Gangliosides act as onconeural antigens in paraneoplastic neuropathies

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Abstract

We describe two patients with progressive neuropathy and lung cancer in whom gangliosides (GS) may represent the oncoantigens. Patient 1 had motor neuropathy, high titers of IgG1 and IgG3 to GD1a and GM1, and expansion of circulating γ-δ T lymphocytes, a T-cell subset responding to glycolipids. Patient 2 presented with Miller-Fisher-like syndrome and IgG3 activity to disialo-GS. In both cases, decreased autoimmune responses and stabilization of neuropathy were accomplished by tumor treatment. By immunohistochemistry, patient 1’s IgG bound to his own tumor and to structures of normal nervous system expressing GD1a or GM1. Infiltration of IgG in the same neural structures was found at his autopsy. Regarding cellular immunity, the proportion of γ-δ T lymphocytes infiltrating carcinoma from patient 1 was significantly higher than in neoplastic controls. These results indicate that GS may represent onconeural antigens in paraneoplastic neuropathy (PNN); their expression on neoplastic tissue may elicit autoimmune responses, which also target neural structures.

Keywords: Gangliosides; Onconeural antigens; Peripheral neuropathy; Autoantibodies; γ-δ T lymphocytes

1. Introduction

Paraneoplastic neurological disorders represent a remote effect of cancer on the nervous system, usually characterized by progressive neurological deterioration; their response to therapy is usually poor and stabilization of the neurological picture is often accomplished only by cancer treatment (Posner, 2002; Keime-Guibert et al., 1999). The involvement of the peripheral nerve or its primary neuron results in paraneoplastic neuropathy (PNN), which can present with sensory, motor or sensorimotor signs (Antoine et al., 1999; Graus et al., 2001). In most cases, these disorders are the result of immune responses to proteins normally present in the nervous system and ectopically expressed by tumors, the so-called onconeural antigens (Giometto et al., 1999; Sutton, 2002). The presence of onconeural antigens on neoplastic cells triggers immune responses with production of IgG autoantibodies and activation of cytotoxic T lymphocytes (Albert et al., 1998, 2000; Benyahia et al., 1999; Plonquet et al., 2002). Extensive studies in the last years have identified a number of onconeural proteins, each associated with the involvement of specific neural structures; despite these efforts, however, autoantibodies are detected in less than 30% of patients with PNN (Sutton, 2002; Antoine et al., 1999). In the remaining cases of suspected PNN, non-proteinic molecules may act as onconeural antigens. In this regard, a patient with melanoma expressing GM2 had polyradiculoneuritis and anti-GM2 IgM (Weiss et al., 1998). Paraneo-
plastic ophthalmoplegia, motor axonal neuropathy and anti-GQ1b antibodies have been reported in association with melanoma (Kloos et al., 2002). However, despite carcinoma cell lines have been shown to express a number of gangliosides (GS) (Brezicka et al., 2000; Fuentes et al., 1997), the association of PNN with anti-GS Ig is still a matter of debate.

Gangliosides are normal constituents of plasma membranes and are particularly concentrated in the nervous system; they are composed of a common lipidic tail and variable extracellular glycidic portions with sialic acid residues (Svennerholm, 1994). The immunogenic potential of GS has been extensively assessed in human post-infectious neuropathies and in experimental models; in both conditions, different clinical pictures have been correlated with autoreactivity to distinct GS, through a T-cell-dependent production of anti-GS IgG1 and/or IgG3 (Quarles and Yuki, 2002). Thus, GQ1b or other disialo-GS are the autoantigens in patients with ataxic neuropathy and ophthalmoplegias (Miller-Fisher syndrome) (Kusunoki et al., 1999; Pestronk, 1988) and axonal motor neuropathy is associated with reactivity to GM1 and/or GD1a (Ho et al., 1999; Nardelli et al., 1988). We describe two patients with lung neoplasia, high titers of anti-GS IgG and axonal neuropathy, in whom several lines of clinical and experimental evidence strongly suggest a causal relationship between GS-expressing neoplasia and peripheral neuropathy, through the production of anti-GS immune responses.

2. Materials and methods

2.1. Patients

2.1.1. Patient 1

A 73-year-old man was admitted to our hospital because of progressive limb weakness over the past 4 years. On neurological examination, weakness, muscular wasting and fasciculation were evident in distal segments of four limbs. Muscle tone was normal, deep tendon reflexes were reduced or absent and plantar responses were flexor. Sensory examination was unremarkable. Blood chemistry and electrophoresis showed no abnormalities, except for increased carcinoembryonic antigen (9.4 ng/ml, n.v.<5) and prostate-specific antigen (9.78 μg/L, n.v.<3.6). Chest X-rays revealed no abnormalities. Lumbar puncture yielded acellular cerebrospinal fluid (CSF) with normal chemical tests. Electroneurography disclosed diffuse axonal motor neuropathy; with normal nerve conductions and no conduction blocks; the electromyographic study showed evidence of muscle denervation. No anti-MAG IgM reactivity was seen by western blot analysis; seric anti-GS Ig reactivity was assessed by ELISA. Prostate biopsy revealed acinar adenocarcinoma, which was treated with local irradiation. The patient was re-admitted 1 year later because of further neurological deterioration. The patient had diffuse muscular weakness and atrophy, mild dysartria and impaired swallowing. Anti-GS Ig and circulating γ-δ T lymphocytes were assessed at admission and repeated every 2 months. Seric prostate-specific antigen was undetectable and no signs of prostate carcinoma were found by CT. Chest X-rays and CT revealed a nodular lung mass in the right superior lobe without local adenopathy. The patient underwent surgical lobectomy, which showed bronchial-alveolar carcinoma. Soon after lung surgery, the patient had acute respiratory failure, requiring respiratory support with mechanical ventilation. High dose steroids (prednisolone 500 mg/day for 5 days) followed, 2 weeks later, by human Ig infusion (0.4 g/kg/day for 5 days) had no effect on his respiratory function. Apart from respiratory failure, neurological and electromyographic evaluations were stable. Cardiac arrest occurred 3 months after lung surgery; the patient remained in deep coma and died 3 months later. At autopsy, no recurrence of lung and prostate carcinoma was observed in tissues or local lymph nodes. In paraffin-embedded sections of the central nervous system (CNS), signs of tissue hypoxia (neuronal loss and gliosis) were detected diffusely in the gray matter of the brain. A relative sparing of gray and white matter in the spinal cord was evident; no pathological information was available for attached roots, dorsal root ganglia and peripheral nerves. In sections taken from cervical, thoracic and lumbar levels, neurons showed no gross quantitative and qualitative changes and inflammatory infiltrates were absent. Spinal cord samples were frozen.

2.1.2. Patient 2

A 71-year-old man was admitted to another hospital in 1998 because of numbness in his hands and feet and unsteady gait, which begun one month earlier. On neurological examination, sensory ataxia and mild diffuse muscle weakness were evident; deep tendon reflexes were absent and sensation of touch, temperature and vibration was impaired. Cranial nerves showed no gross abnormalities; however, he referred the tendency to turn his head to follow objects in the visual field. Blood chemistry was normal. Analysis of CSF revealed increased levels of proteins (0.9 g/l, n.v. 0.15–0.45) and 14 leucocytes/ml. Western blot analysis for anti-Hu IgG and anti-MAG IgM was negative. Seric anti-GS Ig activity was analyzed. Chest radiograph and CT scan showed an ilar mass with mediastinal lymphadenopathy. The patient was treated with two cycles of human Ig infusion (0.4 g/kg/day for 5 days) without improvement of his neurological deficits, which instead slowly worsened over the following months. One year later, a biopsy of paratracheal lymph nodes revealed small cell carcinoma (SCLC). Chest irradiation (36 Gy) was associated with chemotherapy with carboplatin (410 mg) and etoposide (420 mg). Soon after, the patient was admitted to our hospital in September 1999, where the neurological examination revealed trunk and limb ataxia, severe sensory impairment, areflexia and ophthalmoplegia. No recurrence of cancer was detected by CT. The neurophysiological study disclosed...
diffuse, axonal sensory polyneuropathy. Levels of anti-GS Ig were assessed by ELISA at admission and repeated every year. Treatment with azathioprine (150 mg/day) was initiated and continued until today. The neurological follow-up 4 years after SCLC treatment revealed discrete improvement of ocular movements and of trunk and limb sensory ataxia, while superficial sensory deficit was unchanged.

2.1.3. Controls
We analyzed humoral and cellular autoimmune responses to GS in 10 healthy donors (mean age 55 years, healthy controls) and in 7 patients affected by lung adenocarcinoma (mean age 65 years, neoplastic controls); at the time of lung surgery, no clinical and/or neurophysiological signs of peripheral neuropathy were documented. Exclusion criteria from these groups were: diabetes, alcohol abuse, cachexia, CNS metastases or history of previous carcinoma. Serum and a sample of lung carcinoma were obtained from each patient.

2.2. Purification and biotinylation of IgG

IgG from patients 1 and 2 (obtained before cancer treatment) and healthy controls were purified, following the protocol by Goudswaard et al. (1978). Patients’ sera were buffered with 1 M Tris HCl pH 8 (0.1 volume), loaded in protein A- and protein G-sepharose columns (Amersham Pharmacia, Uppsala, Sweden) mounted sequentially; after elution, fractions were collected, quantified and IgG resuspended at the same concentration observed before purification. For biotinylation, purified IgG were treated with 0.1 M N-hydroxysuccinimidoobiotin (Sigma) in dimethylformamide and dialyzed against PBS.

2.3. IgG subclass immunosubtraction

Concentrations of total IgG and IgG1, IgG2, IgG3, IgG4 subclasses were assessed in serum as well as in purified IgG of patients 1 and 2, by commercially available immunonephelometric method on BNII analyzer (DADE-Behring, Marburg, Germany). In order to obtain immunosubtraction of each IgG subclass, samples were diluted 1:10 in the corresponding specific polyclonal antiserum (anti-IgG1, -IgG2, -IgG3 and -IgG4; DADE-Behring), incubated overnight at 4 °C and centrifuged to remove immune complexes. On supernatants, Ouchterlony was performed to demonstrate complete subtraction.

2.4. Detection of seric anti-GS IgG

Measurement of seric anti-GS Ig was performed by enzyme-linked immunosorbent assay (ELISA), according to a standardized technique (Willison et al., 1999), to GM1, asialo-GM1, GM2, GD1a, GD1b, GQ1b (Alexis, Lausen, Switzerland), GM3, GD3 (Matreya, Pleasant Gap, PA, USA), GD2 and sulfatide (Sigma-Aldrich, Steinheim, Germany). Briefly, 96-well microtiter plates were coated with glycolipids (1 µg/10 µl methanol), saturated with 1% bovine serum albumin. After washing, the following samples were incubated overnight at 4 °C using serial dilutions: sera from patients 1 and 2 before and after tumor treatment, sera from healthy and neoplastic controls, purified IgG from patients 1 and 2 with or without IgG subclass immunosubtractions. Wells were washed and incubated with peroxidase-conjugated anti-human IgG or IgM antibodies (Bio-Rad Labs, Hercules, CA, USA) diluted 1:1000. After washing, the substrate solution with 0.2 mg/ml o-phenylenediamine dihydrochloride (Fluka, Buchs, Switzerland) and H2O2 was added and the optical density (OD) measured. After subtraction of background, the titer was assigned as the highest dilution at which the mean OD exceeded the cut-off value; this corresponded to the mean OD plus two standard deviations of sera from healthy subjects at 1:100 dilution. The ability of patient 1’s IgG to bind GM1, asialo-GM1, GD1a, GD1b, GM2, GM3, GD3 or sulphatide was assessed by absorption assays. For this purpose, the serum (1:1000) underwent three consecutive absorptions with each glycolipid (2 h at room temperature, overnight at 4 °C and finally 2 h at room temperature); the residual reactivity to the absorbing glycolipid as well as to GM1 and GD1a was then compared to pre-absorption values by ELISA (OD value after absorption/OD value before absorption×100). Thin layer chromatography (TLC) immunodetection was performed according to a standardized technique (Willison et al., 1993). Each ganglioside (GM1, GM2, GM3, GD1a, GD1b, GD2, GD3 and GQ1b) was loaded on TLC plate (Macherey-Nagel, Düren, Germany) and separated in chloroform/methanol/CaCl2 0.1M (50:42:11). The chromatograms were dipped in polisobutylmethacrylate (Aldrich) 0.25% in n-hexane (Sigma) and incubated with patients’ sera (1:200) overnight at 4 °C. The plate was then incubated with peroxidase-conjugated anti-human IgG (1:500) and the reaction visualized 3,3’-diaminobenzidine and H2O2.

2.5. Immunohistochemistry

Immunohistochemistry was performed on frozen sections from lung cancer of patient 1 and neoplastic controls, from autotopic normal human spinal cord and roots as well as from the spinal cord of patient 1, according to previously described protocols (Bonetti et al., 2000, 2003). Sections were fixed in 4% paraformaldehyde and incubated overnight at 4 °C with primary antibodies: CD68 for macrophages (1:800), CD3 (1:400) for T lymphocytes, CD20 (1:400) for B cells, CD4 (1:400) and CD8 (1:100; all from Dako, Carpenteria, CA, USA), and V61 (1:20; Immunotech, Marseille, France). To assess the expression and cellular localization of GS, we employed IgM monoclonal antibodies (mAbs) to GM1, GD1a, GD1b, GD2, N-acetyl-GM2, GD3, GM3 and GQ1b (all 1:800; Seikagaku, Tokio, Japan). Appropriate secondary biotinylated antibodies and ABC reagent (Vector Labs, Burlingame, CA, USA) were applied.
and peroxidase was used to visualize the reaction, with 3,3′-diaminobenzidine (Sigma) as chromogen; slides were then counterstained with hematoxylin. We then performed a set of experiments to assess the cellular distribution of the antigen(s) recognized by IgG from patient 1. Biotinylated IgG (1:50) from patient 1 and from healthy controls were incubated on frozen sections from normal human CNS tissue as well as from patient 1’s adenocarcinoma to establish the lipid nature of the autoantigen(s), extraction of glycolipids from sections was accomplished by chloroform/methanol (2:1) and 2% Nonidet P40 (Sheikh et al., 1999). In addition, the identification of the cellular structures targeted by patient 1’s IgG was performed by double immunofluorescence. Briefly, samples were incubated with antibodies to: S100 for Schwann cells, neurofilaments (2F11) or neuron-specific enolase (NSE) (1:500; all from Dako), GM1 or GD1α (1:50); the reaction was visualized with appropriate fluorescein-conjugated antibodies (Vector). After extensive washing, biotinylated IgG (1:10) from patient 1 or controls and streptavidin-Texas Red (1:100; Vector) were applied. Double direct immunofluorescence on autopic tissue of patient 1 was performed with the above phenotypic markers and complement membrane lytic complex C5a-C9 (Dako) and fluorescein-conjugated anti-human IgG (Dako) to detect infiltration in nervous tissue.

2.6. Isolation and characterization of circulating T lymphocyte subpopulations

Peripheral blood mononuclear cells from patient 1 and from neoplastic and healthy controls were isolated as previously described (Battistini et al., 1997). The phenotypic analysis was carried out after immunostaining with the following mAbs: fluorescein-conjugated V601 (Endogen, Woburn, MA), V62, CD25, CD69 (Pharminen, San Diego, CA) and phycoerythrin-conjugated CD3 (Caltag, Burlingame, CA). Samples were acquired on FACSCalibur (Becton Dickinson, San Diego, CA) and analyzed with CellQuest software.

2.7. Statistical analysis

For the measurement of anti-GS IgG titers before and after tumor treatment, with or without GS absorption or IgG subclasses subtraction as well as of circulating γ-δ T lymphocytes, each experimental condition was performed in triplicates and repeated twice in patients with suspected PNN, healthy and neoplastic controls; values were compared by repeated analysis of variance and the Bonferroni’s correction was performed between each condition, if a significant ($p\leq0.05$) effect was found by repeated analysis of variance. After the phenotype analysis of tumor infiltrating lymphocytes in patient 1 and neoplastic controls was

Fig. 1. Expression of gangliosides on lung adenocarcinoma. Immunohistochemistry on carcinoma from neoplastic controls showed faint signals for GM1 (A) and more pronounced reactivity for GD3 (B). On adenocarcinoma from patient 1, the majority of neoplastic cells displayed high levels of GM1 (C), GD1α (D) and GD3 (E); immunoreactivity for GM3 (F) and GM2 (G) was detectable with lower intensity, while GD1b (H) and GQ1b (I) were not expressed. Peroxidase, hematoxylin counterstain. Scale bars: A, B, H, I=50 μm; C, E–G=100 μm; D=150 μm.
performed by immunohistochemistry, the number of positive cells for each marker was counted in four randomly chosen fields (1 mm²) and values were compared by repeated analysis of variance.

3. Results

3.1. Expression of gangliosides on lung adenocarcinoma

Immunohistochemistry on carcinoma from neoplastic controls showed absent or faint staining for GM1 (Fig. 1A) and more pronounced reactivity for GD2 and GD3 (Fig. 1B); no immunostaining was observed for GD1a and the other GS (data not shown). At variance, the majority of neoplastic cells from patient 1 expressed high levels of GM1, GD1a, GD2 and GD3 (Fig. 1C–E), low levels of GM2 and GM3 (Fig. 1F,G), and no staining for GD1b and GQ1b (Fig. 1H,I); the signals for GD2 and GD3 were comparable to those detected in control carcinoma. Peritumoral tissue from either patient 1 and control neoplasia showed no expression of GS (data not shown).

3.2. Autoimmune responses to gangliosides in PNN

In both patients 1 and 2, the titers of IgG reacting with all the glycolipids tested by ELISA constantly ranged between 1:1500 and 1:3000 at repeated controls before the treatment of lung carcinoma (Fig. 2); no IgM reactivity to any glycolipid was observed. In healthy and neoplastic controls, no anti-GS IgG activity was detected. The titers of anti-GS IgG significantly dropped in both patients 1 and 2 after the treatment of lung carcinoma (see Table 1). In patient 1, a significant reduction was detected 2 months after tumor removal (Fig. 2A); in patient 2, anti-GS IgG persisted at low titers during the 4 years of follow-up (Fig. 2B). The reactivity of IgG to a wide spectrum of GS observed both in patients 1 and 2 prompted us to investigate whether all these antigens were responsible for the generation of autoantibodies. By immune TLC, a more restricted pattern of recognition of GS was evident in both cases; in fact, IgG from patient 1 specifically reacted with GD1a and GM1, whereas IgG from patient 2 recognized the gangliosides GD1a, GD2, GD1b and GQ1b, but not GM1, GM2 or GM3 (data not shown). These data were confirmed by measuring the capacity of each glycolipid to bind IgG of patient

Fig. 2. Titers of anti-ganglioside IgG in patients with PNN before and after tumor treatment. (A) In the serum of patient 1, high titers (1:3000) of IgG directed to a wide spectrum of glycolipids were repeatedly present before tumor removal (white columns) and significantly decreased two months after lung surgery (gray columns). (B) In patient 2, titers of anti-glycolipid IgG ranged between 1:1500 and 1:3000 in the presence of SCLC (white columns); a significant and permanent decrease of anti-glycolipid IgG titers was observed after SCLC eradication (gray columns). *p<0.001.
as shown in Fig. 3, only GM1 and GD1a were able to significantly reduce IgG reactivity to all GS.

We also investigated in both patients which IgG subclass was responsible for anti-GS activity. After immunosubtraction of each IgG subclass, residual anti-GS reactivity was evaluated by ELISA and compared to the levels obtained before immunosubtraction. In both patients 1 and 2, the subtraction of the IgG3 subclass provided a significant decrease of anti-GS activity (*p < 0.005), while the elimination of either IgG2 or IgG4 fractions failed to modify the residual anti-GS activity in both patients; in patient 1, a significant reduction of anti-GS activity was accomplished also by IgG1 subtraction (data not shown).

3.3. IgG from patient 1 recognized neural structures and his tumor

Biotinylated IgG from patient 1 were employed to assess the distribution of the antigen(s) recognized in situ in normal nervous tissue. By indirect immunohistochemistry with peroxidase and double immunofluorescence with GS and neural phenotypic markers, we found that IgG from patient 1, but not from healthy controls, stained motorneurons and glial cells in normal human spinal cord (Fig. 4A,B) as well as neurons of dorsal root ganglia (Fig. 4C). Regarding the cellular distribution of GM1 and GD1a, we found that both gangliosides were expressed on motorneurons, dorsal root ganglia and glial cells of normal spinal cord (data not shown), as previously described (Gong et al., 2002; Nardelli et al., 1994). In addition to CNS structures, IgG from patient 1, but not from healthy controls, decorated Schwann cells and axonal structures in human PNS. Schwann cells and myelin sheaths were immunostained by patient 1’s IgG showing partial co-localization with GM1 either in posterior (Fig. 4D–F) and anterior (Fig. 4G–I) spinal roots; in addition, axons were stained by IgG from patient 1 either on sensory (Fig. 4E) and motor fibers (Fig. 4H). In the autopic human material available, it was difficult to ascertain the precise localization of IgG reactivity (e.g. whether IgG stained the axolemma or the inner surface of Schwann cells). With this limitation, in longitudinal sections of anterior roots, the signal of IgG from patient 1 appeared to co-localize with GD1a on structures morphologically resembling the node of Ranvier (Fig. 4J–L). When we tested the binding of IgG on tumor from patient 1, we found that his IgG (Fig. 4M), but not from healthy controls (Fig. 4N), recognized the majority of lung neoplastic cells. Delipidation of sections abolished the signals of IgG from patient 1 on both neural structures (data not shown) and on his own tumor (Fig. 4O), thus confirming the lipid nature of the antigen(s) recognized.

The results obtained by indirect immunohistochemistry regarding the cellular targets recognized by IgG from patient 1 were confirmed by the study of his autopic nervous tissue; in the spinal cord and attached roots, infiltration of IgG was detected by direct double immunofluorescence on residual motorneurons (data not shown) as well as on PNS Schwann cells and axons (Fig. 4P–R), without any relevant

| Table 1 Clinical and biological features of patients with paraneoplastic neuropathy and anti-GS IgG |
|-----------------|-----------------|-----------------|
| **Patient 1**   | **Patient 2**   |
| Age (years)     | 73              | 71              |
| Clinical features| Motor neuropathy| Sensory ataxia, |
|                 |                 | ophthalmoparesis|
| Clinical evolution| Chronic, progressive | Subacute, progressive |
| Neurophysiological findings | Axonal polyneuropathy | Axonal polyneuropathy |
| CSF proteins    | Normal           | Increased       |
| Anti-GS IgG     | GM1, GD1a       | Disialo-GS      |
| Circulating γ-δ T cells | Increased | Not performed |
| Tumor           | Lung adenocarcinoma | Small cell lung carcinoma |
| Follow-up (months) | 24; deceased | 60; alive |
| Response to tumor treatment | Normalization of | Slight improvement |
|                 | blood γ-δ T cells | of neuropathy   |
|                 | Stabilization of | Decreased titers of |
|                 | neuropathy       | anti-GS IgG     |

![Fig. 3](image.png)

Fig. 3. Absorption with gangliosides (GS) identified the epitopes able to decrease anti-GS IgG titers. The serum of patient 1 was absorbed with a panel of GS (indicated in the upper case) and the residual IgG activity to glycolipids (lower case) was measured (see Section 2 for details). Only the absorption with GD1a and GM1 significantly reduced the activity of IgG directed to the absorbent GS as well as to the other GS, at variance, GM2 yielded a selective decrease of anti-GM1 IgG reactivity, while the other glycolipids had no influence on anti-GS IgG reactivity. *p < 0.005.
Fig. 4. IgG from patient 1 recognized lipid autoantigens in normal neural tissue and in his tumor. Biotinylated IgG from patient 1 (A), but not from healthy controls (B), immunostained motorneurons in human spinal cord and neurons of dorsal root ganglia (C). Double immunofluorescence for GM1 (D and G) and IgG from patient 1 (E and H) showed a partial co-localization on Schwann cells and myelin sheaths in posterior (F) and anterior (I) roots (resulting in orange signals) but not on axons, which were recognized only by patient 1’s IgG and appeared red. Double staining for GD1a (J) and IgG from patient 1 (K) showed co-localization (L) in anterior roots on focal structures morphologically resembling nodes of Ranvier (arrows). Biotinylated IgG from patient 1 (M), but not from healthy controls (N), immunostained his neoplastic cells and the signal was abolished after extraction of glycolipid antigens (O). In the autoptic nervous tissue of patient 1, double staining for S-100 (P) and IgG (Q) showed co-localization on Schwann cells (orange signals), but not on axons, which were positive only for IgG (R). Scale bars: A–C=150 μm; D–I, P–R=100 μm; J–L=200 μm.
difference between anterior and posterior roots. No evidence of complement activation was detected using the mAb anti-C5a-C9 lytic complex. Infiltration of IgG was absent or very faint in normal spinal cord and roots (data not shown).

3.4. Cellular autoimmune responses in patient 1

The fact that anti-GS activity belonged to IgG1 and/or IgG3 subclasses in both patients 1 and 2 was highly suggestive of a T-cell-dependent B cell activation (Greenlee et al., 2001). Since γ-δ T cells are able to respond to glycolipids and have been involved in acute inflammatory neuropathies (Spada et al., 2000; Ben-Smith et al., 1997), we set out to determine whether this lymphocyte subset played a pathogenetic role in patient 1. As compared to healthy donors and neoplastic controls, in the blood of patient 1, we found a significant increase of the percentage of circulating Vδ1-positive T lymphocytes (Fig. 5A), the majority of them showing high levels of expression of the activation markers CD25 and CD69 (data not shown). The percentage of circulating Vδ1-positive T cells in patient 1 dropped to normal levels 2 months after lung tumor removal (Fig. 5A), together with a decrease of the activation state (data not shown). Such expansion of circulating Vδ1-positive lymphocytes prompted us to investigate the distribution of these T cells in the nervous tissue and in the lung tumor of patient 1. We failed to detect any inflammatory infiltrate in autopic CNS or spinal roots, whereas inflammatory cells were abundant in his lung carcinoma. The phenotypic characterization of inflammatory cells infiltrating the lung neoplasia from patient 1 and neoplastic controls showed a predominance of CD3-positive T lymphocytes, which were mainly composed by CD4 and CD8 T cells. Interestingly, the proportion of Vδ1-positive T cells among tumor infiltrating lymphocytes was significantly higher in patient 1 than in neoplastic controls, where this T-cell subset never exceeded 2% of total T lymphocytes (Fig. 5B).

4. Discussion

We have described two patients with chronic progressive neuropathy and lung carcinoma, who lacked antibodies to known onconeural antigens but had anti-GS IgG; in both cases, several lines of clinical and experimental evidence strongly suggested that GS represented the onconeural targets of humoral and cellular immune responses. In fact, lung adenocarcinoma or SCLC was associated with subacute/chronic axonal neuropathy, which run a progressive course until cancer was treated. Although the time of observation after surgery was short in patient 1, mild improvement of ophthalmoparesis was observed in patient 2 during the four years follow-up. Since immune suppressive therapy in both patients was begun soon after tumor treatment, it is difficult to ascertain whether the positive effect on the neuropathy was due to cancer treatment or whether it was also related to immune suppression. In this regard, however, Ig infusion was performed in patient 2 before SCLC treatment and failed to influence his neurological course.

Similarly to acute post-infectious neuropathies, the different clinical features of these two patients were associated with IgG reactivity to distinct GS. Although IgG reactivity was apparently directed to a wide spectrum of glycolipids by ELISA, a more restricted pattern of GS recognition has emerged with TLC and GS absorption assays. In the patient 2 with ataxic neuropathy and ophthalmoparesis, immune TLC showed that IgG recognized an epitope common to GD1a and disialo-GS, as in patients with acute Miller-Fisher syndrome (Willison et al., 2001). The experiments with TLC and GS absorption indicated that IgG from the patient 1 with progressive motor neuropathy reacted with an epitope common to GD1a, GM1 and probably GM2. In addition, the role of GM1 and/or GD1a as onconeural molecules in this patient is also supported by the expression at high levels of GM1 and GD1a in his lung adenocarcinoma, but not in neoplastic controls. Thus, in line with previous observations (Ho et al., 1999), in both these patients, axonal neuropathy was
associated with anti-GD1a reactivity, while the different clinical pictures reflected distinct fine epitope specificities. Two additional features further indicated a central role for GS as onconeural antigens in these patients with PNN. In fact, examining the anti-GS IgG titers during the follow-up, in both patients, we observed a significant decrease of anti-GS IgG after cancer treatment, which persisted at low levels for years in patient 2. In addition, a causal relationship among lung cancer, anti-GS IgG and neuropathy in was suggested by the binding of patient 1’s IgG to his tumor and to normal neuronal cells, PNS Schwann cells and axons. In line with previous studies (Sheikh et al., 1999; Gong et al., 2002; De Angelis et al., 2001), we found that CNS and PNS structures stained by IgG from patient 1 also expressed GM1 and/or GD1a. In particular, IgG from patient 1 immunostained Schwann cells and axons either in posterior and anterior spinal roots and the immune reactivity was abolished by delipidation of both CNS and PNS sections. Interestingly, a strict correlation was observed between the neural targets recognized by patient 1’s IgG on normal nervous system and those infiltrated by IgG in his autotropic nervous system.

In addition to anti-GS IgG, cell-mediated immune responses to glycolipids might have also played a pathogenetic role in patient 1; in this regard, we focused on γ-δ T lymphocytes, a minor subpopulation of T cells able to respond to lipid antigens (Spada et al., 2000) and involved in other neurologic autoimmune diseases (Borsellino et al., 2000; Ben-Smith et al., 1997). Activation of T lymphocytes and T-cell-dependent production of IgG1 and IgG3 autoantibodies have been demonstrated in patients with autoimmune neuropathies with anti-neural IgG, including PNN (Albert et al., 2000; Ogino et al., 1995; Greenlee et al., 2000; Jean et al., 1994). In patients with Guillain-Barré syndrome, expansion of interleukin 4-producing Vδ1-positive T cells has been demonstrated by our group (Borsellino et al., 2000). In line with these observations, anti-GS activity was restricted to IgG1 and/or IgG3 subclasses in both patients 1 and 2. In addition, a significant increase of activated, circulating Vδ1-positive T lymphocytes was observed in patient 1, as compared to healthy and neoplastic controls; the percentage of Vδ1-positive T cells in patient 1 dropped to normal levels two months after cancer removal, in parallel with the reduction of anti-GS IgG titers. Finally, the phenotypic study of tumor infiltrating T lymphocytes revealed that in patient 1, in parallel with the expansion in peripheral blood, Vδ1-positive T cells represented more than 15% of T lymphocytes; this proportion was significantly higher than in neoplastic controls, where γ-δ T cells never exceeded 1% (Fajac et al., 1992). Whether the activation of these T cells in patient 1 may have cytotoxic (i.e. anti-tumoral) or regulatory (favoring the production of IgG) functions is difficult to ascertain from our investigations.

Taken together, the results presented suggest that the ectopic expression of GS on neoplastic cells elicit autoimmune responses, which may target the nervous system resulting in PNN. Thus, we suggest that anti-GS IgG should also be tested in patients with suspected PNN; in fact, IgG to known oncoantigens are detectable in a minor proportion of PNN cases, while in the remaining sero-negative cases the paraneoplastic origin of the neuropathy remained possible (Antoine et al., 1999). In this regard, the case of patient 1 deserves a comment, since peripheral neuropathy was associated with two distinct adenocarcinomas, of which only one was causally related to PNN. This observation suggests that the co-existence of tumors and neurological disorders is not always sufficient to define a paraneoplastic etiology and enforce the need of further studies to identify target molecules of suspected, sero-negative PNN in order to better approach these patients.

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