The serum of achalasia patients alters neurochemical coding in the myenteric plexus and NO-mediated motor response in normal human fundus

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Abbreviations: ChAT (choline acetyltransferase); ENS (enteric nervous system); IR (immunoreactive); LOS (lower oesophageal sphincter); NSE (neurone-specific enolase); NOS (nitric oxide synthase); SP (substance P); VIP (vasoactive intestinal polypeptide).

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Summary

Background & Aims: Achalasia is a disease of unknown aetiology. An immune mechanism has been suggested on the basis of previous morphological observations. The objective of this study was to test whether the serum of achalasia patients was able to reproduce the phenotype and functional changes that occur with disease progression in an ex vivo human model. Methods: Specimens of normal human fundus were maintained in culture in the presence of serum of patients with achalasia, gastro-oesophageal reflux disease (GORD) or healthy subjects (controls). Immunohistochemical detection of choline acetyltransferase (ChAT), neurone-specific enolase (NSE), vasoactive intestinal polypeptide (VIP), nitric oxide synthase (NOS), and substance P (SP) was carried out in whole mounts of gastric fundus myenteric plexus. In addition, the effects of achalasia serum upon electrical field stimulation (EFS)-induced contractions were measured in circular muscle preparations. Results: Serum of achalasia patients did not affect the number of myenteric neurones. Tissues incubated with serum of achalasia patients showed a decrease in the proportion of NOS (-26% of NSE-positive neurones, p=0.016) and VIP (-54%, p =0.09) neurones, and a concomitant increase of ChAT neurones (+16%, p<0.001), as compared to controls. In contrast, GORD serum did not modify the phenotype of myenteric neurones. The area under the curve of EFS-induced relaxations (abolished by L-NAME) was significantly decreased following incubation with serum of achalasia patients as compared to controls (-7.6±2.6 vs - 14.5±5.0, p=0.036). Conclusions: Serum of achalasia patients is able to induce phenotypical and functional changes which reproduce the characteristics of the disease. Further identification of putative seric factors and mechanisms involved could lead to the development of novel diagnostic and/or therapeutical strategies in achalasia.
Introduction

Achalasia is a primary motility disorder of the oesophagus characterised by the disappearance of oesophageal peristalsis and a poorly relaxing lower oesophageal sphincter (LOS) after swallowing, frequently associated with an increased LOS pressure. Patients with achalasia exhibit a loss of the swallow-induced oesophageal motor inhibitory activity [1] as well as an impaired function of the proximal stomach [2]. The cause of achalasia remains unknown although available data suggest that hereditary, viral, neurodegenerative, or autoimmune factors may be involved [3].

One of the hallmarks of achalasia is a phenotypic switch of myenteric neurones both in the oesophagus and in the proximal stomach. More specifically, a decrease in the number of nitric oxide (NO) synthase immunoreactive neurones has been reported in oesophageal tissues as well as in the proximal stomach of these patients [4,5]. In addition, several observations have shown a decrease or even an absence of myenteric neurones predominantly in the distal oesophagus of achalasia patients [6-8]. Although the pathophysiological factors responsible for alterations of the enteric innervation remain unknown, various observations support a role for an immune/autoimmune component in achalasia: a) activated T-cell lymphocytic infiltrates within the myenteric plexus [9,10], b) the presence of antimyenteric neurones antibodies in the serum [11-15] and finally, c) the association with antigens of the class II major histocompatibility complex (MHC) [14,16]. Indeed MHC II antigens (DR-DQ) have been found to be associated with other autoimmune diseases such as multiple sclerosis, Sjögren’s syndrome, Hashimoto’s thyroiditis, systemic lupus erythematosus [17,18]. In addition an important feature of some autoimmune neuropathies is that the serum as well as IgG of patients is able to alter neurone properties. These alterations range from changes in the electrophysiological properties such as altered calcium flux [19-21], blockade of nicotinic cholinergic receptors [22] or voltage-gated potassium channels [23], and can lead to neuronal cell death (paraneoplastic syndrome with anti-Hu antibodies) [24]. The effects of serum of achalasia patients upon the properties of myenteric neurones are however currently unknown. Therefore, the demonstration of a direct effect of serum of achalasia patients on intact human tissues, and the reproduction of an achalasic phenotype as well as motility dysfunction, could represent an important step in the understanding of the mechanisms of the disease with subsequent potential therapeutic applications.

This study aimed at testing the hypothesis that serum of achalasia patients can affect the phenotype of human gastric myenteric neurones as well as the neurally-mediated motor response in an ex vivo model of normal human fundus. In order, to test the specificity of the serum of achalasia, we also characterized the effects of the serum of Gastro-oesophageal Reflux Disease (GORD) patients upon the neurochemical phenotype of myenteric neurones.

Material and methods

Serum of achalasia patients, GORD patients and controls.

The sera from 18 achalasia patients obtained from our serum bank were used for either neurochemical coding and/or in vitro motility studies (11 males, 7 females, age: 47 ± 18 yrs [mean ±SD]). The duration of the disease was 7 ± 7 yrs, the LOS pressure was 29 ± 23 cmH2O and the percentage of LOS relaxation during swallowing was 27 ± 27%. All but one had a non-vigorous form of achalasia (i.e., amplitude of contraction under 50 cm H2O [25]). Twelve patients had never been treated for their achalasia prior to the start of the study.
to collection of serum samples. Three patients had myotomy and three others had pneumatic dilation.

The sera of 11 healthy volunteers (9 males, 2 females, age: 31 ± 8 yrs) and of 5 GORD patients (3 males, 2 females, age: 57 ± 13 yrs) were used as separate controls. All GORD patients had reflux symptoms requiring continuous proton pump inhibitor treatment.

Blood was collected and centrifuged (7000 g, 15 min). Sera were stored at -80°C.

Ex vivo model of normal human fundus

Specimens were obtained from the intact fundus of patients not previously treated with radiotherapy undergoing surgery for adenocarcinoma of the oesophagus. According to the guidelines of the French Ethics Committee for Research on Human Tissues, these specimens were considered as waste and cannot be used for pathological diagnosis. Neurochemical coding was studied in 10 specimens (7 from male and 3 from female patients, mean age: 66 ± 11 yrs). In vitro motility studies were conducted in 6 specimens (2 from male and 4 from female patients, mean age: 71 ± 12 yrs).

Specimens from the large curvature (about 5 cm away from the LOS), at a distance from the tumour, were resected and immediately processed in the Pathology Department. The tissue was placed in 4°C oxygenated sterile Krebs solution containing (in mM) 117 NaCl, 4.7 KCl, 1.2 MgCl₂ 6.0 H₂O, 1.2 NaH₂PO₄, 25.0 NaHCO₃, 2.5 CaCl₂ 2H₂O, and 11.0 glucose. It was then rapidly transported to the laboratory for dissection.

The piece was pinned flat mucosa up in a dissection dish containing ice-cold sterile oxygenated Krebs solution that was changed every 10-15 min. Preparations were 4-7 x 4-5 cm in size. The mucosa was then carefully removed under a dissection microscope. The tissue was washed 4 times with sterile Krebs solution and pinned back in a sterile Sylgard-coated Petri dish before addition of sterile culture medium (30 ml). The culture medium (Dulbecco’s modified Eagle’s medium/F12; Sigma, Saint Quentin, France) was supplemented with 10% heat-inactivated serum of control, achalasia or GORD patients, 100 IU.ml⁻¹ penicillin, 100 µg.ml⁻¹ streptomycin, 1.1µg.ml⁻¹ amphotericin B, 20 µ.g.ml⁻¹ gentamicin (Sigma), 6mM glutamine, and 2.1 g.l⁻¹ NaHCO₃. Colchicine (40 µM) was added to the medium to enhance nerves immunoreactivity to peptides. The tissue was then maintained at 37°C overnight (16-18 h) in a humidified incubator containing 5% CO₂ and placed on a rocking tray. This time of incubation was chosen because we observed in preliminary experiments that changes in neuronal phenotypes induced by the serum of achalasia patients were similar after 16-18 h and 72 h incubation time.

Immunohistochemistry

The effects of a total of 18 sera of achalasia, 11 of healthy controls and 5 of GORD were tested. An average of three (range 2-6) different sera among which at least one control serum were tested on each fundic specimen (n=10).

After culture, the tissue was stretched and fixed for 4-6 h in 0.1 M phosphate-buffered saline (PBS) containing 4% paraformaldehyde at room temperature. Following several washes in PBS, the tissue was pinned in a dissection dish. Under a dissection microscope, the circular muscle, the oblique layer, and part of the longitudinal muscle were removed in order to expose the myenteric plexus.

Myenteric plexus/longitudinal muscle was then permeabilised for 1-2 h in PBS/NaN₃ containing 0.5% Triton X-100 and 4% horse serum. The tissue was
incubated with the following primary antibodies diluted in PBS/NaNO₃, 4% horse serum and 0.5% Triton-X for 18-20 h at room temperature: goat anti-ChAT (1:200; AB144P, Chemicon, USA), rat anti-substance P (1:1,000; 10-SO15, Fitzgerald, Concord, CA, USA), mouse anti-VIP (1:1,000; Biogenesis; England), and rabbit anti-NOS (1:2,000; R025, Alexis, USA). Following incubation with primary antisera, the tissue was washed with PBS and incubated for 2-3 h with donkey anti-goat IgG conjugated to carboxymethylindocyanine (CY3) (1:500), donkey anti-rat IgG conjugated to 7-amino-4-indodicarbocyanin (CY5) (1:500), donkey anti-mouse IgG conjugated to 7-amino-4-methyl-coumarin-3-acetate (AMCA) (1:50), and donkey anti-rabbit IgG conjugated to fluorescein isothiocyanate (FITC) (1:200).

After the population of neurones immunoreactive for ChAT, SP, VIP, and NOS was identified, some tissues were washed in PBS (4 times, 10 min) and labelled with rabbit anti-NSE (1:2,000; 17437, Polysciences, Germany) for 18-20 h. The tissue was then washed with PBS and incubated for 2-3 h with donkey anti-rabbit IgG conjugated to FITC.

Specimens were viewed under an Olympus IX 50 fluorescence microscope fitted with adequate filter cubes. Pictures were acquired with a black and white video camera (Mod. 4910, Cohu Inc., SL Microtest, Jena, Germany) connected to a Macintosh computer through a frame-grabber card (Scion Image, SL Microtest).

Identification of neuronal cell populations and phenotypical analysis.

In order to determine the general coding of the myenteric plexus, an average of 300±23 neurones were analysed for each condition. The number of ChAT-, VIP-, NOS-, and SP-immunoreactive myenteric neurones was determined by quadruple immunohistochemistry. In 18 tissues, an anti-NSE antibody was used as a general neuronal marker to ascertain the total number of cell bodies in the same previously stained ganglia. The proportion of neurochemically identified populations was then expressed relatively to the number of NSE-immunoreactive neurones, which was assumed to represent the total neurone population [26].

Immunohistochemical identification of anti-neuronal antibodies

Frozen and unfixed sections of primate gut (Euroimmun, Lübeck, Germany) were incubated with the individual serum samples (5 healthy controls and 16 patients with achalasia; dilution: 1:100) for 1 h at room temperature. After washing in PBS-Tween (0.2%) the sections were exposed to FITC-labelled goat-anti-human IgG (Euroimmun) for 30 minutes. The slides were then evaluated for specific staining of neuronal structure under an Olympus IX-50 fluorescence microscope using a semi quantitative rating scale: 0, no staining above background; +, moderate staining; ++, intense staining of neurones.

In vitro motility studies

The effects of a total of 10 sera of achalasia patients and 7 sera of healthy controls were tested. An average of three (range 2-4) different sera among which at least one control serum were tested on each fundic specimen (n=6).

Following a 16-hr incubation with serum, the tissue was transferred into a sterile dish and the submucosa was removed under a microscope. Small strips of circular muscle (approx. 5 x 10 mm) were dissected along the circular axis. These muscle strips were then placed in an organ bath filled with 10 ml of Krebs solution at 37°C continuously bubbled with 95% O₂ and 5% CO₂. Strips were initially stretched with a preload of 10 mN which was maintained during an equilibration period of at least
90 min. After 3 to 4 washes every 10 min, 10 μM guanethidine was added to block sympathetic influence. After 10 min, the preparation was contracted through exposure to carbachol (1 μM). At peak contraction, enteric neurones were electrically field stimulated (EFS) using the following parameters: train duration: 15 s, stimulation frequency: 20 Hz, pulse duration: 400 μs, pulse amplitude: 15 V. This procedure was repeated 2-3 times with a 30-min wash-out period between each stimulation. In order to characterise the NO dependant relaxation, the same protocol was used in presence of L-NAME (50 μM). The duration of the relaxation period, which corresponds to 10% of the maximal relaxation period, and the area under the curve were measured for each EFS-induced response (figure 1). The area under the curve was normalised to the carbachol-induced maximum contractile amplitude.

The contractile response of the circular muscle was continuously recorded using isometric force transducers (Basile no 7005; Comerio, VA, Italy), and acquired onto a PowerMac Performa 7100/80 computer equipped with the MacLab/4s System (ADI, Spechbach, Germany).

**Statistical analysis**

Data are expressed as the mean ± SEM or as median and quartile when appropriate. Results were compared using paired or unpaired t-test. For non-normally distributed data, comparisons were performed using the Mann-Whithney non parametric test. Correlations between patients parameters and observed changes were calculated using linear regression analysis. Differences were considered significant for p<0.05.

**Results**

1. **Effects of serum from achalasia patients on neuronal phenotype.**

   Quadruple immunohistochemical staining was performed on fundic specimens incubated with sera of healthy control (figure 2A-D) and with sera of achalasia patients (figure 2E-H).

   Serum from patients with achalasia induced significant changes in two of the four immunoreactive (IR) populations (figure 3). The proportion of NOS-IR neurones was decreased by 26% on average in presence of achalasia serum as compared to controls (p=0.016). All sera of achalasia patients induced a reduction in the proportion of NOS-IR neurones ranging from –11 to –43%. Conversely, the proportion of ChAT-IR neurones was increased by 16% in presence of achalasia serum as compared to controls (p<0.001). All sera of achalasia patients induced an increase in the proportion of ChAT-IR neurones ranging from 4 to 46%. The proportion of VIP-IR neurones was decreased by 54% as compared to controls (p=0.09). Although the difference did not reach statistical significance, the proportion of SP-IR neurones tended to increase (figure 3).

   Following incubation of fundic tissue with serum from patients with achalasia, no significant change in the number of NSE-positive cells per ganglion was observed as compared to controls (serum of healthy subjects) (median and quartiles : 34 [22-60] vs 33 [16-72] respectively; p= 0.70).

   There was no statistically significant correlation, between the clinical characteristics of patients [age, duration of symptoms, LOS pressure, and percentage of LOS relaxation in response to swallowing] and the changes in the neurochemical coding of myenteric neurones.

2. **Effects of serum from GORD patients on neuronal phenotype.**
Quadruple immunohistochemical staining was performed on fundic samples incubated with sera of healthy control (figure 4A-D) and with sera of GORD patients (figure 4E-H). Serum of patients with GORD did not induce significant changes in ChAT-, NOS-, VIP-, SP-IR populations as compared to controls (figure 5).

3. Effects of serum of achalasia patients and healthy controls on immunolabelling of enteric ganglia.

In the achalasia group 12% of serum samples (2/16 samples) labelled enteric neurones while in the control group no neurone was labelled (5 samples).


In presence of guanethidine, carbachol induced a contraction of the fundic circular muscle. The amplitude of this contractile response was not significantly different between tissues incubated with serum of achalasia patients and controls (respectively 1.6 ± 0.6 N/g vs 1.5 ±0.8 N/g, paired t-test).

The EFS-induced relaxation of the preparation was significantly altered by serum of achalasia patients as compared to controls (figure 6). The normalised area under the curve was significantly decreased in tissues incubated with serum of achalasia patients as compared to controls (7.6±2.6 vs 14.5±5.0, p=0.036, paired t-test) (figure 7A). In addition, the duration of the relaxation at 10% of maximal relaxation value was reduced by serum of achalasia patients as compared to controls, but fell short off statistical significance (23±4 vs 50±15 s, p = 0.09, paired t-test) (figure 7B). The normalised amplitude of EFS-induced relaxation was however not altered by serum of achalasia as compared to controls (33±10 vs 37±5%, respectively, p = 0.71, paired t-test). For the 10 sera tested both for immunochemistry and for motility (6 achalasia patients and 4 controls), there was a statistically significant correlation between the AUC of EFS-induced relaxation and the proportion of NOS-IR neurones (Figure 8).

In presence of L-NAME, the carbachol-induced contractions were not altered in tissues incubated with serum of control or achalasia patients (data not shown). The EFS-induced relaxation was however significantly reduced and almost abolished in all of the preparations (figure 6) (p<0.0004 for serum of controls and p <0.025 for serum of achalasia patients, paired t-test).

Discussion

This work shows that the serum of achalasia patients induces changes in the neurochemical phenotype of myenteric neurones (which are reminiscent of those observed in achalasia), in an ex vivo model of human healthy fundus. These effects seem to be specific of achalasia as serum of GORD patients did not change the neurochemical phenotype of fundic myenteric neurones. Furthermore, ex vivo motility experiments performed on human fundic strips revealed that EFS induced a NO-dependent relaxation that was significantly inhibited in tissues incubated with serum of achalasia patients as compared to control.

This study documents for the first time the effects of serum of achalasia patients both on the neurochemical coding of human myenteric neurones and on one of its functional correlates, i.e., the neurally-mediated motor activity. One of the strengths of this study is the fact that it has been performed in human tissues, thereby avoiding interspecies differences. This is particularly important as high interspecies variability in the organisation, and the neurochemical coding of the enteric nervous system (ENS) as well as in the functions regulated by the ENS has been reported [27]. Drawbacks of using human tissues include relative paucity of tissues, and the fact that specimens are
usually collected from non-healthy older subjects. We tried to minimise this latter point as tissues were taken at distance from the tumours in a macroscopically and histologically normal fundic area. Although control patients were younger than achalasia patients, age is probably not involved in serum-induced alterations, as 1) no correlation between age and changes in the phenotype was observed in achalasia patients and 2) GORD patients had similar age as achalasia patients. In addition, serum of achalasia patients of similar age range as control patients also induced alterations in the neurochemical phenotype.

Incubating human fundic tissues with serum of achalasia patients induced a significant decrease in the proportion of NOS-IR and a decrease (although not significant) in the proportion of VIP-IR myenteric neurones. This observation is consistent with previous studies showing both a decrease in the proportion of NOS-IR myenteric neurones in the human fundus [5] as well as a decrease in VIPergic innervation in achalasia patients [28]. Furthermore, our study revealed an increase in the population of ChAT-IR neurones. Whether the latter phenomenon occurs in achalasia is unknown as previous studies have not thoroughly evaluated the impact of achalasia on cholinergic innervation. However, the cholinergic component of LOS pressure (studied using acetylcholinesterase inhibitors) was found to increase in achalasia patients as compared to healthy controls [29].

However, in our study, we did not evidence clear signs of NOS-IR neuronal cell degeneration as previously reported in achalasia [4,7]. Indeed, no change in the total number of neurones (identified with NSE) was observed after incubation with the serum of achalasia patients. However, the method used could not have been sensitive enough to detect small changes in neuronal population, in particular in our conditions where the variability of the number of neurones per ganglia is large. Consequently, future studies using specific and early markers of cell death are needed to clarify the putative involvement of neurodegeneration. The decrease in NOS-IR neurones would rather be due to a decrease in the expression of nNOS. The rapid occurrence of these changes (16-18h) is consistent with an effect of the serum upon the regulation of nNOS expression at the mRNA and/or protein level. Indeed, in the rat stomach, nNOS expression can be significantly decreased as early as 4h following intraperitoneal injection of endotoxin [30]. Furthermore, IgG of patients with Sjögren’s syndrome are able to increase nNOS mRNA expression as rapidly as 1h after incubation in rat cerebral cortex preparations [31].

These alterations of the neurochemical phenotype could set the basis for the altered motor response to EFS observed in our study. Indeed, and as suggested by the correlation between the proportion of NOS-IR neurones and the amplitude of the EFS-induced relaxation (Figure 8), the decrease in NOS-IR myenteric neurones could explain the decrease in EFS-induced relaxation following incubation with serum of patients with achalasia. This EFS-induced relaxation is NO dependent as it was blocked by L-NAME. These results are consistent with previous studies showing that in the human fundus, EFS-induced relaxation has an important nitrergic component [32]. This decrease in NO-mediated relaxation could be involved in various gastric and oesophageal altered functions observed in achalasia [2,33,34]. Alteration in the expression of NOS in fundic myenteric neurones could also partly explain the increased LOS tone in achalasia as NOS-IR fundic myenteric neurones were shown to innervate and regulate relaxation of the LOS [35].

Both the mechanisms and mediators involved in the changes of phenotype of myenteric neurones induced by the serum of achalasia patients are currently unknown. The putative mediators responsible for the changes in the neurochemical phenotype (as
observed in our study) include inflammatory mediators. Involvement of cytokines is suggested by the following observations: the increase in IL-8 levels in the serum of achalasia patients vs controls (unpublished personal results), the ability of cytokines, such as IL-1β, to regulate gene expression in enteric neurones [36,37], and the presence of inflammatory cells in the myenteric plexus in patients with achalasia [8,38]. Besides cytokines, various neurotrophic factors have been shown to differentially regulate expression of some neuromediators in the ENS. In particular, NT-3 increases the VIP and SP content of enteric or central neurones [39]. Reinforcing the concept that neurotrophic factors could be involved in achalasia is the preliminary observation that a decrease in neurotrophin expression occurs in syndromic forms of achalasia [40].

Changes in neurochemical phenotype could also be associated with the presence of anti-neuronal auto-antibodies in the serum of achalasia patients [12,15]. However, in our study a minority of serum of achalasia patients (12% of the sera tested) labelled enteric neurones further suggesting that presence of neuronal antibodies is rather an epiphenomen than a causative factor in achalasia as first evoked by Moses et al. [15]. In their study they also observed that a large proportion of achalasia sera (49%) did not label enteric neurones [15]. In addition, in our study, the serum of GORD patients (which also contains anti-neuronal antibodies [15]) did not change the neurochemical phenotype of human myenteric neurones.

In conclusion, our study demonstrates that the serum of patients with achalasia is able to change the phenotype of human fundic myenteric neurones and to modulate the EFS-induced NO-dependent relaxation of the human fundus. Identification of this (these) seric component(s) is of pivotal importance to enable the development of novel diagnostic and/or therapeutic strategies. In addition, similar experimental approaches could be used to test the effects of seric components on the ENS and GI functions in other enteric neuropathies [38].
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Competing Interest field
Authors of this manuscript declare no specific competing interest in the field of this work.
References
Figure legends

Figure 1. Illustration of electrically field stimulated (EFS)-induced relaxation of human fundic circular muscle preparations. Following carbachol-induced contraction in presence of guanethidine, myenteric neurones were electrically-field stimulated.

Figure 2. Immunohistochemical detection of transmitter coding in human fundic myenteric plexus incubated with sera of achalasia and healthy subject. Quadruple labelling with antibodies against ChAT (A), NOS (B), VIP (C) and SP (D) performed on a myenteric ganglion following incubation with serum of control patient. Quadruple labelling with antibodies against ChAT (E), NOS (F), VIP (G) and SP (H) performed on a myenteric ganglion following incubation with serum of achalasia patient. Two fragments of gastric fundus from the same patient were incubated with serum of control or achalasia patients. Scale bar: 25µm.

Figure 3. Proportion of NOS-, ChAT-, VIP-, and SP-immunoreactive myenteric neurones in human fundic specimen after incubation with the serum of healthy controls (empty bars) and patients with achalasia (filled bars). Results are presented as percentages of the total neuronal population, as determined by NSE immunostaining. * :p<0.05 ; ** :p<0.001.

Figure 4. Immunohistochemical detection of transmitter coding in human fundic myenteric plexus incubated with sera of GORD and healthy subject. Quadruple labelling with antibodies against ChAT (A), NOS (B), VIP (C) and SP (D) performed on a myenteric ganglion following incubation with serum of control patient. Quadruple labelling with antibodies against ChAT (E), NOS (F), VIP (G) and SP (H) performed on a myenteric ganglion following incubation with serum of GORD patient. Two fragments of gastric fundus from the same patient were incubated with serum of control or achalasia patients. Scale bar: 25µm.

Figure 5. Proportion of NOS-, ChAT-, VIP-, and SP-immunoreactive myenteric neurones in human fundic specimen after incubation with the serum of healthy controls (empty bars) and patients with GORD (filled bars). Results are presented as percentages of the total neuronal population, as determined by NSE immunostaining.

Figure 6. Typical traces illustrating the decrease of EFS-induced NO dependent relaxation in human fundic muscle strips incubated with the serum of a healthy control subject and of a patient with achalasia. Serum of achalasia patient was found to decrease electrical field stimulation (EFS)- induced, NO-dependent relaxation in human fundic circular muscle strips. Following carbachol-induced contraction, EFS [……_] induced a relaxation in tissue preincubated with controls serum (A). In presence of L-NAME (100 µM), EFS [……_]–induced relaxation was precluded (B). Following carbachol-induced contraction, EFS [……_] also induced relaxation in tissue preincubated with serum of achalasia patients (C). Relaxation was however reduced as compared to control. In presence of L-NAME (100 µM), EFS [……_] induced relaxation was also precluded (D). Effects of L-Name were studied on the same muscle strips (panels A and B, and C and D). Data were normalised to the amplitude of the contraction prior to EFS.
Figure 7. Effect of preincubation with control serum (empty bars) and serum from achalasia patients (filled bars) on the area (A) and the duration (determined at 10% of the maximal relaxation amplitude) (B) of the EFS-induced relaxation of human fundic circular muscle strips (individual results of 6 separate experiments and mean of the experiments are shown).

Figure 8. Correlation between the AUC of EFS-induced relaxation of human fundic muscular strips and the proportion of NOS-immunoreactive neurones. The correlation was calculated in the 4 control sera (open circles) and 6 achalasia patients sera (closed circles) for which both the neurochemical coding and the \textit{ex vivo} motility study were performed.
A scatter plot showing the relationship between NOS immunoreactive neurones (as a percentage of NSE) and the AUC of EFS-induced relaxation. The data points are differentiated by circles for control subjects and filled circles for achalasia patients. The line of best fit is represented by the equation $Y = 0.33x + 27.01$, with a correlation coefficient $r = 0.69$ and a p-value of $p = 0.035$. The x-axis represents the AUC of EFS-induced relaxation, and the y-axis represents the NOS immunoreactive neurones (%NSE).
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