepts, Berkeley, CA
 - 16 Carlsson, J., Armstrong, V. W., Reiber, H., Felgenhauer, K. and Seidel, D. (1991) *Clin. Chim. Acta* **196**, 167–176
 - 17 Basu, S. K., Ho, Y. K., Brown, M. S., Biheimer, D. W., Anderson, R. G. W. and Goldstein, J. L. (1982) *J. Biol. Chem.* **257**, 9788–9795
 - 18 Pitas, R. E., Boyles, J. K., Lee, S. H., Hui, D. and Weisgraber, K. H. (1987) *J. Biol. Chem.* **262**, 14352–14360
 - 19 Weiler-Güttler, H., Sommerfeldt, M., Papandrikopoulou, A., Mischek, U., Bonitz, D., Frey, A., Grupe, M., Scheerer, J. and Gassen, H. G. (1990) *J. Neurochem.* **54**, 444–450
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