ELSEVIER

Biochimica et Biophysica Acta 1362 (1997) 177–182

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Identification of an active site in the antisecretory factor protein

Ewa Johansson [a], Stefan Lange [b], Ivar Lönnroth [a,*]

[a] *Department of Medical Microbiology and Immunology, Gothenburg University, Guldhedsgatan 10, S-41346 Göteborg, Sweden*
[b] *Department of Clinical Bacteriology, Gothenburg University, Guldhedsgatan 10, S-41346 Göteborg, Sweden*

Received 2 May 1997; revised 4 September 1997; accepted 5 September 1997

Abstract

The antisecretory factor (AF) is a new regulatory protein, produced in the human pituitary gland, which reverses intestinal fluid secretion induced by cholera toxin. We have previously described the cDNA-cloning and characterization of the expressed gene. The aim of this study was to identify the region responsible for the antisecretory activity in the AF-molecule. The recombinant full-length AF has an increased ability to inhibit hypersecretion after treatment with trypsin, indicating that the activity of AF is achieved by smaller peptide fragments. To localize the active region of AF, we expressed truncated forms of the recombinant protein and examined their antisecretory activity against cholera toxin-induced fluid secretion in rat. Nine recombinant AF peptides and four smaller peptides made by solid phase synthesis were tested. Five of the peptides lacked all activity, whereas seven of them were highly active, a dose between 4 and 15 pmol causing a half-maximal inhibition. All the active peptides contained amino acid 36–42 of the AF sequence, whereas none of the inactive peptides contained this sequence. Our results suggest that the site of the antisecretory activity resides in a small region (I)VCHSKTR between position 35 and 42 of the AF molecule. © 1997 Elsevier Science B.V.

Keywords: Peptide; Gastrointestinal; Cholera toxin; Fluid transport; Diarrhea; Intestinal mucosa; Rat

1. Introduction

The permeability of the intestinal epithelium to water and electrolytes is regulated by peptides, which are expressed both in the gut and in the central nervous system [1,2]. Some of them including vasoactive intestinal peptide (VIP), substance P, secretin, pituitary adenylate cyclase activating polypeptide, natriuretic peptide and endothelin increase the transport from the serosal to the mucosal side of the intestinal epithelium [2,3]. Bacterial enterotoxins seem to mimic the effect of neuropeptides on the enteric nervous system, an action causing fluid secretion and diarrhea. Thus, cholera toxin from *Vibrio cholerae* mimic the action of VIP [4], whereas toxin A from *Clostridium difficile* mimics the action of substance P [5]. Furthermore, both VIP and substance P might be involved in the action of each enterotoxin on the enteric nervous system [4,5].

Other peptides, such as neuropeptide Y, peptide YY, somatostatin, enkephalines and sorbin, are known to increase the absorption of electrolytes and fluid in the gut [2,6]. Of these, somatostatin, peptide YY and enkephalins have been shown to inhibit cholera

Abbreviations: AF, antisecretory factor; PCR, polymerase chain reaction; VIP, vasoactive intestinal peptide; GST, glutathione *S*-transferase

* Corresponding author. Fax: +46 31 824122; E-mail: ivar.lonnroth@microbio.gu.se

PII S0925-4439(97)00066-5

toxin-induced fluid secretion [7–9]. Recently, we isolated and sequenced cDNA expressing a new 41 kDa protein called antisecretory factor (AF), which inhibits cholera toxin-induced fluid secretion in the small intestine [10]; 10^{-12} mol of AF causes a half-maximal inhibition in rats and 10^{-11} mol in pigs exposed to cholera toxin [11]. The AF protein is found in the pituitary gland, central nervous system and intestinal mucosa and is secreted into the blood after challenge with bacterial enterotoxins. AF is a potent antagonist of chloride ion transport across nerve cell membranes and probably influences the intestinal permeability via the enteric nervous system [12].

The aim of the present study is to determine the active site of the AF molecule by which it inhibits intestinal hypersecretion. The capacity of truncated forms of AF to inhibit cholera toxin-induced fluid secretion in intestinal loops in rat is tested.

2. Material and methods

2.1. cDNA cloning of AF-fragments

Sense and antisense oligonucleotides corresponding to the human AF-sequence were used in PCR amplification of a set of overlapping products with the previously isolated AF-1 cDNA clone [10]. The fragments were obtained by using primer pairs A/B, A/C, F/C, D/E, F/E, D/G and H/I (Table 1), which have *Eco*R1 sites in frame with the pGEX-1λT expression vector (Pharmacia, Sweden). The seven resulting PCR-products were digested with *Eco*R1, purified with Sephaglas BandPrep Kits (Pharmacia), ligated to the *Eco*R1-site of pGEX-1λT and transfected into Epicurian Coli XL1-Blue cells (Stratagene) as previously described [10]. The inserted DNAs were verified by the dideoxy chain termination sequencing [13] with Sequenase version 2.0 (US Biochemical) using the pGEX forward and reverse universal primers (Pharmacia).

2.2. Expression and purification of fusion proteins

Glutathione *S*-transferase (GST) fusion proteins were expressed in *Escherichia coli* after induction with 0.1 mM isopropyl-β-D-thiogalactopyranoside [14]. Cells were harvested 4 h after induction. After sonication and centrifugation, the supernatant containing the expressed fusion proteins was purified by passing the lysates through glutathione–agarose (Pharmacia). The recombinant AF proteins were released from GST (26-kDa) by bovine thrombin treatment (10 units) for 2 h at 22°C and the pure AF peptides were collected and their concentration determined [10].

2.3. Trypsin-digestion

Purified fusion protein AF-1 (100 ng) was incubated with 10 ng trypsin (type XI: from bovine pan-

Table 1
Sequences of the oligonucleotide PCR Primers

Primer	Sequence	Position
A	5′-CGCGAATTCATGGTGTTGGAAAGCAC-3′	1–16
B	5′-GACGAATTCTGATTCTTGCCTTGTCGG-3′	315–297
C	5′-GCGAATTCGGGTTGGACAGTATGTAGCTTG-3′	240–218
D	5′-GGCGAATTCTTACCCACCAGGCTG-3′	61–78
E	5′-GAGAATTCAAGGCCCACGTTGTTCTCAGGG-3′	153–132
F	5′-AGCGAATTCGTTTGTCATTCAAAGACCCGCAG-3′	106–128
G	5′-CGGAATTCAACTATGTTGACAGCATCCTGC-3′	108–87
H	5′-CGGAATTCCGCAGCAACCCTGAGAA-3′	124–140
I	5′-CGGAATTCAGTGTCTGGGGTGAGTGTGGT-3′	207–184

These oligonucleotide primers were used for PCR amplification of AF cDNA. The position refers to the sequence within the open reading frame of AF cDNA ([10] and Fig. 1).

Table 2
Biological effects of peptides made by recombinant DNA-technique or solid phase synthesis

Code	Oligonucleotide	Peptide	Inhibition of cholera secretion (pmol)
AF-1	1–1131	1–376	1–10
AF-2	186–1131	63–376	> 100
AF-3	361–1131	121–376	> 100
AF-4	1–315	1–105	6
AF-5	1–240	1–80	4
AF-6	106–240	36–80	6
AF-7	61–153	21–51	5
AF-8	61–108	21–36	> 100
AF-9	124–207	42–69	> 100
AF-10	(106–153)	36–51	5
AF-11	–	35–46	14
AF-12	–	35–42	15
AF-13	–	36–41	> 100

The peptides AF-1 to AF-9 were produced in *E. coli* transfected with constructs made from AF-cDNA fragments (see Fig. 1) and pGEX-1λT plasmid. For each oligonucleotide fragment, the start and end is related to the corresponding basepairs in the open reading frame of AF (= AF-1). The positions of amino acid-residues in AF at the start and end of each peptide is also given. The four smallest peptides AF-10 to AF-13 were produced by solid phase organic synthesis. The purified AF-peptides were tested in rat for inhibition of cholera secretion as described in Fig. 2; the amount (pmol) causing halfmaximal inhibition is noted for each peptide.

creas, Sigma) for 1 h at 22°C before the biological activity was tested in the rat loop assay. A similar sample but without AF-1 was prepared as a control in the animal test. The trypsin was not inactivated due to a possible risk of thermolability of the AF protein.

2.4. Sequence analysis

The nucleotide sequence and the deduced protein sequence were analyzed with the MacVector DNA and protein analysis program (Eastman). Hydropathy measurements were determined by using the algorithm of Kyte and Doolittle [15].

2.5. Organic chemical synthesis

The shortest tested recombinant AF peptide showing bioactivity, AF-10 and three shorter peptides AF-11, AF-12 and AF-13 (Table 2) were synthesized by organic chemical synthesis on solid phase using a 430A peptide synthesizer (Applied Biosystems, Foster City, CA). The peptides were purified to > 95% purity on reversed phase HPLC, using a Delta Pak C18 300A column (Waters, Milford, MA) with a mobile phase of water/acetonitrile containing 0.1% trifluoric acid (linear gradient).

2.6. Antisecretory activity

The antisecretory activity was measured in a previously described rat intestinal loop model [16]. In brief, a jejunal loop was challenged with 3 μg of cholera toxin. Various amounts of AF peptides dissolved in 2 ml of PBS or buffer alone (control) were injected intravenously 20–30 s before challenge with cholera toxin. The weight of the accumulated fluid in the intestinal loop (mg/cm) was recorded after 5 h. All samples were tested blindly in the dose range 1–10 pmol; peptides with no activity in this range were further tested in a 10-fold higher dose. Each preparation was tested in at least six rats, except for the trypsin-digested AF-1 and its trypsin control,

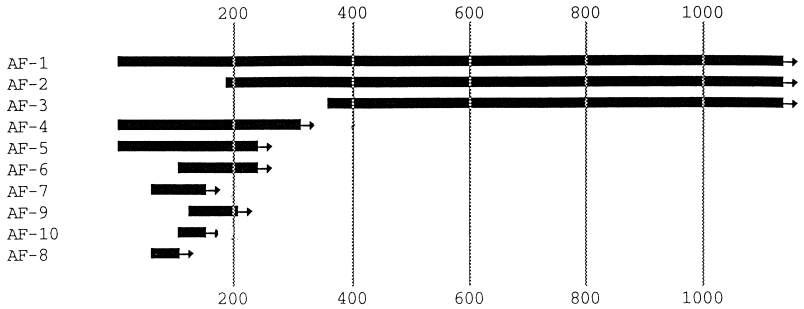

Fig. 1. Horizontal map of AF cDNA as template. The figure shows the expressed part of ten different cDNA fragments generated by PCR. The primers used for PCR are given in Table 1. The number of base pairs are marked.

which were tested in four plus four animals. Fisher's protected least significant difference was used for statistical analysis of the data.

3. Results

3.1. Construction and expression of AF cDNA

Ten different AF-cDNA constructs were used (Fig. 1), of which three AF-1, AF-2 and AF-3 have been described in a previous publication [10]. The additionally seven AF-cDNA fragments (AF-4 to AF-10) were generated by PCR with AF-1 as template (Fig. 1, Table 2). The expression constructs were ligated into the *Eco*R1-site of the pGEX-1λT-vector so that the ORF was in frame with the GST protein. The constructs were transformed into *E. coli*, and overexpression of the fusion proteins was induced with isopropyl-β-D-thiogalactopyranoside, giving moderate levels of recombinant proteins. The fusion proteins were purified on glutathione–agarose, and the AF peptides were cleaved from the bound GST with thrombin.

3.2. Trypsin treatment

The biological activity of the whole AF-1 protein produced in *E. coli* was tested in a rat loop model. When the protein was enzyme-digested with trypsin, the capacity to inhibit cholera toxin-induced secretion was enhanced two-fold compared with the untreated AF-1 (Fig. 2(a)). No change in activity was registered after injection of a trypsin control sample. The increased antisecretory capacity of AF after incubation with trypsin indicated that the activity was exerted by a peptide fragment of the AF molecule.

3.3. Testing of recombinant AF peptides

Nine truncated AF recombinant peptides were tested in the rat model. First, two peptides containing the C-terminal part of the AF molecule, AF-2 and AF-3 were tested (Table 2). Not one of them had activity when tested in the dose interval 1–100 pmol, i.e. 1–100 times the dose of AF giving half-maximal inhibition. In contrast, AF-4 containing the N-terminal part of the AF molecule (residues 1–105), caused a dose-dependent inhibition of cholera secretion (Fig. 2(b)). One pmol of AF-4 reduced the response by

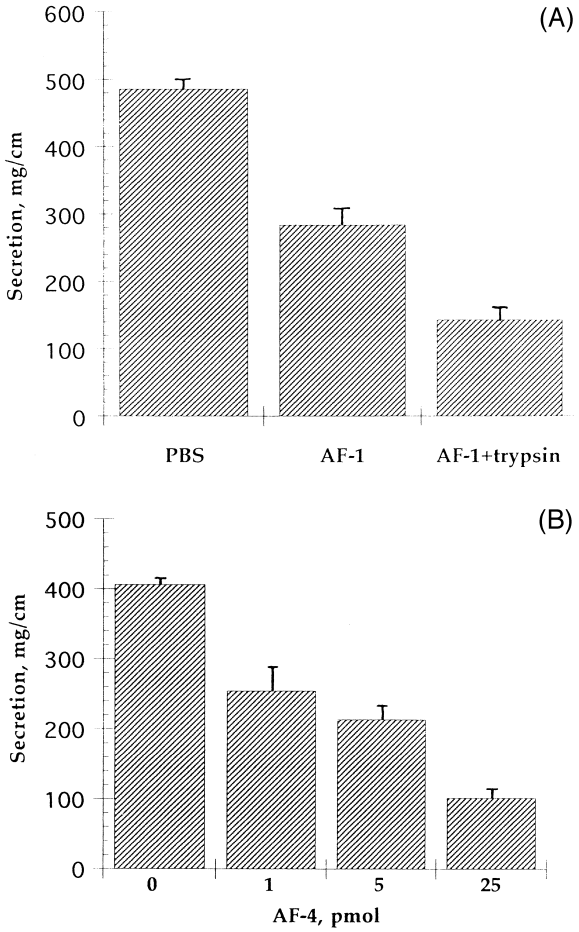

Fig. 2. Biological activity of recombinant AF-1 (3 nmol) before and after trypsin-incubation (a) and of AF-4 (b). Recombinant peptides or buffer controls were injected intravenously in rat; 3 μg of cholera toxin was injected into an intestinal loop; after 5 h, the accumulated fluid (mg/cm intestine) was measured. Each value represents the mean ± S.E. of a group of four to six animals.

37% ($p < 0.01$, $n = 6$), whereas 5 and 25 pmol reduced it by 48 and 76%, respectively. The N-terminal AF-4 peptide was further truncated into smaller peptides: AF-5, AF-6, AF-7, AF-8, AF-9 and AF-10 (Table 2). AF-5, containing residue 1–80 and AF-6, containing residue 36–80, were both active in doses between 1–10 pmol. Similar doses of AF-7 (21–51) and AF-10 (36–51) also exerted activity, whereas AF-8 (21–36) and AF-9 (42–69) had no activity. Thus, the activity of AF-1 seems to reside in the 16-meric peptide AF-10, having the sequence VCH-SKTRSNPENNVGL.

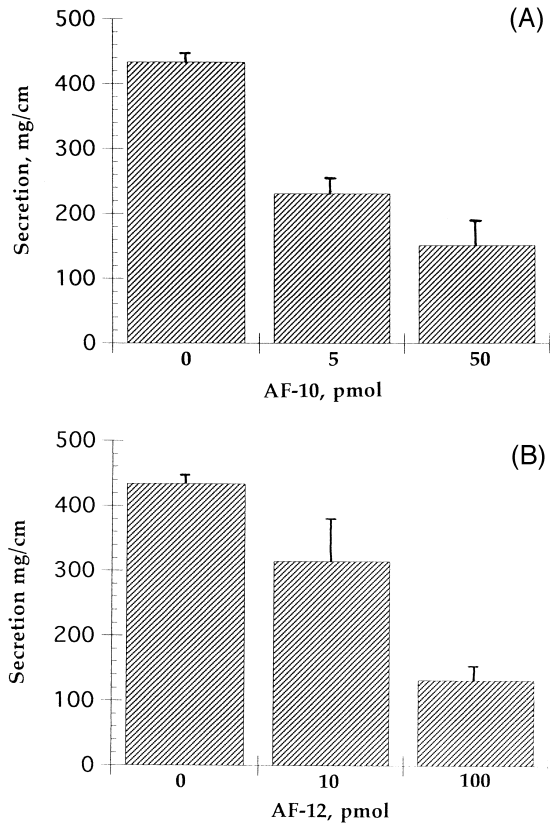

Fig. 3. Biological activity of AF-10 (a) and AF-12 (b) peptides produced by solid phase synthesis. The experimental details are given in Fig. 2. Each value represents the mean ± S.E. of a group of six animals.

3.4. Testing of peptides made by solid phase synthesis

The activity of the AF-10 peptide was confirmed by testing a peptide made by means of solid phase synthesis. The biological activity of the produced AF-10 peptide was similar to that made by the recombinant technique, the dose exerting a half-maximal inhibition being about 5 pmol (Fig. 3(a)).

AF-10 was further truncated into three small peptides AF-11, AF-12 and AF-13 which were produced by solid phase technique (Table 2). These peptides are all relatively hydrophilic and have isoelectric points between 8 and 10. AF-11 (IVCHSKTRSNPE) and AF-12 (IVCHSKTR) were both potent inhibitors of cholera secretion, however, less potent than AF-10 (Fig. 3). In contrast, the hexapeptide AF-13 (VCHSKT) had no activity when tested in the interval 1–100 pmol.

4. Discussion

The results suggest that the antisecretory activity of human AF resides in a short sequence between amino acids 35 and 42 having the sequence (I)VCHSKTR. The peptides containing this sequence (with or without a N-terminal isoleucine) were active, whereas the AF peptides outside this sequence were inert. In most peptides known to inhibit intestinal secretion, the activity seems to reside in short sequences: in sorbin the active site is a heptapeptide [17]; in peptide YY a 15-peptide [18]; in somatostatin an octapeptide [19]; and in opiates a pentapeptide [20]. The activity of AF was increased after treatment with trypsin, suggesting that AF also in vivo might be activated by hydrolyses. Such a conversion into an active peptide occurs in like manner with the precursor proteins to opiates and somatostatin.

Like AF, somatostatin and enkephalin have been used to reverse cholera toxin-induced secretion [7,9]. Whereas somatostatin is active at nanomole and enkephalin at micromole concentrations, AF and its peptide derivatives are active at picomole levels. There is, however, no homology between AF or its active peptide with any other of the antisecretory peptides and, accordingly, their receptors should also differ. Like other peptide receptors mediating antisecretory activity, the AF receptor appears to reside in the enteric nerve system [2]. The active N-terminal part of the AF molecule contains four cysteine groups, whereas no cysteine is present in the remaining two-thirds of the molecule. Intrachain and interchain disulfide cross linkage are likely to occur in the secreted form of the AF peptide. The free sulfide group of the active heptapeptide might be essential for the activity and its susceptibility to oxidation should be tested.

A second activity of the AF molecule is its anti-inflammatory quality observed in the rat's intestine after exposure to toxin A from *C. difficile* [21]. Toxin A binds to receptors resembling the glycolipid B5 or X2 [22], whereas cholera toxin binds to receptors resembling GM1–ganglioside [23]. After binding, the two toxins are internalized into the cells where they exert enzymatic activities: cholera toxin catalyzing ADP-ribosylation of adenylate cyclase [24] and toxin A transferring glucose residues to the rho protein

[25]. The permeability changes of the two toxins are mediated by the enteric nervous system through which system AF seems also to exert its effects [11,12]. To determine whether the same receptor is involved in the antagonistic action of AF on both types of toxicity, it is crucial to investigate the activity of the various truncated AF peptides on toxin A-mediated inflammation and fluid secretion.

The cellular receptor for AF and the possible intracellular signal transduction triggered by the receptor–agonist interaction are unknown. However, the C-terminal part of AF has been shown to bind to polyubiquitin and was therefore suggested to be a regulatory protein in the polyubiquitin–proteasome complex [26]. The transfection of a polyubiquitin gene into the intestinal mucosa of calves is known to be the cause of the lethal bovine virus diarrhea [27]. Because ubiquitin is a stress protein known to be induced during neurological and inflammatory bowel diseases [28,29], it might mediate the action of enterotoxins on the enteric nerve system that lead to intestinal secretion and diarrhea.

Acknowledgements

We thank Ingela Jonson for her excellent technical assistance.

References

[1] M.R. Brown, Ann. N. Y. Acad. Sci. 579 (1990) 8–16.
[2] M.B. Hansen, E. Skadhauge, New aspects of the pathophysiology and treatment of secretory diarrhoea, Physiol. Rev. 44 (1995) 61–78.
[3] T.D. Nguyen, G.G. Heintz, J.A. Cohn, Gastroenterology 103 (1992) 539–544.
[4] A. Sjoqvist, J. Fahrenkrug, M. Jodal, O. Lundgren, Acta Physiol. Scand. 133 (1988) 289–295.
[5] I. Castagliuolo, J.T. LaMont, R. Letourneau, C.P. Kelly, J.C. O'Keane, A. Jaffer, T.C. Theoharides, C. Pothoulakis, Gastroenterology 107 (1994) 657–665.
[6] M. Vagne-Descroix, D. Pansu, H. Jörnvall, M. Carlquist, H. Guignard, G. Jourdan, A. Desvigne, M. Collinet, C. Caillet, V. Mutt, Eur. J. Biochem. 201 (1991) 53–60.
[7] M. Yoshioka, H. Asakura, Y. Hamada, S. Miura, K. Kobayashi, T. Morishita, A. Morita, M. Tsuchiya, Digestion 36 (1987) 141–147.
[8] G.H. Ballantyne, J.R. Goldenring, FX Fleming, S. Rush, J.S. Flint, L.P. Fielding, H.J. Binder, I.M. Modlin, Am. J. Physiol. 264 (1993) G848–854.
[9] D.R. Morgan, J. Sellin, L. Gutierrez, H.L. DuPont, L.V. Wood, Infect. Immun. 48 (1985) 754–758.
[10] E. Johansson, I. Lönnroth, S. Lange, I. Jonson, E. Jennische, C. Lönnroth, J. Biol. Chem. 270 (1995) 20615–20620.
[11] I. Lönnroth, S. Lange, E. Skadhauge, Comp. Biochem. Physiol. 90A (1988) 611–617.
[12] S. Lange, I. Lönnroth, A. Palm, H. Hyden, Pflugers Arch. 410 (1987) 648–651.
[13] F. Sanger, S. Nicklen, A.R. Coulson, Proc. Natl. Acad. Sci. U.S.A. 74 (1977) 5463–5467.
[14] D.B. Smith, K.S. Johnson, Gene 67 (1988) 31–40.
[15] J. Kyte, R.F. Doolittle, J. Mol. Biol. 157 (1982) 105–132.
[16] S. Lange, FEMS Microbiol. Lett. 15 (1982) 239–242.
[17] P. Nicol, R. Vienet, G. Jourdan, C. Dumas, F. Abou-el-Fadil, H. Benech, J.M. Grognet, T. Tarrade, D. Pansu, M. Descroix-Vagne, Peptides 16 (1995) 1343–1350.
[18] B. Eto, M. Boisset, P. Eden, A. Balasubramaniam, J.F. Desjeux, Peptides 16 (1995) 1403–1409.
[19] P.E. Battershill, S.P. Clissold, Drugs 38 (1989) 658–702.
[20] M.P. Primi, M.J. Fargeas, L. Bueno, Regul. Pept. 21 (1988) 107–115.
[21] J. Torres, E. Jennische, S. Lange, I. Lönnroth, Gut 32 (1991) 791–795.
[22] S. Teneberg, I. Lönnroth, J.F. Torres, U. Galili, M. Ölvegård-Halvarsson, J. Ångström, K.A. Karlsson, Glycobiology 16 (1996) 599–609.
[23] J. Holmgren, I. Lönnroth, J.E. Månsson, L. Svennerholm, Proc. Natl. Acad. Sci. U.S.A. 72 (1975) 2520–2524.
[24] D.M. Gill, Proc. Natl. Acad. Sci. U.S.A. 72 (1975) 2064–2068.
[25] I. Just, M. Wilm, J. Selzer, G. Rex, C. Von Eichel-Streiber, M. Mann, K. Aktories, J. Biol. Chem. 270 (1995) 13932–13936.
[26] K. Ferrell, Q. Deveraux, S. van Nocker, M. Rechsteiner, FEBS Lett. 381 (1996) 143–148.
[27] N. Tautz̄, G. Meyers, H.J. Thiel, Processing of poly-ubiquitin in the polyprotein of an RNA virus, Virology 197 (1993) 74–85.
[28] J. Lowe, R.J. Mayer, Neuropathol. Appl. Neurobiol. 16 (1990) 281–291.
[29] D. Reumaux, C. Meziere, J.F. Colobel, P. Duthilleul, S. Mueller, Clin. Immunol. Immunopathol. 77 (1995) 349–357.