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Autoantibodies in complex regional pain syndrome bind to a differentiation-dependent neuronal surface autoantigen

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ABSTRACT

Complex regional pain syndrome, which is characterised by pain and trophic disturbances, develops frequently after peripheral limb trauma. There is an increasing evidence of an involvement of the immune system in CRPS, and recently we showed that CRPS patients have autoantibodies against nervous system structures [6]. Therefore we tested the sera of CRPS patients, neuropathy patients and healthy volunteers for surface-binding autoantibodies to primary cultures of autonomic neurons and differentiated neuroblastoma cell lines using flow cytometry. Thirteen of 30 CRPS patients, but none of 30 healthy controls and only one of the 20 neuropathy sera had specific surface binding to autonomic neurons ($p < 0.001$). The majority of the sera reacted with both sympathetic and myenteric plexus neurons. Interestingly, 6/30 CRPS sera showed binding to undifferentiated SH-SY5Y neuroblastoma cells. However, differentiation of SH-SY5Y into a cholinergic phenotype induced a surface antigen, which is recognised by 60% of CRPS sera (18/30), but not by controls ($p < 0.001$). Our data show that about 30–40% of CRPS patients have surface-binding autoantibodies against an inducible autonomic nervous system autoantigen. These data support an autoimmune hypothesis in CRPS patients. Further studies must elucidate origin and function of these autoantibodies in CRPS.

1. Introduction

Complex regional pain syndromes (CRPS) may develop particularly after peripheral limb trauma, either without (CRPS 1) or with clinically evident peripheral nerve lesions (CRPS 2). The main clinical symptoms are pain and hyperalgesia, vasomotor, sudomotor and trophic changes on the affected extremity. In addition, motor symptoms are usually present from the beginning [1,34], and may progress with ongoing duration [32].

Studies on the pathophysiology of CRPS revealed that there are two major mechanisms responsible for the clinical signs of acute CRPS: Exaggerated inflammation after trauma [19], in particular the neurogenic inflammatory component [22,38]; and dysfunction of the sympathetic nervous system [36].

Recent evidence led one focus of research on the involvement of the immune system in CRPS pathogenesis. Some studies revealed that HLA alleles DQ1 [20], DR13 [33], DR15 [25] and the centromeric regions of the HLA class 1 antigens [30] were linked to CRPS. Furthermore, acute CRPS cases can be sufficiently treated using systemic corticosteroids [7,9]. Accordingly, in a recent study we have been able to demonstrate autoantibodies specifically targeting autonomic (e.g. sympathetic) nervous system structures [6]. These autoantibodies, which were detected by immunofluorescence, have been directed against intracellular antigens of sympathetic ganglia neurons. Additionally, Goebel et al. could also demonstrate increased binding of CRPS sera to various peripheral and central nervous system structures [12]. However, the underlying antigens and pathophysiological role are yet unknown. In well-characterised autoantibody-mediated neuroimmunological diseases, such as myasthenia gravis or Lambert–Eaton myasthenic syndrome, pathogenically relevant autoantibodies are directed against surface structures (review in [21]).

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In the present study, we therefore screened for autoantibodies against surface epitopes in CRPS patients. We here show that about 45% of CRPS sera have surface-binding autoantibodies against primary cultured autonomic neurons, and that this autoantigen is inducible in neuroblastoma cells by differentiation with a combination of retinoic acid and bone morphogenetic protein (BMP)-2.

2. Patients and methods

2.1. Patients

Serum was obtained from 30 patients with clinically defined CRPS (20 CRPS 1, 10 CRPS 2, according to the IASP diagnostic criteria [16]; mean age 48.5 ± 11.8 years; 19 women) after informed consent and approval of the local ethical committee. Sera of 30 healthy controls (HC), 20 patients with non-inflammatory neuropathy (NP; 10 with diabetes type II, 5 with hereditary sensorimotor neuropathy, 5 with alcoholic neuropathy) and 20 patients with peripheral nerve lesions (NL; 6 with carpal tunnel syndrome, 6 with radial nerve lesion after upper arm fracture, 4 patients with idiopathic plexus neuritis, and 4 with pressure palsy of the peronaeal nerve) served as controls. The control groups were age- and sex-matched (HC 52.6 ± 12.4 years, NP 58.4 ± 14.0, NL 54.5 ± 17.4). None of the healthy controls or NL and 11/20 NP patients had a history of chronic neuropathic pain. The mean duration of CRPS symptoms in our patients' group and the NP group was 25.1 ± 23.8 weeks and 287.4 ± 155.5 weeks, respectively. The clinical and epidemiological data of the CRPS patients are given in Table 1. Twelve of the CRPS patients have been recently described elsewhere [6].

2.2. Methods

To test for surface autoantibody binding, we used the neuroblastoma cell line SH-SY5Y, which originates from a tumour of the sympathetic nervous system. The SH-SY5Y cells were used undifferentiated or differentiated according to the protocol given below. The epithelial cell line HEK 293 served as a non-neuronal control cell line. Additionally primary cultures of sympathetic neurons and gut myenteric plexus neurons from rats have been isolated and tested as described below.

2.2.1. Differentiation of neuroblastoma cells

For differentiation experiments, SH-SY5Y cells were plated at 20,000 cells/cm^2 and cultured for 2 days prior to differentiation. Cells were differentiated by incubation with the following agents for 4 days: all-trans retinoic acid (RA, Sigma, St. Louis, MO, USA) at 10 μM; BMP-2 (BioVision, Mountain View, CA, USA) at 10 ng/mL; transforming growth factor (TGF)-β1 (Sigma, St. Louis, MO, USA) at 10 ng/mL. We also used combinations of RA and BMP-2 or RA and TGF-β1 under the same conditions.

Table 1
Clinical and epidemiological data of the CRPS patients.

Age	52.8 ± 13.8
Sex	25f/15m
CRPS I/CRPS II	20/10
Affected limb (upper/lower)	24/6
Surgery	17/30
Motor impairment	21/30
Sensory impairment	20/30
Trophic changes	24/30
Time to serum analysis[a] (weeks)	25.1 ± 23.8

[a] Time between onset of symptoms and obtaining of serum.

2.2.2. Isolation of sympathetic neurons

Adult Wistar rats were anaesthetised with halothane and sacrificed by cervical dislocation. The whole vertebral column was then removed and the sympathetic trunk on either side was dissected over its full length including cervical and lumbar ganglia. All ganglia were cut out of the trunk, cleaned from connective tissue and placed into calcium- and magnesium-free phosphate-buffered saline (PBS). The ganglia were incubated in 6 mg/mL collagenase Worthington type CLS II (Biochrom, Berlin, Germany) and 2 mg/mL trypsin type III-S (Sigma, (St. Louis, MO, USA) for 40 min in a shaking water bath at 37 °C. Afterwards, the ganglia were washed thrice with plating medium (minimum essential medium (MEM) supplemented with 10% fetal calf serum, 1% penicillin/streptomycin and 1% glutamine). All chemicals for the plating medium were obtained from Sigma (St. Louis, MO, USA). Cells were then dissociated by repeated passages through three Pasteur pipettes with decreasing diameter, and then stored in plating medium under a humidified 5% CO_2 atmosphere at 37 °C.

2.2.3. Isolation of gut myenteric plexus

As described previously [29], myenteric plexus from 12- or 13-day-old Wistar rats was obtained. After decapitation the whole gut was removed and stored on ice-cold MEM eagle (Sigma). In the small intestine the smooth muscle layer was separated from the submucosal layer under microscopical control (Wild Heerbrugg) at 15× magnification. The colon was flushed, opened and incubated as a whole in 1 mL of a Collagenase solution (Worthington CLS II, 1 mg per mL in: Hank's balanced salt solution, Gibco) at 37 °C for 1.5 h. After the digestion step the vials were mechanically agitated using a vortex. The already visible plexus pieces that were cut were collected and transferred into another petri dish with ice-cold MEM. The remaining undigested tissue was re-digested with a fresh collagenase solution at 37 °C for another 15 min. The procedure was repeated until all the muscle pieces were digested. Finally, the plexus pieces were collected and centrifuged at 1000 rpm for 2 min. The supernatant was decanted and the pellet was incubated with 1 ml trypsin (Life Technologies) for 5 min. After centrifugation at 1000 rpm for 10 min, the trypsin solution was replaced by Dulbecco's Modified Essential Medium (DMEM, Gibco) containing 10% fetal calf serum (FCS), 1% penicillin/streptomycin and 1% glutamine. Then the plexus was dissociated by aspirating through a 27-gauge needle.

2.2.4. Autoantibody detection by flow cytometry

We recently introduced flow cytometry (FACS) to detect autoantibodies in neuroimmunological diseases [5]. To avoid unspecific binding to ubiquitous antigens we pre-absorbed the sera by incubating in PBS containing 1% FCS and 0.1% NaN_3 (FACS buffer) with HEK 293 cells for 24 h at 4 °C (serum dilution 1/50) prior to incubation of the primary cell cultures. The different cell lines and primary cultures were then incubated with the pre-absorbed patients' sera for 30 min at 4 °C, washed, and incubated again with FITC-conjugated anti-human IgG (DAKO) (30 min, 4 °C in dark). After washing again, binding was analysed in a FACScalibur (Beckton–Dickinson, Heidelberg, Germany) using CellQuest® software. After measurement of the mean fluorescence intensity (mfi), the mean mfi of the controls of each experiment has been set as 100%. For each serum, the percentage of difference from the controls was calculated. This standardisation is necessary since the binding, given as mfi, of patients sera to a mixed primary cell culture can vary between the experiments. A percentage above mean + 2.5 standard deviations of the controls was considered positive. To avoid irrelevant binding to intracellular antigens, we tested the primary cell cultures with trypan blue for viability and also measured binding of anti-neurofilament antibody (Dako, Hamburg, Germany) to non-permeabilized cells.

2.2.5. Identification of neuronal cells

To show neuronal-specific binding, cells were incubated with the patients sera and FITC-conjugated anti-human IgG as described above, fixed with 2% paraformaldehyde (20 min, 4 °C), permeabilized with FACS-buffer containing 0.1% saponine and then incubated with the neuronal-specific anti-HuD antibody (Molecular Probes). Cells were then gated according to their reactivity to the neuronal anti-HuD protein and only positive (neuronal) cells were analysed in flow cytometry.

2.2.6. PCR experiments

Total RNA was isolated using RNeasy Mini Kit (Qiagen, Heidelberg, Germany) as per manufacturer's instructions. RNA quantity was determined with a BioRad spectrophotometer (Munich, Germany). Total RNA (1 μg) was subjected to reverse transcription using Oligo(dT)18 Primer. The RNA/primer mixture was denatured at 70 °C for 5 min before the addition of a MasterMix containing 1 μL RiboLock™ RNase Inhibitor (20 μ/μL), 2 μL of 10 mM dNTP mix, 4 μL of 5× Reaction buffer and 1 μL RevertAid™ M-MuLV Reverse Transcriptase (200 μ/μL) in diethylpyrocarbonate treated H2O (Fermentas GmbH, Germany). The reactions were incubated for 60 min at 42 °C before quenching for 10 min at 70 °C. cDNA was stored at 4 °C until further use. Primer sequences used for PCR were designed using Primer Premier 5® software. Expression of dopamine-β-hydroxylase (DβH) and vesicular acetylcholine transporter (VAChT) was assessed by polymerase chain reaction (RT-PCR). Primer sequences used are DßH: F: 5′-ACTGTCCAC TTGGTCTACGGG-3′ and R: 5′-CGGTCGGGTTTCATCTTG-3′; VAChT: F: 5′-GGGGCATCCTCTATGAGTTCG-3′ and R: 5′-GTGGCAATGGTG GGTTCG-3′; and porphobilinogen deaminase (PBGD): F: 5′-TGCAA CGGCGGAAGAAAAC-3′ and R: 5′-GGCTCCGATGGTGAAGCC-3′. PBGD was used as a housekeeping gene because its level is not affected by the differentiation protocol used. Each PCR amplification contained 1 μL cDNA, 2.5 μL of 10× reaction buffer, 1 μL each of forward and reverse primers for gene of interest and 0.5 μL Taq polymerase. The reaction was supplemented with nuclease-free H_2O to bring the volume to 25 μL. Thermal cycling conditions used are as follows: initial denaturation at 95 °C for 5 min, followed by 30 cycles at 95 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s and a final extension at 72 °C for 5 min. A negative template control containing 1 μL nuclease-free H_2O was included for each set of primers. To confirm the presence of product and amplicon size, PCR products were separated on 1% agarose gel and stained with ethidiumbromide.

2.2.7. Statistical analysis

Frequency of autoantibodies and comparison with clinical data were analysed by Fisher's exact test. Means of fluorescence intensity were compared using one-way analysis of variance (ANOVA), followed by the Tukey's multiple comparison test for post hoc comparison. All statistical analysis was performed using Prism® 4.02 Software (Graph Pad Inc.). The level of significance was set at 5%.

3. Results

3.1. Surface binding to primary cultured autonomic neurons

Sera have been tested for surface binding to sympathetic neurons (SNs) and myenteric plexus neurons (MPNs). Only binding to neuronal cells identified by anti-HuD antibody was considered positive. Flow cytometry analysis (Fig. 1A) revealed autoantibody binding to SN or MPN in 13/30 (43.3%) of the CRPS patients, but only in 1/20 NP patients, 1/20 NL patients and none of the healthy controls ($p < 0.0001$). Nine patients showed binding to both SN and MPN, the sera of two patients bound exclusively to SM, but not to MPN, and two CRPS sera contained autoantibodies against MPN, but not against SN (shown for binding to sympathetic neurons, Fig. 1B).

3.2. Binding to undifferentiated SH-SY5Y neuroblastoma cells

Only 20.0% (6/30) of the CRPS patients, one of the NP patients and none of the other control groups showed binding to undifferentiated SH-SY5Y neuroblastoma cells (one-way ANOVA, $p < 0.01$, Fig. 2).

3.3. Binding to differentiated SH-SY5Y neuroblastoma cells

Binding to primary cultured (and well-differentiated) autonomic neurons was detectable in more than twice the patients compared to undifferentiated SH-SY5Y. Therefore we differenti-

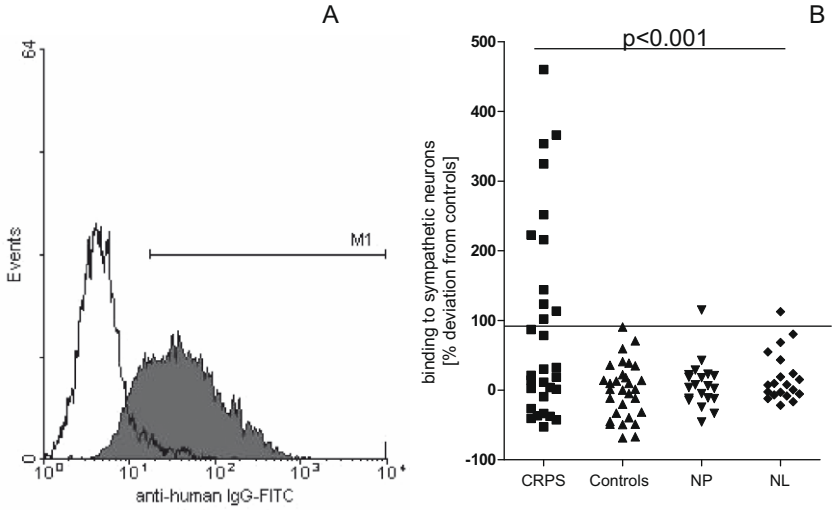

Fig. 1. (A) Binding of a CRPS serum (filled graph) and a control serum (line graph) to sympathetic neurons. (B) Binding to sympathetic neurons (expressed as percent difference to the mean binding of the controls) after pre-incubation of the sera with HEK 293 cells to remove unspecific binding. Cut-off, marked as horizontal line, was determined as mean of the controls +2.5× std dev. CRPS patients have more and higher binding to sympathetic neurons than controls, neuropathy patients (NPs) or patients with peripheral nerve lesions (NLs).

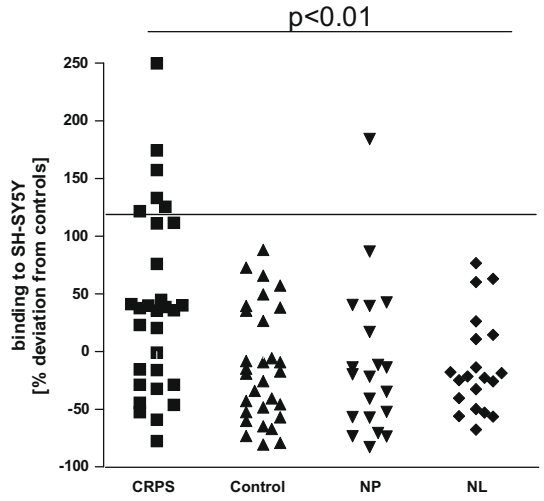

Fig. 2. Binding of CRPS and control sera to undifferentiated SH-SY5Y cells measured by flow cytometry. Cut-off, marked as horizontal line, was determined as mean of the controls +2.5× std dev.

ated SH-SY5Y neuroblastoma cells into different neuronal phenotypes to test whether the recognised antigen is differentiation-dependent or not.

We used a variety of differentiation agents (RA, TGF-β1, TGF-β1/RA, BMP-2 or BMP-2/RA) and checked the differentiated neurons for cholinergic and catecholaminergic markers. Dopamine-β-hydroxylase was induced by differentiation with TGF-β1, but strongly down-regulated by BMP-2/RA differentiation (Fig. 3). In contrast, the cholinergic marker vesicular acetylcholine transporter (VAChT) was down-regulated using TGF-β1 and slightly up-regulated by RA and BMP-2/RA differentiation. This indicates that TGF-β1 induces a more catecholaminergic neuronal phenotype, whereas BMP-2/RA induces a cholinergic neuronal phenotype.

Then CRPS and control groups were incubated with the differentiated neurons to detect specific surface binding. CRPS and controls did not show significant binding to either TGF-β1-differentiated, RA-differentiated or BMP-2-differentiated SH-SY5Y cells (Fig. 4A–C). There was even a tendency to a lower binding of the CRPS sera to the TGF-β1- and the TGF-β1/RA-differentiated cells (Fig. 4B and C). However, if SH-SY5Y cells are differentiated with a combination of RA and BMP-2-inducing a cholinergic neuronal phenotype, 60.0% (18/30) of CRPS sera, but only one of the NL patients and none of the other control patients showed any significant surface binding (one-way ANOVA, $p < 0.001$, Fig. 4D).

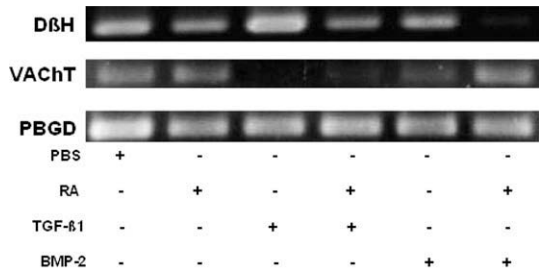

Fig. 3. PCR characterization of differentiated SH-SY5Y cells. Different agents induce differentiation of SH-SY5Y cells into two different neuronal phenotype. SH-SY5Y cells were differentiated using TGF-β1, RA, BMP-2 alone or in combinations. After differentiation total RNA was prepared and subjected to cDNA synthesis. This cDNA was used for PCR amplification of VAChT, DβH and PBGD.

3.4. Association with clinical data

We compared surface binding in CRPS with clinical and epidemiological data. Patients with and without surface-binding autoantibodies to primary cultured autonomic neurons or differentiated cells did not differ in any epidemiological or clinical features tested (Table 2).

4. Discussion

A significant number of CRPS patients have autoantibodies against a surface epitope of autonomic neurons. Our results further indicate that this surface antigen is weakly expressed in undifferentiated neuroblastoma cells, but can be induced by differentiation into a distinct phenotype, preferentially cholinergic. These results provide more evidence that autoimmunity against the autonomic nervous system may be involved in the pathogenesis of CRPS.

Peripheral trauma, in particular if it is accompanied by partial peripheral nerve lesion, causes a rapid release of cytokines and neurotrophins [27,28], which activate and sensitize primary and probably also spinal afferents [24]. Activation and sensitization of primary afferents also cause an increase in neuropeptide release into the affected body region (mainly substance P (SP) and calcitonin gene-related peptide (CGRP)). Chronic release of neuropeptides might be responsible for the clinically evident inflammatory CRPS symptoms [38]. On the other hand, impairment of sympathetic function has been repeatedly shown in CRPS – regarding vasoconstriction [3,36] and sudomotor function [2]. The origin of these sympathetic nervous system symptoms in posttraumatic CRPS is still widely unclear.

There has been little information about a possible involvement of the immune system in CRPS. CRPS has been associated to different HLA alleles [20,25,26,30,33]. Although association with HLA alleles does not necessarily implicate an immune etiology of a disease, many autoimmune diseases have distinct HLA associations. Further arguments for an involvement of the immune system in CRPS are studies showing altered monocyte reactivity to interferon-γ stimulation [17], and recent reports about the increased prevalence of parvovirus B19 IgG in CRPS patients [31]. Our group have been able to confirm the latter results [15]. Parvovirus B19 infection induces autoantibody production and might lead to autoimmune diseases [18]. Accordingly, we and others have been able to demonstrate autoantibodies against autonomic nervous system in CRPS [6,12]. These autoantibodies might be transferable since IgG derived from CRPS serum samples, which was injected in mice, led to abnormal behaviour [11].

Most autoantibodies with pathogenic effects are directed against surface epitopes, mostly receptors or ion channels. In the Lambert–Eaton myasthenic syndrome (LEMS), the underlying autoantigen is the presynaptic voltage-gated P/Q-type calcium channel. Autoantibodies block these channels and thereby induce neuronal dysfunction in myenteric plexus neurons, which also explain the clinical symptoms of autonomic dysfunction in LEMS patients [21,37]. Although in our patients there was no difference in clinical autonomic symptoms between surface-binding antibody-positive and -negative patients this does not exclude more subtle differences which would need differentiated autonomic testing in forthcoming studies. Autoimmunity against the autonomic nervous system has been reported in paraneoplastic neurological syndromes, scleroderma, pure autonomic neuropathies and the Lambert–Eaton myasthenic syndrome (LEMS). In the majority of these autoimmune disorders pathogenic surface-binding autoantibodies could be demonstrated [13,37]. Pathogenic effects of autoantibodies on autonomic neurons have also been shown in autonomic neuropathies [13,35]. The surface binding to autonomic

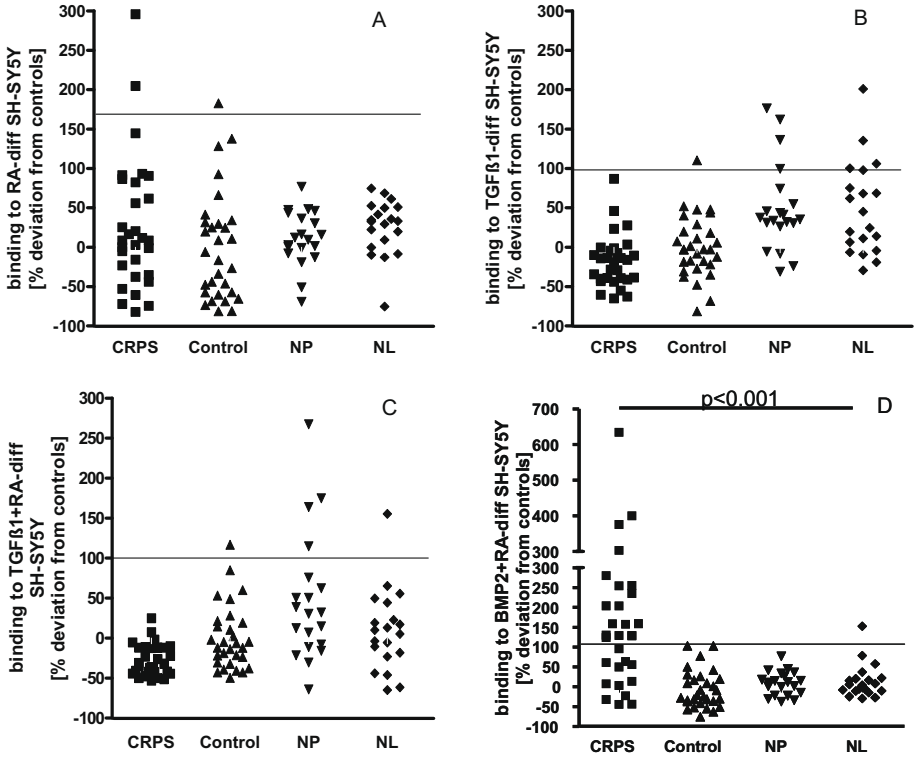

Fig. 4. Surface binding of autoantibodies to differentiated SH-SY5Y neuroblastoma cells. SH-SY5Y cells differentiated with (A) RA, (B) TGF-β1, (C) TGF-β1/RA, or (D) BMP-2/RA were incubated with CRPS and control sera and their binding was analysed by flow cytometry. Only BMB-2/RA-differentiated cells express a surface autoantigen recognised by CRPS sera.

neurons therefore supports our assumption of a pathogenic role of these autoantibodies in CRPS.

In our study, only about 43% of the CRPS patients were positive for surface-binding antineuronal autoantibodies. One reason might be the flow cytometric test system due to unspecific background binding to the primary cells of both controls and patients. However, another reason might well be the inclusion of patients according to the IASP criteria. Although all these patients meet the criteria of CRPS, it is unclear, whether they all have the same physiopathology. Even by clinical criteria, there seem to be subtypes within these groups. For instance, patients with "warm" and "cold" CRPS do not only differ in the temperature of the affected limb, but also show a distinct clinical pattern including differences in sensory impairment measured by quantitative sensory testing or differences in the frequency of associated dystonia [10]. Therefore, the patients with autoantibodies may represent a special pathophysiological subgroup of CRPS.

Table 2
Clinical data of the surface-binding-positive and -negative patients.

Feature	Surface binding (+) (n = 13)	Surface binding (−) (n = 17)	p
Age	52.5 ± 15.6	50.6 ± 10.8	n.s.
Sex	8f/5m	11f/6m	n.s.
CRPS type	6 CRPS I 7 CRPS II	14 CRPS I 3 CRPS II	n.s.
Affected limb	12 upper/1 lower	12 upper/5 lower	n.s.
Operation	6/13	11/17	n.s.
Motor impairment[a]	8/13	13/17	n.s.
Sensory impairment[b]	8/13	12/17	n.s.
Skin Temperature[c]	7/10[e]	10/16[e]	
Hair growth[c]	5/10[e]	6/16[e]	n.s.
Sweating abnormalities[c]	4/10[e]	8/16[e]	n.s.
Time to serum analysis[d] (weeks)	37.6 ± 35.3	20.9 ± 19.2	n.s.

[a] Any paresis in the affected limb, not related to nerve lesion.
[b] Any sensoric disturbances, not related to nerve lesion.
[c] Increased or decreased.
[d] Time between onset of symptoms and obtaining of serum.
[e] Information not available from all patients.

Neuroblastoma cells are a well-established model for the differentiation of dedifferentiated cells into a variety of neuronal subtypes [14]. The high binding to differentiated autonomic neurons in comparison to neuroblastoma cells was surprising, since other autoantibodies (i.e. anti-VGCC in LEMS) show preferential binding to undifferentiated neuroblastoma cells in a high percentage. We could obviously induce the autoantigen by differentiation of neuroblastoma cells into a cholinergic phenotype, whereas differentiation into a catecholaminergic phenotype by TGF-β1 decreases the surface binding of CRPS sera. These data suggest that the underlying autoantigen is highly restricted to distinct neuronal phenotype and may be located on special cholinergic neurons. The mutual use of BMP-2 and RA in the presence of fetal calf serum has been proved to play a trophic positive role in the differentiation of immature cells like SH-SY5Y to cholinergic phenotype neurons that express choline acetyltransferase [14]. We could additionally show an up-regulation of vesicular acetylcholine transporter (VAChT) expression associated with a down-regulation of catecholaminergic markers in BMP-2/RA-differentiated cells. Cholinergic neurons are all central autonomic neurons and the peripheral parasympathetic and sudomotor neurons [23]. Thus, autonomic disturbances induced by anticholinergic autoantibodies might be very different in CRPS patients and include vasomotor [36], sudomotor [4], cardiovascular [26] and urogenital [8] symptoms.

The origin of the autoimmune process and its relevance for the pathophysiology of CRPS remains to be finally elucidated. It further remains unclear whether the autoantibodies are already present before the clinical manifestation of CRPS. We could not detect

these autoantibodies in controls, NL and NP indicating that a trauma or nerve lesion alone does not induce specific autoimmunity. Regarding the signs of inflammation and the association with antimicrobial serology, one may speculate that in CRPS the autoimmune process may be the result of a cross-reactive immunity between viral or bacterial epitopes and structures of the autonomic nervous system. A comparable molecular mimicry causing autoimmune inflammation of the nervous system has been described in campylobacter-associated polyradiculitis. Interestingly, an association of campylobacter-positive serology to CRPS has been recently described (review in [12,39]).

Taken together, our findings of surface-binding autoantibodies specifically directed against autonomic neurons suggest that there might be some autoimmune etiology of CRPS – at least in certain patients. Our results should trigger further investigations including identification and cloning of possible autoantigens, and detailed investigations of functional effects in order to further specify the role of the immune system in CRPS pathophysiology.

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