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The localization and specificity of guinea pig inner ear antigenic epitopes

MING-YU CAO, M.D.*†, MICHEL GERSDORFF, M.D.*, NAÏMA DEGGOUJ, M.D.*,
JEAN-PAUL TOMASI, M.D.†

Abstract

In this study, we investigated the relative localization of some antigenic epitopes in the inner ear. The inner ear protein antigens were extracted from various parts of the guinea pig inner ear. Brain, kidney, lung, heart and liver extracts were also obtained. We found by SDS-polyacrylamide gel electrophoresis that total inner ear extracts separated into three high concentration polypeptide bands with molecular weights of approximately 30, 42, 58 kd and three low density bands of 20, 25 and 35 kd. The 30 kd band was found mainly in the extract of the spiral ganglion and the acoustic nerve in the modiolus. The 42 and 58 kd bands were detected in the extract of the spiral ligament and the stria vascularis. The Organ of Corti and the basilar membrane extract gave rise to three bands of 30, 42 and 58 kd. Twenty-eight of the 75 sera from patients with inner ear disease reacted with the 30 and 58 kd bands of the inner ear protein extracts by immunoblotting. Sixteen of these 28 positive sera were then used to probe immunoblots of the brain, kidney, lung, heart and liver extracts. The 58 kd band was also found in protein extracts of the brain, the lung and the liver. This study suggests that the 30 kd antigenic epitope may be mainly related to the acoustic nerve and that the 58 kd antigenic epitope is not cochlear specific.

Key words: Cochlea; Hearing loss, Sensorineural; Autoantibodies; Immunoblotting

Introduction

More and more experimental and clinical evidence indicates that some inner ear diseases may be related to autoimmunity. It has been demonstrated that the inner ear can be a target organ in animal experiments (Yoo *et al.*, 1983, 1984; Harris, 1987; Yamanobe and Harris, 1992). Moreover, the inner ear may share a common antigen (or cross-reacting antigen) with other organs or tissues, such as collagen, which is related to autoimmune inner ear disease (Yoo *et al.*, 1983, 1984; Yoo, 1984; Joliat *et al.*, 1992) and is widespread. These common antigens may also be responsible for a more general expression of the disease. However, the characterization of a unique inner ear antigenic epitope with which the autoantibodies of patients react, is uncertain (Harris and Sharp, 1990; Joliat *et al.*, 1992; Lejeune and Charachon, 1992; Veldman *et al.*, 1993; Gersdorff *et al.*, 1994). There is no doubt that research in this domain will enhance our understanding of the mechanisms of autoimmune inner ear disease.

Some inner ear extracts from humans (McCabe, 1979; Hughes *et al.*, 1984; Arnold *et al.*, 1985; Joliat *et al.*, 1992) and various animals, such as cattle (Harris, 1987), guinea pig (Soliman, 1989), chick (Orozco *et al.*, 1990) and swine (Veldman *et al.*, 1993), have been used for experimental and clinical studies in order to investigate the presence and the character of autoantibodies in the serum of patients and to elucidate the mechanism of some idiopathic progressive sensorineural hearing loss. All extracts were obtained either from the entire inner ear tissue or from some location in the inner ear. Using immunoblotting, it has been found that several protein bands of these inner ear extracts have a reactivity with autoantibodies of patients with inner ear disease (Harris and Sharp, 1990; Joliat *et al.*, 1992; Veldman *et al.*, 1993; Gersdorff *et al.*, 1994), although the extracts themselves may be derived from different sources. Nevertheless, whether these antigenic epitopes have a relative distribution in the various parts of the inner ear, and are specific to the inner ear and relevant to the pathogenesis of autoimmune inner ear disease, remains unclear.

In this study, the protein antigens were extracted from the inner ear, brain, kidney, heart, lung and liver of guinea pigs. Sera from patients with inner ear disease were used to probe the various tissue immunoblots. The particular localization in the inner ear of the different molecular weight protein antigens was investigated. Cochlear specificity of the inner ear antigenic epitopes was evaluated.

Materials and methods

Sera

The sera from 75 patients with Ménière's disease, idio-

From the Department of Otorhinolaryngology* and Laboratory of Autoimmunity†, Université Catholique de Louvain, Cliniques Universitaires St-Luc, Brussels, Belgium.
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pathic progressive sensorineural hearing loss, otosclerosis, sudden deafness or benign positional vertigo were collected prior to any corticosteroid treatment. A detailed medical history was established for all patients. Forty-two control sera were obtained from unrelated donors with normal hearing.

Antigen preparation

Inner ear disease was obtained from 10 Hartley guinea pigs by microdissection (Cao *et al.*, 1988). During the dissection, the inner ear tissues were separated into different pools: (1) total inner ear; (2) the vestibular organ (semicircular duct, ampulla, utricule, and saccule); (3) the spiral ligament and the stria vascularis; (4) Corti's organ and the basilar membrane; and (5) the acoustic nerve in the modiolus. The brain, the kidney, the lung, the heart and the liver of guinea pigs were also acquired separately. Each of these pools was homogenized by ultrasonification at 20 kHz in 10 volumes of sample buffer (10 mM Tris-HCl; 1 mM EDTA; 2.5 per cent SDS; and five per cent beta-mercaptoethanol; pH 7.4) containing the following protease inhibitors: 10 mg/ml antipain, 2 mg/ml pepstatin, 40 mg/ml phenylmethylsulphonyl fluoride (PMSF), 10 mg/ml chymostatin and 10 mM *N*-ethylmaleimide (Sigma Chemical Co., St Louis, MO). The homogenate was then centrifuged at 3000 rpm for 10 min; the supernatant was decanted and filtered through a 0.22 μm filter, and then placed for five minutes in a water bath at 100°C. When cooled, the protein concentration was adjusted to 1 g/l. The resultant antigen preparations were frozen at −30°C until required for electrophoresis.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Each of the tissue extracts (inner ear, brain, kidney, lung, heart and liver) was prepared for electrophoresis by adding an equal volume of sample buffer, containing 25 per cent sucrose. A standard protein sample (Pharmacia LKB, Uppsala) to determine the molecular weights of the bands, was also prepared. Electrophoresis was performed on a 10–15 per cent SDS-polyacrylamide gel (Pharmacia, Uppsala) in an automated electrophoresis apparatus (Phastsystem, Pharmacia) by applying 70 volthours. Protein bands were visualized by Coomassie blue staining.

Immunoblotting

Immediately after electrophoresis, the separated proteins from unstained SDS-PAGE gels were transferred onto nitrocellulose sheets (Millipore Corp., Bedford, MA), by using a trans-blot cell (Phastsystem) and a transfer buffer (48 mM Tris base, 39 mM glycine, 0.037 per cent SDS, and 20 per cent methanol) for one hour. The quality of the transfer was checked by staining the gels. The gels should not show any coloured bands after the transfer. The nitrocellulose was dried and then cut into strips. One of the strips was stained with amido black (0.1 per cent amido black B-10, 45 per cent methanol and 10 per cent acetic acid solution) and destained with 25 per cent methanol and seven per cent acetic acid solution, to visualize the total transferred protein. The remaining nitrocellulose strips were used for the immunoblot assays.

The strips were washed with TBS (20 mM Tris-HCl; 500 mM NaCl; pH 7.5) for 10 minutes and incubated for two hours at room temperature with five per cent non-fat dry milk in TTBS (TBS and 0.05 per cent Tween-20; pH 7.5), followed by three hours of incubation with a 1/50 dilution of the test sera in the five per cent milk at room temperature. The strips were washed twice in TTBS, and incubated again for two hours with a 1/600 dilution of an alkaline phosphatase-conjugated second antibody (rabbit anti-human IgA, IgG, IgM, (DAKO, Denmark) in TBS. Finally, the strips were washed twice in TTBS and once in TBS, and developed with a freshly prepared solution of alkaline phosphatase-conjugate substrate (Bio-Rad). The strips were rinsed and read.

Results

Analysis of inner ear proteins by SDS-PAGE

The total inner ear protein extract separated into six main bands: three high concentration polypeptide bands with a molecular weight of approximately 30, 42, 58 kd and three low density polypeptide bands of 20, 25 and 35 kd (Figure 1). The relative distribution of the polypeptide bands in the different sites of the inner ear are shown in Table I. These bands could be associated with different parts of the inner ear. The 30 kd band was mainly found in the extract of the spiral ganglion and the acoustic nervous fibre in the modiolus. The 42 kd and 58 kd bands were related to the spiral ligament and the stria vascularis. In the extract of Corti's organ, there were three bands with molecular weights of 30, 42 and 58 kd, but their concentration was low in this preparation. The extract of the vestibular organ did not show any detectable presence of these bands, possibly due to a very low concentration of antigen protein in the extract.

Western immunoblotting patterns

In immunoblots of the guinea pig inner ear extracts, two protein bands were detected by sera from patients with inner ear disease (Figure 2): one with a molecular weight of 30 kd and the other of 58 kd. Twenty-eight of the 75 sera (37 per cent) reacted with the inner ear protein extract. Twenty-four of these 28 sera bound only to the 30 kd band. Of the remaining four sera, two reacted only with the 58 kd band and two reacted with both the 30 and the 58 kd bands (Table II). Two of the 42 control sera (4.8 per cent) had a very faint reaction with the 30 kd band. The data from the patient and control groups were statistically compared using the chi-square test ($p<0.001$).

The patient groups consisted of individuals with idiopathic progressive sensorineural hearing loss, Ménière's disease, otosclerosis, sudden deafness, and positional vertigo. The results of the immunoblots of the inner ear extracts with sera from various diseases are shown in Table III. The antibody against the 58 kd inner ear protein band was detected in four sera from the patients with idiopathic progressive sensorineural hearing loss. The 30 kd band was found in various patient groups.

Sixteen sera were randomly selected from 28 anti-cochlear positive sera. The 16 sera and 10 control sera were used to probe the brain, kidney, lung, heart and liver extracts respectively. The 58 kd band was also found on the immunoblots of the brain, the lung and the liver. Two of the 16 sera reacted with the 58 kd band of the brain

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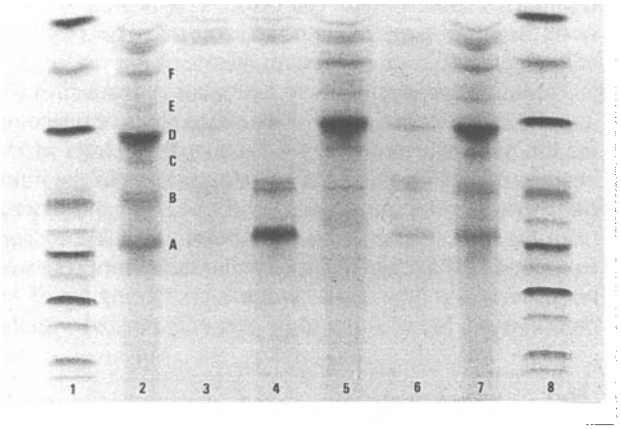

FIG. 1

SDS-PAGE patterns of the inner ear protein extracts from guinea pigs with Coomassie blue staining. Lane 2 and lane 7 show the extracts from total inner ear tissue. The molecular weights of the bands are 58(A), 42 (B), 35(C), 30 (D), 25(E), 20 (F) kd. Lane 3: shows the extract from the vestibular organ; lane 4: the extract from the spiral ligament and the stria vascularis; lane 5: the extract from the acoustic nerve in the modiolus; lane 6: the extract from Corti's organ and the basilar membrane. Lane 1 and lane 8 show the co-migration of standard proteins. The molecular weights of the 11 standard protein bands are 14.4, 20.1, 30, 43, 53, 67, 76, 94, 116, 170, and 212 kd respectively.

extract. Three sera reacted with the 58 kd band of the lung extract. Seven sera reacted with the 58 kd band of the liver extract. Moreover, three sera bound to the 52 kd band of the brain extract (Figure 3). No specific band was found in the immunoblots of the kidney and the heart. None of these bands (30, 52, and 58 kd) was found in the 10 control sera.

Discussion

It has been shown that the endolymphatic sac plays an important role in the generation of inner ear immune responses and the host defence of the inner ear (Tomiyama and Harris, 1987). The inner ear is capable of responding to antigen challenge (Harris, 1983, 1984). On the other hand, the immunoglobulins in the perilymph could be derived mainly from the blood vessels of the perilymphatic space by ultrafiltration (Mogi *et al.*, 1982). When sequestered inner ear antigens are released or self-antigens in the inner ear are modified by various causes, there exists a cross-reactivity of the inner ear antigens with some microorganism antigenic epitopes or other tissue antigenic epitopes, and the normal surveillance mechanism of self-tolerance breaks down, resulting in self-reactive antibodies and/or activated lymphocytes reacting with the relevant self-antigen and causing damage to the inner ear tissue, and thus autoimmune inner ear disease may arise. Our data show that antibodies against inner ear protein extracts were detected by Western blot in the sera of patients with inner ear disease. It suggests that humoral immunity may be involved in some inner ear diseases.

Veldman *et al.* (1993) investigated 76 sera from patients with rapidly progressive sensorineural hearing loss and sudden deafness, using swine inner ear blots. All antigenic epitopes with a molecular weight of 27, 45, 50, 68, 80 kd were found to be not specific for the cochlea. In this study, 26 of the 75 sera from patients with inner ear disease recognized the 30 kd band of the inner ear extract. This band was not found in the extracts of the brain, the kidney,

TABLE I

THE DISTRIBUTION OF THE INNER EAR PROTEIN BANDS IN THE DIFFERENT PARTS OF THE INNER EAR

	20 kd	25 kd	30 kd	35 kd	42 kd	58 kd
TIE	+	+	+++	+	+++	+++
AN	+	+	+++	+	+	–
OC	–	–	+	–	+	+
SL&SV	–	–	–	–	+++	+++
VO	–	–	–	–	–	–

TIE: Total inner ear. AN: The spiral ganglion and the acoustic nervous fibre in the modiolus. OC: The organ of Corti and the basilar membrane. SL&SV: The spiral ligament and the stria vascularis. VO: The vestibular organ. +: Relative density of the protein band. –: No band.

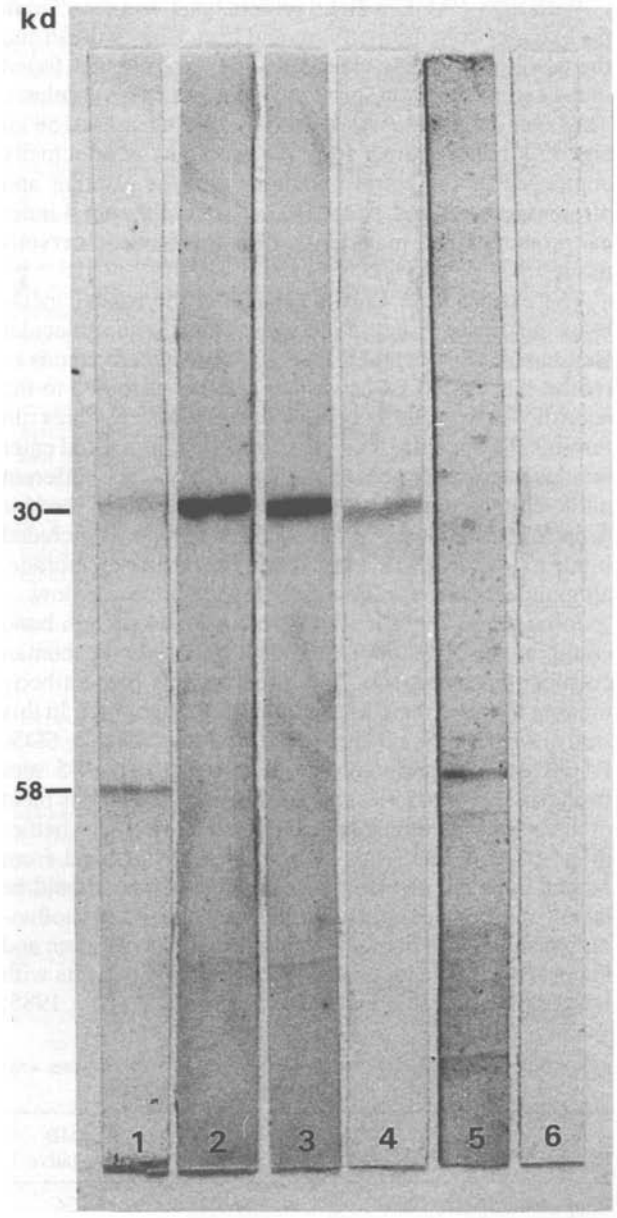

FIG. 2

Immunoblot of inner ear extract with the sera from patients with inner ear disease (lanes 1–5), and control (lane 6). In lane 1, the serum from a patient reacts with both the 30 and 58 kd bands. Lanes 2–4 show three sera binding to the 30 kd band. Lane 5 shows the pattern of inner ear disease serum reactivity with the 58kd protein band. A control serum (lane 6) does not react with any of these bands.

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TABLE II

FREQUENCY OF DETECTION OF REACTION OF VARIOUS PROTEIN BANDS WITH INNER EAR DISEASE AND CONTROL SERA

Protein bands	Inner ear disease sera (n = 75)	Control (n = 42)
30 kd	24	2
58 kd	2	0
30 and 58 kd	2	0
No bands	47	40

the lung, the heart and the liver. However, the 58 kd band could be found in the inner ear, the brain, the lung and the liver. Some sera could bind to the 58 kd antigenic epitope in the different tissues, so the 58 kd antigenic epitope was not specific to the inner ear.

With SDS-PAGE, a 30 kd protein band was detected in the extracts from the modiolus and from Corti's organ and the basilar membrane, whereas no 30 kd band was found in the extract from the spiral ligament and stria vascularis. The latter extracts separated into two main bands of 58 kd and 42 kd. The extract from the modiolus is principally composed of the spiral ganglions and the afferent and efferent nerve fibres, so the 30 kd band of the total inner ear protein extract may derive from the acoustic nervous tissue.

The extract from Corti's organ and the basilar membrane also gave rise to three weak bands with molecular weights of 30, 42 and 58 kd on SDS-PAGE. It stands to reason that the 30 kd band may also be attributed to the acoustic nerve tissue, because the acoustic nerve fibres run through the opening of the osseous spiral lamina and enter the basilar membrane, and then afferent and efferent nerve-ending boutons attach to the lower ends of the hair cells. The component of the cochlear nerve was included in the extract of Corti's organ and the basilar membrane, although its concentration in this preparation was low.

Joliat *et al.* (1992) reported that a 30 kd protein band could be discerned on gel electrophoresis of human cochlear tissue extracts, and three patients had antibody binding to this protein as detected by Western blot. In this study, we also found the 30 kd protein band on SDS-PAGE gel and immunoblot. Twenty-six of the 75 sera from patients with inner ear disease reacted with this band of the guinea pig total inner ear extract. However, whether there exists a difference between the 30 kd band from human inner ear and from guinea pig inner ear should be taken into consideration. Furthermore, an immunofluorescence was also demonstrated in the spiral ganglion and the nervous tissue incubated with sera from patients with inner ear disease in previous studies (Arnold *et al.*, 1985; Gersdorff *et al.*, 1994). The 30 kd polypeptide derived from the spiral ganglion and the acoustic nerve might be an antigenic epitope in these tissues.

TABLE III

RESULTS OF IMMUNOBLOTTING IN VARIOUS PATIENT GROUPS AND CONTROLS

Diagnosis	No. of samples	IMB positive	IMB negative
Idiopathic PSNHL	39	15	24
Ménière's disease	21	10	11
Otosclerosis	5	2	3
Sudden deafness	6	1	5
Positional vertigo	4	0	4
Controls	42	2	40

PSHL: Progressive sensorineural hearing loss. IMB: Immunoblotting.

We think that a proteolytic degradation product of the larger molecular weight 58 kd antigen does not account for the presence of the 30 kd band in the extract of the inner ear, because this band was found only in the inner ear extracts containing nervous tissue, like the extract from the acoustic nerve in the modiolus and the extract from the Corti's organ and the basilar membrane, but was not observed in other tissue extracts containing the 58 kd protein band. Meanwhile, some sera only reacted with the

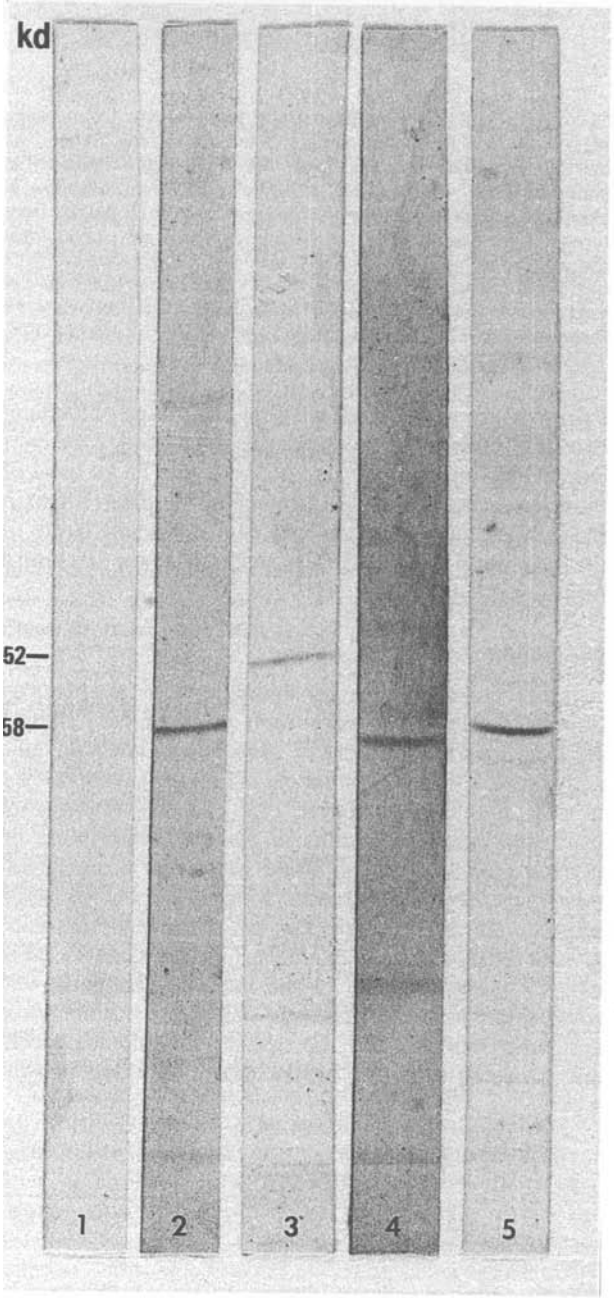

FIG. 3

Immunoblots of the brain, liver and lung extracts with sera from patient and control. The cross-reacting antibody in a serum from the patient with inner ear disease bound to the 58 kd protein band in the extracts from the brain (lane 2), the liver (lane 4) and the lung (lane 5). Lane 3 shows a 52 kd band of the brain extract which reacted with the serum from a patient. Lane 1 is an immunoblot of the brain extract which reacted with a control serum.

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30 kd protein band or with the higher molecular weight protein band in the same extract. On the other hand, however, when the inner ear protein was extracted, the protease inhibitors were added to the sample buffer, and thus the activity of some proteases would have been inhibited.

The 58 kd antigenic epitope in the inner ear mainly originated from the lateral wall of the cochlear duct. This antigenic epitope was also detected in the brain, the lung and the liver. Seven of the 16 positive sera against the inner ear extract was also reacted with the 58 kd band of the liver extract. It showed that there was a high concentration of the 58 kd antigenic epitope in the liver extract.

Conclusions

Our data show that the various molecular weight inner ear antigens detected on the SDS-PAGE, were distributed in different sites of the guinea pig inner ear. The 30 kd protein band may be mainly related to the acoustic nerve tissue in the modiolus. Sera from patients with inner ear disease reacted with the 30 kd band but some also reacted with the 58 kd band. The 30 kd antigenic epitope was only found in the inner ear in this study, and the 58 kd antigenic epitope was not specific for the inner ear. Circulating autoantibodies were found in 37 per cent with inner ear disease. Whether these antigenic epitopes have any direct bearing on the pathogenesis of autoimmune inner ear disease requires further study.

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Address for correspondence:
Dr Ming-Yu Cao,
Laboratoire d'Auto-immunité,
Université Catholique de Louvain,
Cliniques Universitaires St-Luc,
Ave. Hippocrate 53,
1200 Brussels,
Belgium.

Fax: +32–2–76 9428.