Auto-antibodies in Myeloma Patients with Peripheral Neuropathy

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Abstract

Background - Peripheral neuropathy is a known complication of multiple myeloma. Incidence of peripheral neuropathy increases with the use of certain drugs (eg: Thalidomide, Bortezomib). It was hypothesised that the reason for neuropathy in myeloma due to autoantibody damage on the nerve tissue. Aim of the study was to determine the specific types of paraneoplastic auto-antibodies and anti-ganglioside antibodies and the presence of such auto-antibodies using antigenic targets in monkey cerebellum.

Patients and Methods – 377 myeloma patients with peripheral neuropathy were selected from MRC myeloma trial IX. The age range was 31 to 90. The sex ratio was 241: 136 Blood samples were collected at the time of recruiting and six years later. There were only 269 patients at the end of six years. Serum samples were stored following a precise laboratory procedure till the tests were done. Samples were tested for paraneoplastic auto-antibodies (Ma2, Yo) and antigen targets in monkey cerebellum using indirect immunofluorescence and western blots. Serum samples were also screened for seven types of anti-ganglioside antibodies using western blots.

Results – 43 patients (11%) had antibodies against cerebellum. Five were positive to paraneoplastic auto-antibodies (Ma2). Anti-ganglioside antibodies were detected in 30 (8%). Only IgM were found in 26, IgG and IgM in 3 and IgG in 1.

Conclusion – Significant number of myeloma patients with neuropathy has antibody against nerve tissues.

Discussion – Sample consists of elderly, neuropathy is a common occurrence in elderly. Research is ongoing with different sample of myeloma patients without neuropathy, who are tested for the specific auto-antibodies. Detected unidentified antibody patterns need further investigation.

Key Words: *Auto antibodies, Indirect immunofluorescence, Monkey Cerebellum, Myeloma, Peripheral Neuropathy, Western Blots*

Introduction

Peripheral neuropathy is one of the most observed undesirable clinical manifestations in tumourgenicity. Early studies show some evidence that there is a correlation between peripheral neuropathy and auto-antibodies against nervous system whether these auto-antibodies directly cause peripheral neuropathy in cancer patients is not yet identified. Myeloma is a carcinoma originating from plasma cells. Therefore, it directly affects the immune system which produces immunoglobulins that help to fight infections and disease. [1] There is no cure for myeloma but low dose of chemotherapy is used for particular patients to stabilize the disease. Yet there is a risk of worsening of neuropathy with chemotherapy such as thalidomide and bortezomide for myeloma patients. These are known to be associated with carcinoma related neuropathy. We also identified other antigen markers on monkey cerebellum. They may be derived from malignant plasma cells or from patients' own intact immune system against tumour.

Multiple Myeloma

Multiple myeloma is a cancer of plasma cells, which are produced mainly in bone marrow. Plasma cells are specials B-lymphocytes and produce immunoglobulins. Myeloma begins with a single cell mutation which continues cloning into a large number of identical malignant cells. In normal situation immunoglobulins which are produced from plasma cells vary and can react with a range of antigens. Due to monoclonality of plasma cells a large quantity of monoclonal antibodies are produced. These are also known as para-protiens, are presented in blood and urine in about 99% of myeloma patients. There is a decreased amount of normal antibodies and most of them are dysfunctional. Therefore association with neutropenia, there is an increased risk of infections in myeloma patients and this can be fatal. Bone marrow involvement leads to impaired haemopoiesis and anaemia and a high concentration of monoclonal plasma cells in the circulating blood which can be detected. [2]

Oestoeporosis and bone lesions are two other significant features of myeloma. The neoplasm in bone marrow causes multiple fractures in bones. The malignant cells of myeloma imbalance the osteoclast (which destroy bones) and oesteoblasts (which rebuild bones) and leads to lytic bone lesions. Due to fractures calcium released into blood resulting hypercalcemia in myeloma patients. This leads to destruction of the patients' renal system. Such damage to kidney promotes higher concentration of calcium in blood. [1, 2]

Each year, about 3000 new multiple myeloma patients are diagnosed in the United Kingdom. There is a lesser incidence of myeloma in young adults under aged of 40, but it gradually increases with the age. Myeloma is twice as common in Afro-Caribbean ethnic groups compared to Caucasians. Also, occurrence of myeloma is commoner in males. Reason for such epidemiology is not specifically understood. [1, 3]

Peripheral neuropathy (PN)

PN results from damages to peripheral nerves. Causes of PN are multifactorial. In Europe, the main cause for peripheral neuropathy is Diabetes mellitus and in Africa and Asia it is caused by leprosy [4]. These patients can experience altered sensation, pain and autonomic weakness.

Disease	Incidence of (PN)
Multiple Myeloma	3- 13%
MGUS	8-37%
POEMS	50-85%
Waldrenstrom's macro-globulinemia	5-10%
Amyloidosis	15%-20%

Table 1: Incidence of PN in myeloma and myeloma associated diseases. [8]

PN is very commonly associated with myeloma, yet the reason has not been identified. It is known that treatments for Myeloma can cause PN. It has been observed due to biological treatments such as thalidomide, bortezomib (Velcade) and lefunomide. In patients receiving thalidomide, PN is observed to be in the range of 25%-75%. Bortezomib also cause PN in higher percentage of Myeloma patients (35%). [9, 17]

Immunological basis for neurological disorders in cancer patients was found in 1950s and in 1980s Posner *et* all showed how immunity to tumours is related to neurological disorders [5]. They reported that paraneoplastic neurological antibodies ie. Auto antibodies which have been produced to both neuronal and oncogenic antigens cause neuronal damage. [7] This damage is known as paraneoplastic syndrome and these specific types of antibodies are seen in both blood and CSF (Cerebrospinal fluid) [16]. In this study we looked for specific paraneoplastic antibodies (Figure 1) namely, Yo antibodies (PCA-1), Tr antibodies (PCA-Tr) , Hu antibodies (ANNA-1), Ri (ANNA-2), Amphiphysin antibodies, Ma antibody [11,15,16].

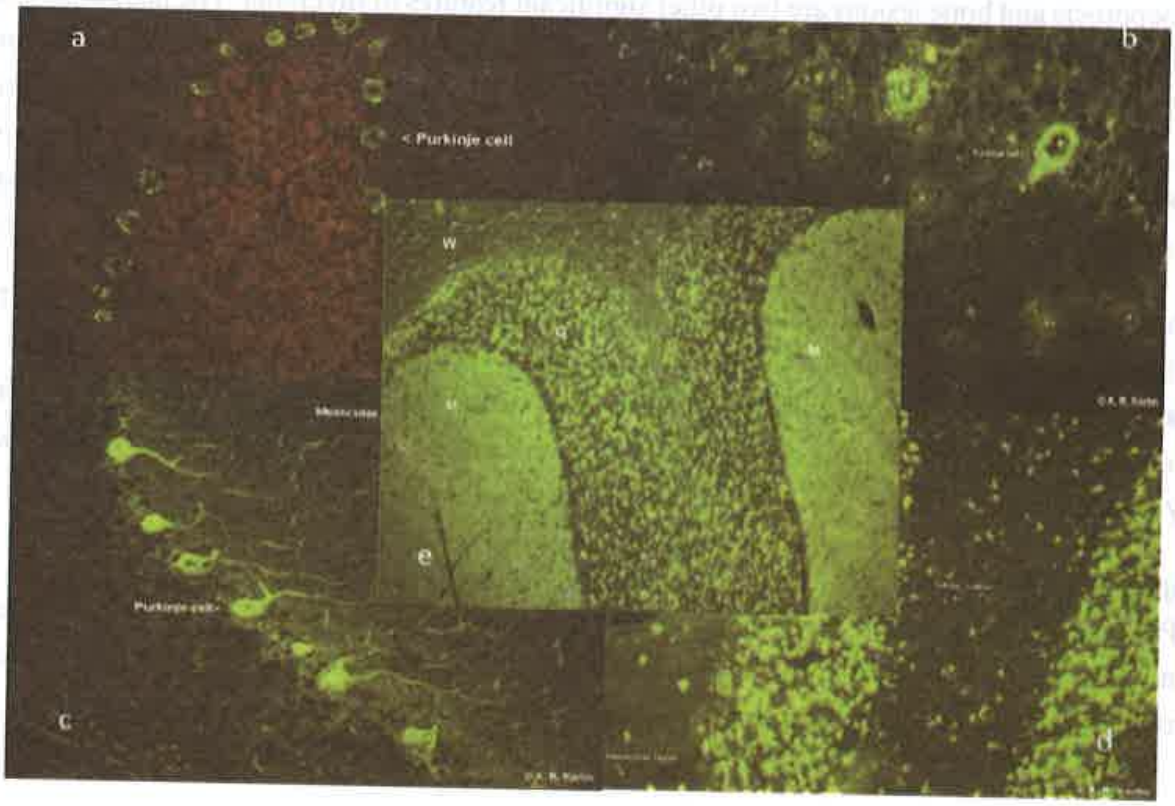

Figure 1: (a) Yo antibodies (PCA-1); stains ribosomes , rough endoplasmic reticulum and Golgi apparatus of Perkinjie cells (b) Ma antibodies; stains nucleoli of neuronal cells (c) Tr antibodies (PCA-Tr); stains Purkinjie cell cytoplasm as well as the dnetrites and also it is shown as dots over the molecular layer of cerebellum section. (d) Hu antibodies (ANNA-1) ; stains the nuclei of nerone in central nervous system as well as peripheral nervous system, also stains cytoplasm of the neurones but faint staining compared to nuclei staining (e) Amphiphysin antibodies; stain neuropil in molecular layer and granular layer

Associations of Antigangliosides antibodies were recognised with the peripheral neuropathy. Gangliosides are complex glycosphingolipids that contain an aliphatic anime (ceramide) and at least one sialic acid residue. Each different type of ganglioside is identified as a formula: G refers to ganglio and M (mono), D (di), T (tri) and Q (quad) refers to the number of sialic acid residues [6]. Gangliosides are components of plasma membrane and are also found in central and peripheral nervous systems. It is also known that gangliosides are appeared on microorganisms and therefore neuropathies can be seen in patients with diseases such as *Campylobacter jejuni*, *Mycoplasma pneumonia* and *Cytomegalovirus* or *Haemophilus influenzae*. We studied seven Antigangliosides antibodies such as GM1, GM2, GM3, GD1a, GD1b, GT1b and GQ1b [12]. It is known that tumour cells express gangliosides as the mutation of glycosylation. This reaction leads to produce gangliosides in tumour cell as it cannot be observed in mature normal cells. These gangliosides are similar to the gangliosides that are produced by the nervous system namely, GM1, GM2, GM3, GD2 and GD3. These gangliosides which are on cancer cell surface and blood are detected from immune system of the body and produce anti gangliosides antibodies which is a target for anticancer immunisation [7]. PN occurs in cancer patients as the anti gangliosides antibodies attack not only the gangliosides which are produced by the tumour but also the ones that has been produced by the nervous system [13].

Aim of the study

- To assess whether PN in myeloma is related to neuro-oncological factors
- To assess whether PN is caused by cancer related factors or treatment related factors
- To identify the presence of paraneoplastic antibodies and anti ganglioside antibodies in myeloma serum
- To identify antigen markers in monkey cerebellum for other antibodies from myeloma serum

Patients and Methodology

Patients

Patients for the study were selected from MRC myeloma IX trial that has recruited with newly diagnosed myeloma (2002- 2008). They were randomised to different therapies and will be followed up to death. (40% have died). 377 of these patients were selected for this study because they had developed peripheral neuropathy in some point in their follow up (Male 241, Female 136 and median age 64 years, range 31- 90) These patients were selected due to their level of PN that was graded according to National Cancer Institute common toxicity criteria scale. Patients with both motor and sensory neuropathy were selected and they were graded into 0 (None or change), 1 (Subjective weakness;

no objective findings), 2 (Mild objective weakness without significant impaired of function), 3 (Objective weakness with impairment of function), 4 (Paralysis). These patients have been treated with conventional dose of chemo-therapy and with ABCM (Adriamycin, BCNU, cyclophospahmide and melphalan), high dose of treatment, thalidomide, bortezomide (Velcade), allogenic transplantation and bisphosphonates. This analysis is blinded as we are unable to detect the treatment that those patients had received especially the biological treatments such as thalidomide, bortezomide (Velcade) which is given a high influence to peripheral neuropathy. However we examined both serum samples from diagnosis and the last sample and were sent to test for paraneoplastic antibodies [10]. Both patients' latest and presentation samples were tested. Initially latest samples were tested for paraneoplastic antibodies on monkey cerebellum tissue.

Method

Detecting paraneoplastic antibodies

Indirect immunofluorescence on cerebellum tissue

IF 16+ automated immunofluorescence analyzer (Binding Site, UK) is used to detect auto antibodies in patient's serum samples on Monkeycerebellum attached slides (Binding site, UK). Positive PCA control (Purkinjie cell Assay positive control –Binding site) and as the negative control Binding site, code CON92 is used. Diluted (1:100) antihuman IgG (H+L) monkey absorbed AFF FITC (Binding Site, code AF003.M) is added. Slides are read under UV light microscope.

Confirmation of paraneoplastic antibodies

Patients' samples that were positive for paraneoplastic antibodies on indirect immunofluorescence were confirmed by using direct company was made Western blots (EUROIMMUN AG, Seekamp, Lubec) to determine the specificity to the neurones. Three specific bolts were performed; Blots for anti-Hep-2 cell antigens to confirm immunofluorescence positive ANA patterns on monkey cerebellum tissue are brain specific auto-antibodies, Blot for antibodies against neuronal antigens (IgG) (Recombinant blot/ BB2) to detect human antibodies of the IgG class to six different antigens namely amphyphysin, CV2/CRMP5, Ma2, RI, Yo and Hu, Blots for anti neuronal antibodies to identify human antibodies against neuronal antigens including Hu, Yo and Ri. Competitive Assay is performed to confirm Ma2 antibodies on patients' serum samples. As for positive controls ANA positive control and Ma2 positive patient's sample was used.

Detecting anti-ganglioside antibodies

Blot for antibodies against ganglioside was performed in-vitro assay detects human antibodies of class of IgG and IgM to seven GM1, GM2, GM3, GD1a, GD1b, GT1b and GQ1b. (EUROIMMUN AG, Seekamp, Lubec)

Results

Detecting and confirming Paraneoplastic antibodies

49 patients' samples had positive staining on monkey cerebellum tissue and this staining has been categorised into six sections namely, Ma2 antibodies, Atypical Yo antibodies, Fibres in White matter (WM), Granular layer (GL) and Molecular layer (Ml), Antinuclear antibodies, mitochondrial antibodies and cytoskeleton staining.

Ma2 antibody

There were six samples were positive for Ma21 antibody staining on the tissue and they were confirmed with blots and five out of six came as positive. We categorised these staining of Ma2 as definite paraneoplastic antibodies (Figure 2a).

Atypical Yo antibody

There were nine samples which were positive for atypical Yo antibody staining and these are confirmed as non-neuronal specific antibodies with neuro-blots. These specific staining was found to be probable paraneoplastic antibodies (Figure 2b).

Staining on WM, GL and ML

More frequent positive staining on cerebellum tissue was the staining in WM, GL and ML (n=20, 40.8%) The type of antibodies in unknown but later in this study we found there were higher number of positive samples (n=8) for anti-ganglioside antibody could be seen among the samples that are positive to these type of staining (Figure 2d, 2e). They were categorised into possible paraneoplastic antibodies yet they show rare positivity on neuro-blots.

Figure 2: (a) Ma2 antibody: identified of the purkinjie cell is stained (b) Yo antibody: Ribosomes, Rough Engoplasmic reticulum of purkinjie cells were stained (c) Cytoskeleton straining: Mesh staining on the neurones (d) ML staining : Dendrites staining in the Molecular Layer of the monkey cerebellum (e) Dendrites staining in the white matter of the monkey cerebellum

ANA staining

Antineuclear antibodies (ANA) were another main category of staining on the cerebellum tissue. There were four positive samples for ANA as they stain every neuron cytoplasm as well as the nucleus.

MT staining

Mitochondrial (MT) staining was another pattern which has been seen on cerebellum tissue. There were less of staining on mitochondrial staining (n=2) which similar to Hu staining.

Cytoskeleton staining

There were nine positive samples for cytoskeleton staining. These were found to be staining in and out the Purkinjie cell and astrocytes as well as the in the dendrites (Figure 2c).

Summary of the staining pattern is shown in table bellow (Table 2),

Types of patterns	No. of positive results
Ma2 antibdoy (paraneoplastic)	5
Atypical Yo antibody (Probable paraneoplastic)	9
Fibres in WM, GL and ML (May be paraneoplastic)	20
ANA	4
MT	2
Cytoskeleton staining	9

Table2: Summary of the paraneoplastic auto antibodies which has been detected.

Positive stainings were visible in the latest from the latest samples.

GL and Staining WM, ML

Compared to the latest sample there were many positives to the known (n=89) and there were most of them are staining in white matter and granular layer and molecular layer. There were all together 52 positive sera to the particular staining most of them are found to be as staining on white matter. The specific antibodies have stained fibres of the white matter of them weak positive (n=33) but some strong staining were observed (n=6). Others were speckles on the Granular layer and molecular layer and most of them are GAD type staining.

Atypical Yo

And there were 12 positive samples for atypical Yo but they were overlap with other staining patterns mainly with the speckles granular layer staining those patterns cannot be confirmed as atypical Yo.

Ma2 antibody

Ma2 antibody type like staining pattern was another main pattern in those samples; there was less number of positive samples for Ma2 was found (n=3). Positive samples as well as the presentation samples of the samples that have the positive latest sample on Ma2 antibody on recombinant western blot yet all those tests came as negative.

MT staining

There were several mitochondrial positive staining as well. The results for mitochondrial staining mostly overlapped with the Granular layer and Molecular positive staining.

ANA staining

ANA positive staining samples were also significant in presentation samples and there were seven positive samples. Most ANA positive samples do not overlap with other type of positive staining. Overall there was no correlation between presentation sample and latest samples for positive staining pattern on monkey cerebellum.

Detecting anti-ganglioside antibodies

377 of the latest samples of patients of neuropathy have been screened for anti-ganglioside antibodies and there were 29 patients' samples which were positive (7.69%). Most of them (26) were positive for IgM immunoglobulins and 4 were positive for IgG immunoglobulins. (Table 3) These positive samples exhibited different intensity for each anti ganglioside antibodies and each positive sample showed the positivity for several types of anti-ganglioside antibodies. (Figure 3a) Serum anti-ganglioside antibodies IgM anti GM1 was most frequent (n=14, 48.27%) among anti-ganglioside antibodies. Among IgM anti GM1 (Figure 3b) most of them were positive with low intensity but there are two important higher intensity GM1 positive samples. IgM-GM2 anti-ganglioside antibodies were second most positive antibody type compared to others (n=13, 44.37%) and these positive samples were categorised according to their intensity; there were low intensity samples, 4 medium intensity samples and 1 higher intensity sample. There were 7 positive samples for IgM-GM3 anti-ganglioside antibodies and there are 4 low intensity of and 3 medium intensity samples. For IgM there are other type anti-ganglioside antibodies yet they were having low frequency. 3 positive patients' samples have IgM-GD1a yet they were in low intensity. IgM anti GD1b was observed in two positive samples.

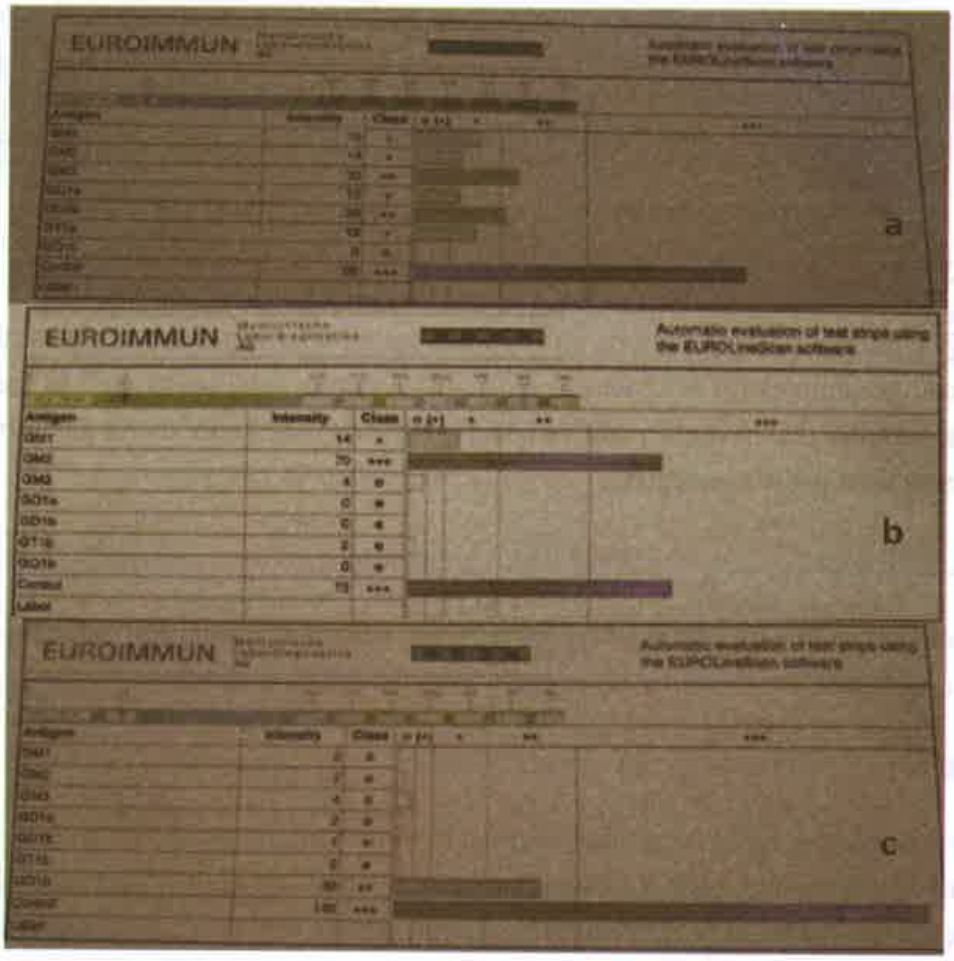

Figure 3: (a) Patient's sample positive to multiple anti-gangliosides (b) Patient's sample positive to both GM1 and GM2 with different intensities (c) Patient's sample positive to GQ1b

One sample had lower intensity where as the other serum samples have the medium intensity. One patient's serum sample had low positivity to IgM-GT1b anti-ganglioside antibodies and IgM-GQ1b anti-ganglioside antibodies can be seen in one patient sample which has a medium intensity. This was a significant feature because normally GQ1B anti-ganglioside antibodies (Figure 3c) were positive to IgG there were four samples which are positive to IgG GM3 anti-ganglioside antibodies and all were appeared in lower intensity. Also there was a low intensity positive GD1b anti-ganglioside antibody for IgG immunoglobulin. The most highlighted result from anti-ganglioside antibodies that there are only two patients had the anti-ganglioside antibodies for both IgM and IgG immunoglobulins.

IgM					IgG				
Antigen	Intensity			Total	Antigen	Intensity			Total
	+	++	+++			+	++	+++	
GM1	12		2	14	GM1				
GM2	8	4	1	13	GM2				
GM3	4	3		7	GM3	4			4
GD1a	3			3	GD1a				
GD1b	1	1		2	GD1b	1			1
GT1b	1			1	GT1b				
GQ1b		1		1	GQ1b				

Table 3: Result summary of the anti-gangliosides both IgM and IgG immunoglobulins

Clinical features from Myeloma trial positive for positive patients for auto-antibodies

Peripheral neuropathy can be categorised into mainly sensory neuropathy and motor neuropathy. In this study among 377 patients there were only 309 patients who are positive to sensory neuropathy and 54 patients are negative to sensory neuropathy and 17 patients' details are missing. Considering about the motor neuropathy there are 255 patients who are positive to motor neuropathy and 110 patients were negative. 27 patients' motor neuropathy details are missing. The patients who were positive to paraneoplastic antibodies, the concentration of IgG were detected. Normal concentration for IgG in human is known to be as 8-17 mg/ml and in these patients we found there was vast distribution in the level of IgG concentration. The range for IgG to 0.57mg/ml to 85.2mg/ml and their median is 10.2 mg/ml which was included in the range of normal IgG concentration. Also we looked at the para-protein that has been detected in each patient, and it has been described as follows. (First letter A, G, M, D is known as immunoglobulin type; L- λ, Lambda; K- κ, kappa; LC- light chain; np-no para protein)

The patients' states of cancer (progression, plateau etc.) according to their treatment were analysed. Summary is shown in below. Most of the patients were in plateau state (43%) and second most common state was as progression state (34%). There were 2 patients in 2nd maximum response to treatment and only 1 patient shows 1st maximum response to the treatment.

Discussion

PN results from damage to peripheral nerves and is associated with many cancers. This can be due to an auto immunological effect tumour presence or due to the damage to tissues as a result to treatment. In this research we studied 377 myeloma patients with peripheral neuropathy and we screened their serum samples for two specific auto antibodies namely, paraneoplastic autoantibodies and anti ganglioside antibodies. We hypothesised the damage to peripheral nerves may be caused by anti ganglioside antibodies. Our hypothesis was that the damage to peripheral nerves may be caused by anti-ganglioside antibodies and paraneoplastic auto-antibodies.

Peripheral neuropathy also occurs in myeloma patients as a side effect of anti cancer drugs. This is supported by early studies, which show that peripheral neuropathy was found in a tenth of myeloma patients at diagnosis increasing to a third in patients treated with thalidomide or bortezomide [3].

We used indirect Immunofluorescence on monkey cerebellum section to identify paraneoplastic antibodies. In addition to know paraneoplastic antibodies like Ma, Yo antibodies there were other antibody patterns that bound to other targets in the cerebellum. We used indirect Immunofluorescence to detect paraneoplastic antibodies as they are commercially less expensive and it is the best way to eliminate the negative samples [19]. The positive control has been changed in monkey cerebellum assay from normal PCA positive control to ANA positive control [20]. The reason for changes in controls is some monkey cerebellum slides were missing nuclei in cells. PCA control only detects the cytoplasm antigens whereas ANA control detects not only the cytoplasm antigens and also it stains nuclei. This was important to identify the nuclei specific paraneoplastic antibodies.

Our studies showed there was no correlation between in paraneoplastic antibodies in presentation

samples and latest samples. Hence we are unable to show there is an association between treatment for myeloma patients and particular antibodies.

Company made western blots were only used to confirm the anti ganglioside antibodies. Positive for Anti-ganglioside antibodies were found in 8% of samples. Most common were IgM antibodies against GM 1-3. Most of the positive samples for anti gangliosides antibodies showed low intensity. Only three samples with GM1 and GM2 for IgG immunoglobulins had higher intensity.

Even though it is known that paraneoplastic antibodies and anti-ganglioside antibodies cause peripheral neuropathy in other tumour patients, our results cannot support the hypothesis that these similar antibodies detected in myeloma patients are responsible for their peripheral neuropathy. This is because antibodies were found only in a very small percentage of our patients with PN compared to other solid tumours where the proportion of antibodies has been much higher (84% for paraneoplastic antibodies in solid cancer) [22]. Additionally we have not tested patients without PN to see if they also carry these antibodies. Therefore to test this hypothesis further, myeloma patients without peripheral neuropathy should also be screened for the same auto-antibodies.

We cannot totally discard the idea that PN is not due to an immune reaction in myeloma patients. We have detected a large number of antibodies in this study and this may individually or in combination be capable of producing neuronal damage. This has to be investigated by further research.

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