

Immunological study of hereditary motor and sensory neuropathy type 1a (HMSN1a)

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Objectives: Fifty three patients were studied to investigate whether autoimmune or inflammatory mechanisms could explain the phenotypic heterogeneity of patients with hereditary motor and sensory neuropathy type 1a (HMSN1a). **Methods:** Serum samples were examined for antibodies to peripheral nerve myelin protein 22 (PMP22), ganglioside GM1 and cauda equina homogenate, and interleukin-6 (IL-6) and soluble tumour necrosis factor receptor 1 (sTNF R1) concentrations. Serological results were compared with those from patients with other neuropathies (ONPs, n=30) and with normal subjects (n=51).

Results: In the group as a whole, no relation emerged between clinical severity and any immune parameters. Immunohistochemical examination of four sural nerve biopsies did not show significant inflammatory infiltration. In a subset of 12 patients who experienced stepwise progression of disease, there was a trend towards a higher proportion having anti-PMP22 antibodies (33% v 15% of those with gradual disease progression, 3% ONPs, and no normal controls) and complement fixing antibodies to human cauda equina (25% v 5% with gradual progression, 8.6% ONPs, 3.9% normal controls, p=0.07).

Conclusions: Patients with HMSN1a and a stepwise disease progression may have an inflammatory, autoimmune component superimposed on the genetic condition.

The early literature acknowledged great variation in age of onset, rapidity of progression, nerve conduction velocity, and neuropathological abnormalities in the hereditary motor and sensory neuropathies (HMSNs). The division of the commonest HMSNs into types 1 and 2 on the basis of nerve conduction velocities¹ was invaluable for future classification studies and suggested some clinical differences between these types, but did not explain the phenotypic variation. Even within the commonest genotype for HMSN, a 1.5 Mb duplication in c17p11.2, (the gene for the myelin protein PMP22^{2–6}), clinical variability both within and between families is marked and not yet explained.^{7–10}

We have shown that recombinant, homologous PMP22 induces experimental autoimmune neuritis (EAN) and that these animals develop antibodies against the immunising antigen.¹¹ We have recently identified anti-PMP22 antibodies in 52% of patients with Guillain-Barré syndrome (GBS) and 35% of those with chronic inflammatory demyelinating polyradiculoneuropathy (CIDP).¹² Using a different assay others have detected anti-PMP22 antibodies in 70% of patients with HMSN1a and 60% with HMSN2 as well as in those with other neuropathies.¹³

We have now studied 55 patients with HMSN1a to establish whether immunological mechanisms might contribute to the phenotypic heterogeneity of this group.

METHODS

Fifty five patients known to have the chromosome 17p11.2-p12 duplication were interviewed and examined by one of us (CMG). Serological studies were performed on 53 and neurophysiological assessment on 33. Patients not tested declined.

Clinical data

Patients were recruited between January 1995 and January 1997 from the neuromuscular disease clinic at Guy's Hospital, the Departments of Neurology and Neurogenetics at the National Hospital for Neurology and Neurosurgery, and the

Charcot-Marie-Tooth Society. The first 55 patients recruited by any of these means were included. All were interviewed with a standard questionnaire and underwent clinical examination. Three clinical scoring systems were used: The Medical Research Council (MRC) sum score,¹⁴ a modification of the neurological disability score (NDS)¹⁵ comprising reflex, sensory and weakness scores and the the upper and lower limb sections of the Guy's neurological disability score (GNDS).^{16, 17}

Nerve conduction studies

The compound motor action potentials (CMAPs) of abductor pollicis brevis and abductor hallucis, the sensory nerve action potentials (SNAPs) of the right median and sural nerve, and the motor nerve conduction velocities (NCVs) of the right median and tibial nerve were measured (Nicolet Compass Portabook).

Serology

Serum samples from the 53 patients above, together with those from 30 patients with other neuropathies (ONPs) and 51 normal control serum samples were collected and stored at -70°C. The ONP group included patients with idiopathic sensorimotor neuropathy, HMSN2, granulomatous neuropathies,

Abbreviations: HMSN1a, hereditary motor and sensory neuropathy type 1a; PMP22, peripheral nerve myelin protein 22; IL-6, interleukin-6; sTNF R1, soluble tumour necrosis factor receptor 1; ONPs, other neuropathies; EAN, experimental autoimmune neuritis; GBS, Guillain-Barré syndrome; CIDP, chronic inflammatory demyelinating polyradiculoneuropathy; NDS, neurological disability score; GNDS, Guy's neurological disability score; CMAP, compound motor action potentials; SNAPs, sensory nerve action potentials; NCVs, nerve conduction velocities; ECD1 and ECD2, first and second extracellular domain peptides; AIDP, acute inflammatory demyelinating polyradiculoneuropathy; IDP, inflammatory demyelinating polyradiculoneuropathy; DAB, 3,3'-diaminobenzidine

hereditary neuropathy with liability to pressure palsies, idiopathic lumbar plexopathy, and vasculitis.

Anti-PMP22 antibodies

Titres of anti-PMP22 antibodies were measured by ELISA and western blot in the serum samples of all patients and controls as we have previously described.¹² For the ELISA, MP22 first and second extracellular domain peptides (ECD1 and ECD2) were cross linked to bovine serum albumin (BSA) and used to coat ELISA plates. Plates were incubated with patients' serum and bound antibody detected with horse radish peroxidase (HRP) conjugated rabbit anti-human IgM, IgG or anti-pan Ig (anti-IgG/Ig/IgA). Plates were developed with *o*-phenylenediamine dihydrochloride and H₂O₂ and the optical density read at 490 nm. For the western blot, suspended frozen sections of human cauda equina were fractionated by SDS-PAGE, transferred to nitrocellulose, incubated with patient's serum and then with HRP conjugated rabbit anti-human IgG or IgM and developed with diaminobenzidine hydrochloride and cobalt-nickel enhancement.

Interleukin-6 and soluble tumour necrosis factor receptor 1
Serum interleukin-6 (IL-6) and soluble tumour necrosis factor receptor 1 (sTNF R1) concentrations were measured in 52 HMSN1a subjects, 51 normal subjects, 10 ONPs and also in eight patients with GBS, with a quantitative sandwich enzyme immunoassay technique (Quantikine™, R and D Systems, Minneapolis, USA). Optical densities were read at 450 nm with 570 nm wavelength correction and concentrations calculated by creating a standard curve with the standards provided.

Complement fixation test

Serum samples were heat inactivated at 56°C for 30 minutes and tested for complement fixing activity to a predetermined optimal dilution of human cauda equina homogenate.¹⁸ This was performed on the serum from 52 patients with HMSN1a, 51 normal controls, and 23 patients with ONPs.

Anti-ganglioside antibodies

Serum samples from 52 patients with HMSN1a, 51 normal controls, and 23 patients with ONPs were tested by enzyme linked immunosorbent assay (ELISA) using methods previously described.^{19, 20} Immulon-3 ELISA plates were coated with ganglioside:cholesterol, blocked, and incubated overnight with patients' serum. Bound antibody was detected with anti-human IgM or anti-IgG, plates were developed with *p*-nitrophenylphosphate, and the optical density was read at 405 nm.

Histology

Paraffin wax embedded sural nerve biopsies of four patients with the c17p11.2 duplication were available. Controls were biopsies of three patients with vasculitis (one with polyarteritis nodosa, two with non-systemic vasculitic neuropathy), three with inflammatory demyelinating neuropathy (one with acute inflammatory demyelinating polyradiculoneuropathy (AIDP), two with CIDP), and six with chronic idiopathic axonal polyneuropathy. Transverse skip serial sections (5 µm) were heated at 60°C overnight to assist adherence. Antibodies against CD68 (monoclonal PGM-1), CD3 (polyclonal) and CD20 (monoclonal L26) in EPOS (enhanced polymer one step staining, DAKO) form were used. Sections were dewaxed through xylene, rehydrated, and blocked with 1% H₂O₂ in methanol. Before application of anti-CD68 and anti-CD20, sections were microwaved. Pretreatment for anti-CD3 was incubation for 30 minutes in 0.1% trypsin at 37°C. Sections were blocked with normal goat serum, incubated with the EPOS antibodies for 1 hour, and visualised with 3,3'-diaminobenzidine (DAB) counterstained with haematoxylin.

Areas of the endoneurium and epineurium/perineurium were measured using a Freelance software image analysis programme. Numbers of positive cells in both areas were counted by an observer blind to the identity of the section. The upper limit for increased cellular infiltration in each compartment was defined as the mean +3SD of that seen in patients with chronic idiopathic axonal polyneuropathy.

Statistical analysis

Calculations were performed using GraphPad Prism® software with two tailed tests of significance. Differences in proportions were made using a χ^2 test or Fisher's exact test. Differences between two groups were determined by a *t* test or the Mann-Whitney test. Other differences between larger numbers of groups were made with one way analysis of variance (ANOVA, with Bonferroni's multiple comparison test for post hoc analysis) if the groups were normally distributed, or the Kruskal-Wallis (with Dunn's multiple comparison test for post hoc analysis) if they were not. Correlations between two groups were performed with the Pearson test if groups were normally distributed and the Spearman test if they were not.

RESULTS

Disease progression

Most patients gave a history of slowly progressive disease. However, in 12 patients progression was in a stepwise rather than gradual fashion. Five of these were sporadic cases and none of those with a family history were in the same family. One patient had an IgM paraprotein,²¹ and three others had a history of improvement with immunosuppression. One had worsened significantly during each of her pregnancies.

Clinical neurophysiology

Sural nerve SNAPs were absent in all patients and the mean median SNAP amplitudes reduced at 0.7 µV (range 0–13.6 µV). The mean CMAP amplitude of the abductor pollicis brevis was 3.1 mV (range 0.1–12.7 mV) with a mean median motor NCV of 23 (12–32) m/s. The CMAP amplitude of the abductor hallucis was more severely reduced, mean 0.19 mV (range 0.03–1.7). The maximum tibial motor NCV in the lower limb was reduced to a similar extent as in the upper limb (mean 21 m/s, range 15–28).

Serological results

Anti-PMP22 antibodies

Fourteen out of 53 patients with HMSN1a had antibodies detected by ELISA against either of the PMP22 extracellular domains compared with two of the 51 normal subjects (*p*=0.002) and three of those with ONPs (*p*=0.09, table 1). The diagnoses in the ONP group with positive anti-PMP22 antibodies were alcohol related axonal neuropathy, adrenomyeloneuropathy, and idiopathic axonal neuropathy (with a history of sarcoidosis). One of the normal subjects had worked with myelin proteins in the laboratory for many years.

Table 1 Details and ELISA results in each group examined for anti-PMP22 antibodies.

	HMSN1a	Normal	ONP
No of patients	53	51	30
Age (mean (SD))	43 (18)	33 (17)	45 (23)
Sex (M/F)	30M/23F	23M/28F	15M/15F
Any antibodies	14	2	3
IgM antibodies	4	1	0
IgG antibodies	9	1	1
Polyvalent antibodies	5	1	3

All antibody results are for absolute numbers of patients. Some patients had more than one class of antibody.

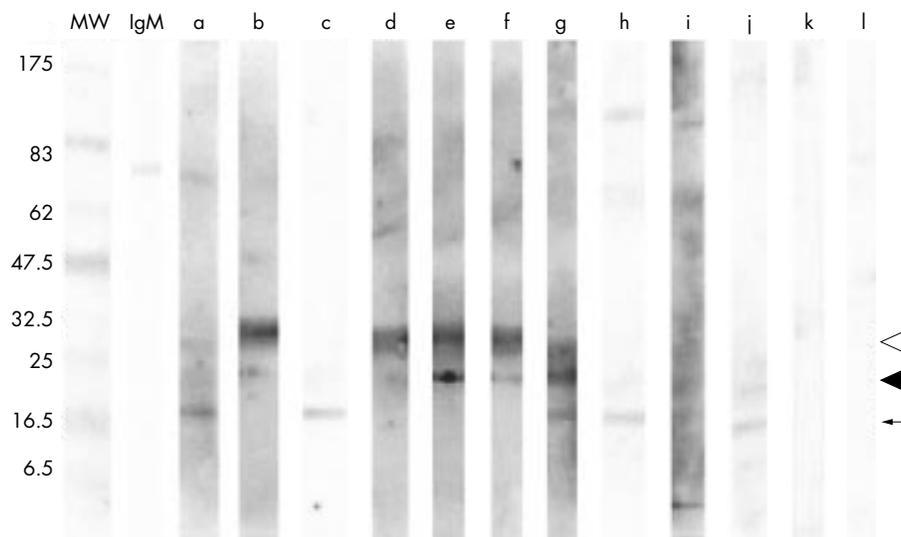


Figure 1 Western blot of human cauda equina incubated with serum from representative patients with HMSN1a. The blot was incubated with the serum of patients with HMSN1a (lanes a-g) or normal control subjects (lanes h-l). The lane marked IgM represents a blot with the IgM secondary antibody alone (no primary). Bands indicating a protein of 22 kDa are indicated by the filled arrowhead (lanes b, e, f, g). Some patients (lanes b, d, e, f, g) had antibodies against proteins of about 30 kDa (empty arrow head). Some subjects (lanes a, c, g, h, i) had antibodies directed against a smaller protein of 16 kDa (arrow).

Western blotting confirmed 10 out of 14 of the positive ELISA results from the group of patients with HMSN1a (fig 1). Five patients had IgG antibodies alone, two had IgM antibodies alone, and three had both IgM and IgG antibodies confirmed by both ELISA and western blot. Western blotting did not confirm the presence of anti-PMP22 antibodies in the two normal control subjects with a positive ELISA but did identify a 22 kDa band with the serum from one other normal subject.

By western blotting, serum also bound to a protein band of about 30 kDa (range 30–36 kDa) in six patients (five IgG antibodies, one IgM) with HMSN1a and one (IgG) with ONP which was not bound by the serum of any normal subjects. A band of approximately 16 kDa was also detected in the serum of four patients (all IgM) with HMSN1a and eight normal controls (four IgM, four IgG), but not that from any of the patients with ONPs.

There were no significant differences in any of the measures of severity or neurophysiological indices in the 10 patients with HMSN1a with anti-PMP22 antibodies confirmed by western blot and ELISA compared with the rest.

TNF R1

There was no significant increase in the concentration of sTNF R1 in patients with HMSN1a compared with normal subjects (fig 2 A). Four patients had particularly high sTNF R1 concentrations (arrowed). The patient with HMSN1a was a woman aged 54 with mild disease although moderate resting tremor, who did not have anti-PMP22 antibodies. The patients with ONPs had polyarteritis nodosa, inactive sarcoidosis, and chronic idiopathic axonal polyneuropathy.

IL-6

There were no significant increases in the concentrations of IL-6 in patients with HMSN1a compared with normal subjects (fig 2 B). Five patients had particularly high concentrations, three with severe GBS and two normal subjects. Two of the patients with GBS had antibodies to PMP22.¹² The normal subjects with high IL-6 concentrations were healthy women, neither with anti-PMP22 antibodies.

Anti-GM1 antibodies and complement fixation test against human nerve

Antibodies to ganglioside GM1 and a positive complement fixation test against human cauda equina were not more common in patients with HMSN1a. However, two patients with HMSN1a had very high titres by complement fixation test. One of these (titre 1/16364) was known to have an IgM paraprotein²¹ the other (titre 1/65456) was one of the patients with a stepwise disease progression who had responded to immunosuppression.

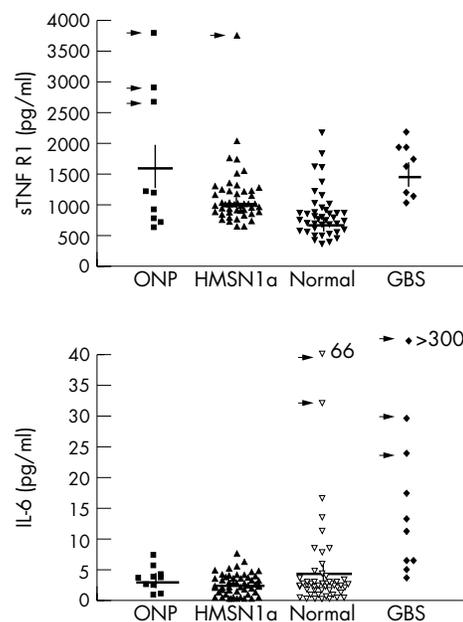


Figure 2 (Top) Graph showing sTNF R1 concentrations. Arrows indicate patients discussed in text. (Bottom) Graph showing IL-6 concentrations in all groups examined. Arrows indicate patients discussed. Bars on both graphs indicate mean (SE)

Table 2 Serological results in patients with a stepwise disease progression compared with those with a gradual progression

	Patients with stepwise progression	Patients with gradual progression
Total number of patients	12	40
sTNF R1 concentrations (mean (SD))	896 (500)	1097 (562)
IL-6 concentrations (mean (SD))	2.7 (1.9)	2.9 (1.6)
Positive CFT (n)	3	2
Anti-GM1 antibodies (IgM) (n)	1	4
Anti-PMP22 antibodies* (n)	4 (1 IgM, 2 IgG, 1 both)	6 (1 IgM, 3 IgG, 2 both)

*Patients positive by both ELISA and western blot. (n), absolute numbers of patients.

Serological results in the group reporting stepwise disease progression

Serological results of these 12 patients were compared with those in whom progression was gradual (table 2). There were no significant differences with regard to anti-PMP22 antibodies, sTNF R1, or IL-6 concentrations. Three (25%) patients with a stepwise progression had a positive complement fixation test compared with two out of 40 (5%) of those with gradual disease progression ($p=0.07$).

Histological studies

Patients with HMSN1a had no evidence of increased infiltration of T lymphocytes (CD3). Three patients with HMSN1a, one with IDP and one with vasculitis had small numbers of endoneurial B cells (from $0.7-3.3/\mu^2$). Epineurial B cells were also seen in small numbers (from $0.2-5.7/\mu^2$) in two biopsies of patients with HMSN1a, two with IDP and one with vasculitis. Biopsies from two patients with HMSN1a who reported stepwise deterioration were examined. One showed no evidence for cellular infiltration, the other was known to have an IgM paraprotein and his biopsy did show some epineurial macrophage infiltration and a few epineurial B cells. Electron microscopic examination of this patient's biopsy had previously shown a demyelinating neuropathy with hypertrophic changes (concentric Schwann cell proliferation), widely spaced myelin, and IgM deposition on the myelin sheath.²¹

CONCLUSIONS

Clinical,²²⁻²⁴ serological,^{25, 26} electrophysiological,²⁷ and histological²⁸⁻³² studies have suggested the existence of an inflammatory component in HMSN. Immune deficiency in a mouse model of a hereditary demyelinating neuropathy results in a less severe neuropathy.³³ We have shown that antibodies to important nerve constituents or increased cytokine concentrations are present in the serum of only a minority of patients. For all serological measurements, there were no differences in severity of disease between those with positive results (or high concentrations) and those without.

Anti-PMP22 antibodies

We hypothesised that an increase in PMP22 protein expression in HMSN1a^{34, 35} might lead to immune responses directed against this protein. We previously showed that anti-PMP22 antibodies are found in 35%-52% of patients with inflammatory neuropathies.¹² Recently, using different techniques, a high proportion of patients with different types of peripheral neuropathy including HMSN were found to have anti-PMP22 antibodies.¹³

Ten patients had antibodies against PMP22 detected by both ELISA and western blot. Of these, six also had antibodies against a 30 kDa protein, likely to represent antibodies against P0, which have been described in other peripheral neuropathies.^{36, 37} Antibodies against PMP22 were more

common in patients with HMSN1a than in normal subjects, but not more than in those with other neuropathies. These results are similar to those of Ritz *et al.*¹³

As no clinical differences were detected between those with anti-PMP22 antibodies and those without, it seems unlikely that clinical phenotypic differences can be ascribed to a humoral response directed towards PMP22. It is possible that the finding of anti-PMP22 antibodies in HMSN1a is usually a non-specific reaction to nerve damage rather than of primary pathological importance. It would be interesting to determine whether patients with HMSN1a have antibodies directed towards P0 in similar numbers and titre to confirm this.

Cytokine concentrations

Previous studies had shown increased plasma concentrations of a leukotriene, decreased concentrations of arachidonic acid, and raised levels of inflammatory cells in HMSN^{25, 26} suggesting a potential for immune mediated damage by cellular mechanisms. Neither the "Th2 cytokine" IL-6 which stimulates B cells, nor the "Th1 cytokine" sTNF R1, which corresponds to raised TNF concentrations,³⁸ were raised, suggesting that neither pathway is activated in HMSN1a.

Histology

Demyelination in HMSN 1a is most active in childhood when macrophage associated myelin removal ("active" demyelination) has been described,²⁸⁻³⁰ similar to that seen in GBS³⁹ and CIDP.⁴⁰ In adults, isolated clusters of mainly T cells have been found in 12% of patients with HMSN1a. Endoneurial oedema, which may be prominent in CIDP,²² has been described in 21% of patients with HMSN1a³⁰ and onion bulbs may be seen in CIDP as well as HMSN1a.^{30, 41}

Our histological study did not confirm previous reports of increased numbers of T cells in HMSN,^{30, 31} as none of the patients with HMSN1a had more epineurial or endoneurial cells than in chronic idiopathic axonal polyneuropathy. Only one patient with HMSN1a had increased numbers of macrophages,^{28, 32} and he was known to have an IgM paraproteinaemic neuropathy.²¹ Although numbers of B cells were small in all biopsies, they were found most often in HMSN1a, although as the finding was marginal we think that it would be inappropriate to advance hypotheses for isolated B cell infiltration in HMSN1a.

Patients with stepwise disease progression

In 12 patients, all from different families, the progression of the disease was stepwise, with months in which progression in symptoms was rapid, followed by months or years of seemingly quiescent disease. One patient was known to have an IgM paraprotein,²¹ but none of the others had any additional known causes for their "relapses".

Three patients had been treated with immunosuppression (IVIg in one patient and this together with plasma exchange in the other two patients) as the pattern of their illness fitted

that of either AIDP (in one case, similar to recently reported cases^{42,43}) or CIDP (two cases). All responded favourably. Two groups have described beneficial responses to prednisolone in patients with HMSN type 1^{22,23} but it is not known whether these patients had the HMSN1a genotype. Partial steroid responsiveness has also been recently reported in a family with a mutation in the myelin protein zero gene.⁴⁴

The characteristics of the group with a stepwise progression with regard to weakness, areflexia, sensory loss, or disability were no different from the group as a whole, although only half of them were examined during what they described as a relapse. There was a trend towards more patients having anti-cauda equina antibodies in this group compared with those with a gradual progression. Although not significant, twice as many patients with stepwise progression had anti-PMP22 antibodies (33% compared with 15% with gradual progression). This compares with 52% of those with GBS and 35% of those with CIDP.¹²

The subgroup with stepwise progression may represent patients in whom a heightened humoral immune response occurs, directed against myelin proteins. There are several possible explanations for this. It could be that these patients have an additional immunosusceptibility to an inflammatory demyelinating neuropathy. Because they have altered or over-expressed PMP22, which already renders their peripheral nerves liable to demyelination, a superimposed inflammatory demyelinating disorder may be more likely to occur in them than in genetically normal subjects. Similar susceptibilities have been implicated in adrenoleukodystrophy^{45,46} and facio-scapulothoracic dystrophy^{47,48} and have been reported for EAN in inbred strains of rats.⁴⁹ Alternatively the inherited neuropathy may expose myelin antigens, or the c17p duplication may contain genes that modify the immune response in some patients. In mice heterozygously deficient in the myelin protein zero gene, T cells show enhanced reactivity to myelin components and immune deficiency results in less severe peripheral nerve disease.³³ It is plausible but seems less likely that these patients represent a subgroup of pure HMSN1a responsive to immunosuppression. Of course, it remains possible that these patients with a stepwise disease progression simply have coincidental inflammatory neuropathy and HMSN1a, but our results suggest that, in this group, immune mediated mechanisms relate the two conditions.

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