Pathogenesis of some neurological immune diseases: ultrastructural and morphometrical observations on rat thymus

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Numerous studies on neuro-immuno-modulation indicate that the thymus is involved in many neurological diseases, including experimental allergic encephalomyelitis (EAE). Twenty Lewis rats were induced for EAE. At X, XII, XX and XXX days post-inoculation the animals were killed, and the thymus was recovered and harvested. Specimens of thymus were submitted to morphological light microscopy analysis (1% toluidine blue) and ultra-structural analysis (transmission electron microscopy). Significant morphometric data were collected by examining the images quantitatively and by statistically analysing the values. Our results show that the microenvironment of the thymus is severally involved in acute EAE. Thymocytes and reticular epithelial cells show many changes which are closely related to the pathogenesis of EAE. In particular we observed: (1) inside the cell an increase in intra-cytoplasmic vacuoles, and changes in the thickness of the nuclear membrane, mitochondria, rough endoplasmic reticulum, cellular inter-digitations and cellular electron-density; (2) outside the cell an increase in pericellular translucent halo, intercellular spaces, intercellular contacts and apoptotic and necrotic figures. The evidence of a thymic role in MS may suggest the intriguing therapeutic concept of thymectomy in the management of this neurological disease. [Neurol Res 2005; **27**: 41–46]

Keywords: Experimental allergic encephalomyelitis; multiple sclerosis; demyelinizing diseases; rat; thymus

INTRODUCTION

Experimental allergic encephalomyelitis (EAE) is a cell-mediated autoimmune disease of the central nervous system (CNS) characterized by inflammatory infiltrates and demyelination of the nervous tissues[1,2]. Lewis rats are genetically predisposed for the induction of EAE[3]. This experimentally induced disease represents an animal model for the study of the human demyelinating disease multiple sclerosis (MS)[4,5]. Pathological changes of the thymus have been reported in cases of myasthenia gravis, MS and other neurological disorders[6,7]. In fact, the thymus plays an essential role in the stepwise process of differentiation and maturation of T cell subsets. The most recent results reveal the presence in EAE and/or MS patients of several alterations in the number and function of immune organs. One hypothesis is that a segregation of T-suppressor lymphocytes in CNS may trigger a cell-mediated autoimmune reaction against cellular elements of the same CNS[8,9]. Studies on the role of thymus in auto-immunity start around the years 1968–1969[10,11]. Recently it re-entered into fashion[12–15] owing to the discovery of new antibodies which recognize particular antigens implied in these neurological diseases. In order to clarify the possible role of thymus microenvironment in the pathogenesis of EAE, an ultra-structural analysis of thymus architecture in rats experimentally induced for EAE was made. Moreover, as new data, the quantitative analysis of images and the statistical analysis of data were performed.

MATERIALS AND METHODS

EAE was induced in adult Lewis rats by inoculation of an emulsion containing spinal cord tissue and *Mycobacterium tuberculosis* in complete Freund's adjuvant. The animals were treated according to the Convention of Helsinki on the utilization of animals in biomedical research. All procedures performed in this study were in accordance with the ethical standards of the responsible committee on animal experimentation and with the Declaration of Helsinki (1964) of the world Medical Association (amended in 1975 and 1983),

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published in *Philosophy and Practice of Medical Ethics* (British Medical Association, 1988). Most animals developed clinical signs of EAE within 12 days. Thymectomies were performed at X (pro-dromic symptoms), XII (early stage), XX (middle stage) and XXX (late stage) days post-inoculation (d.p.i.) of emulsion in rats and similarly in controls. Small pieces of the thymus were fixed in 2% glutharaldehyde for transmission electron microscopy analysis.

Male Lewis rats (220 g) and female Denkin Hartley guinea pigs (300 g) were obtained from Messrs Morini (S. Polo D'Enza, Italy) and kept under standard conditions, treated and stalled in authorized laboratories of our University. The control rats were injected with the vehicle alone (Freund's adjuvant in saline). EAE was induced, in male Lewis rats, by a single s.c. injection of 100 µg in 500 µl of an emulsion of guinea pig spinal cord into the hind foot pad. Local anaesthesia was performed with a saline solution of Novocain 0.5%. The ratio of the contents of the emulsion was 100 mg of spinal cord of guinea pig, homogenized in 4 ml saline, emulsified with 1 ml of complete Freund's adjuvant (CFA) (Difco, Detroit, MI) together with 10 mg *Mycobacterium tuberculosis* (H37 RA Difco). The first clinical signs of EAE began day 12 post-immunization.

Clinical assessment

Rats were weighed and examined daily for the presence of neurological signs. Clinical signs: 1, partial or complete loss of tail tonus; 2, paresis of the hind limbs; 3, complete paralysis of the hind limbs; 4, paralysis of the complete lower part of the body; 5, death due to EAE. Grades 2–4 are often accompanied by urinary and fecal incontinence[16].

Tissues sampling

The rats were killed by decapitation at various times. The thymuses were rapidly removed and small specimens were used for each different technique.

Usual laboratory staining

The anatomical details of thymus specimens were analysed after staining with toluidine blue (1% solution) or hematoxylin and eosin.

Transmission electron microscopy

Thymus specimens were pre-fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer. These pre-fixed tissue samples were post-fixed in 1% buffered osmium tetroxide for 1 h, processed by conventional methods and embedded in epoxy resin Epon 812. Semithin sections were stained with 1% toluidine blue. Ultrathin sections were stained with uranyl acetate lead citrate and examined with a Philips CM 10 electron microscope.

Quantitative analysis of images

Quantitative analysis of the intensity of the staining samples was performed by means of a Quantimet Analyser (Leica). The control values from samples incubated without dye were considered as "zero". Quantitative analysis of images (QAI) may provide incorrect results, because the main choices (i.e. the instructions for software) are made by each operator, according to his/her personal preferences. Therefore, it is mandatory to follow the rules carefully: the counts must be repeated at least three times using the double-blind technique and should be performed by different people, on different analysers and with samples identified by a number or a letter. Another scientist, who identifies each sample and attributes specific values that then undergo statistical analysis, must obtain the final results. The values reported in our experiments represent the intensity of staining for each type of tissue and are expressed as Conventional Units (CU) $\pm$ standard error of the mean; further details on QAI, including the definition of CU, are reported in the manual of the Quantimet Leica 500[17].

Statistical analysis of data

The statistical analysis performed included basic statistical methods such as mean values, maximum and minimum limits, variations, standard deviation (SD), standard error of mean (SEM) and correlation coefficients. Finally, by comparing the significant differences for each group with the corresponding values in the other homogeneous groups, a correlative analysis was obtained. Correlation coefficients denote a significant level when $p<0.001$[18].

RESULTS

Our results are shown in *Figures 1–5* and are tabulated in *Tables 1* and *2*. At 10 d.p.i. it is probable that no ultra-structural modification of the immunological reaction has a corresponding ultra-structural modification. However, large lymphocytes with nuclear and cytoplasmic characteristics resembling an activated population were present. The epithelial cells did not show any degenerative changes (*Figure 1*). The clinical and pathological signs of cerebral injury started at

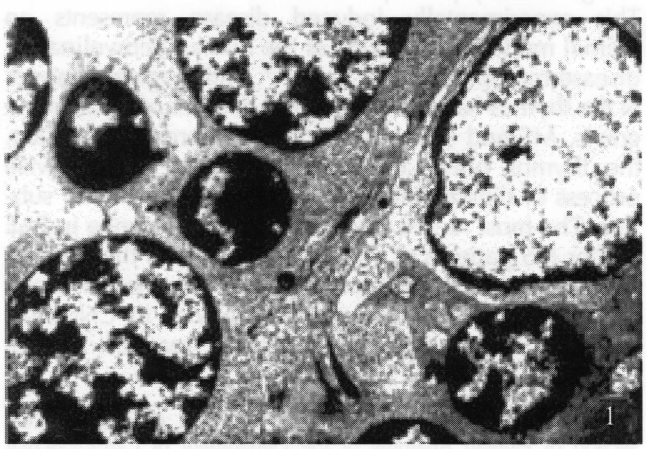

Figure 1: Transmission electron microscopy. Normal rat thymus. The normal morphology of reticular epithelial cells is shown. Magnification ×3000 and reproduced here at 60%)

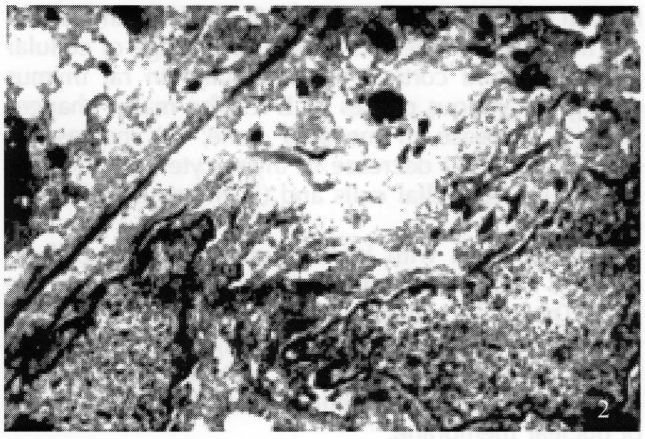

Figure 2: Transmission electron microscopy. Treated rat thymus at an early stage of EAE showing scattered degenerative vacuoles in the reticular epithelial cells. Magnification ×3000 and reproduced here at 60%)

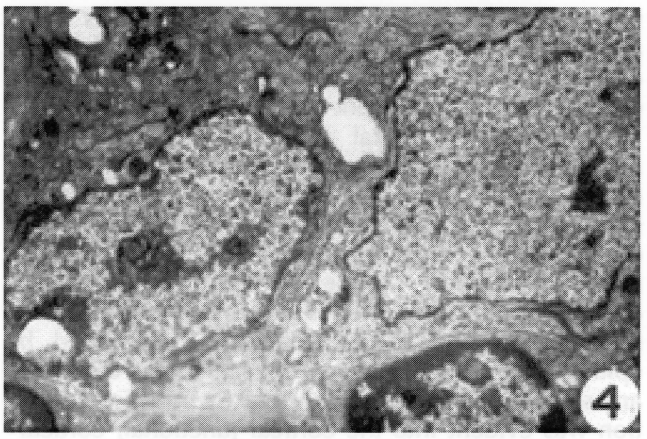

Figure 4: Transmission electron microscopy. Treated rat thymus at a late stage of EAE with reticular epithelial cells appearing severely altered and showing chromatin dispersion in their nuclei and fibrillar aggregates in their cytoplasm. ×3000

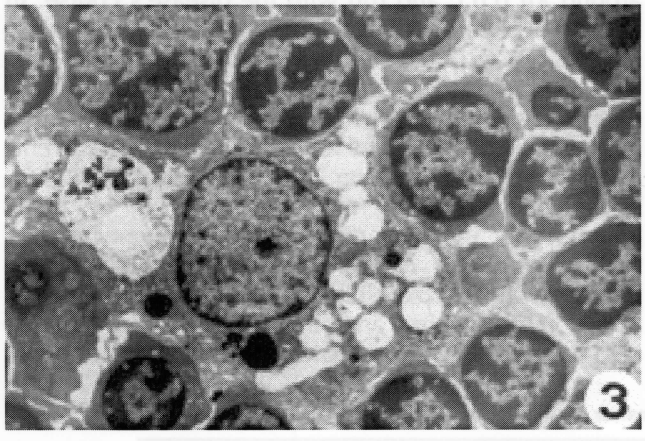

Figure 3: Transmission electron microscopy. Treated rat thymus at the middle stage of EAE showing more frequent and larger degenerative vacuoles in the reticular epithelial cells. ×3000

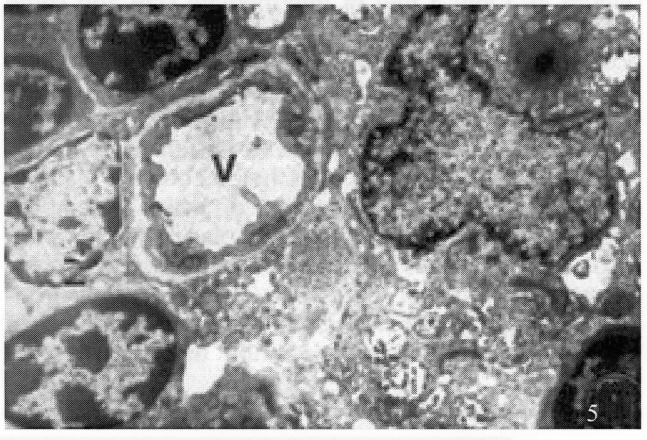

Figure 5: Transmission electron microscopy. Treated rat thymus at a late stage of EAE. Note the sharp contrast between reticular epithelial cells, which show many degenerative characters and a blood vessel which appears intact. ×3000

10–12 d.p.i. As stated in Materials and methods, clinical signs were observed in all rats immunized with emulsion of spinal cord in CFA during the course of the disease. The EAE-affected rats showed clinical signs of disease at about 12 d.p.i. At 10–12 d.p.i. distal tail weakness ascended the tail and 2 days later the tail had complete flaccid paralysis. At 12–14 d.p.i. the weakness reached also the distal hind-limbs up to the stage of complete flaccid paralysis. Then, at 18–20 d.p.i. the clinical index decreased up to the almost complete recovery of the affected rats. At 25–30 d.p.i. a moribund status (80% of the treated animals) or a remission of the disease could be observed (20% of the treated animals). No clinical signs were observed in control animals immunized with CFA alone. At 12 d.p.i. a polymorphism of thymic cell populations was seen by electron microscopy. Furthermore the intercellular space increased and morphological structural changes of the epithelial cells appeared. This process may suggest a cellular depletion from the thymus and an alteration of the relationships between epithelial cells and thymocytes. Ultra-structural changes in the thymus consisted of the presence of degenerative vacuoles in reticular epithelial cells (*Figure 2*).

At 20 d.p.i. the contacts between epithelial cells and thymocytes were irregular with dilated intercellular spaces. This fact was more evident in the cortical–medullar and medullar areas. Epithelial cells maintained a star appearance and the inter-digitating cytoplasm projections still made a sort of envelope circumscribing lymphoid cells. In these areas the epithelial cells had made some cytoplasmic modifications, such as: an increase of electron-density material, a dilatation in rough endoplasmic reticular profile, a re-assembling of microfilaments and, finally, an increase of small electron-dense mitochondria and multivesicular bodies. The nucleus had regular shape and size, contained some marginal hetero-chromatin. The thymocytes were various sizes and shapes. Large thymocytes with an irregular nucleus were abundant in the areas

where the connections with epithelial cells were altered. These thymocytes had a nucleus with abundant chromatin and a nucleolus, and presented an increase of cytoplasm compartment (*Figure 3*).

At 30 d.p.i. epithelial cells had a more evident increase in intracytoplasmic vacuoles, and thymocytes showed some apoptotic figures. The epithelial cells showed an intense cytoplasmic vacuolisation with electron-lucent formations of different sizes and a massive dilatation of the nuclear membrane was evident. The thymocytes had variable irregular nuclei, some of these were apoptotic and surrounded by epithelial cells. The intercellular space was decreased and sometimes disappeared. A total thymic disorganization was evident into the cortical junctional, cortical–medullar and medullar areas.

The thymocytes were heterogeneous in shape and size. The small thymocytes were characterized by an extreme electron-dense nucleus, nuclear membrane alterations, scant cytoplasm and an irregular plasma membrane, surrounded by an electron-lucent zone (*Figure 4*). Only a few of intermediate size and some large thymocytes were present, with a nucleus composed of abundant chromatin and scant etero-chromatin and some mitochondria in the cytoplasm. Many necrotic figures and thymocytes with serious alterations of the nuclear membrane were visible. The intercellular space was filled with heterogeneous material of variable electron-density part of which resembled portions of cytoplasm with deleted plasma membranes. Also, epithelial cells had many degenerative changes: alterations of nuclear membrane, decrease of electron-dense cytoplasm granules, increase of number of vacuoles and mitochondria, while the vessels were normal in size and shape (*Figure 5*). *Table 1* summarizes our morphometric results on the percentage of cellular population and cortical–medullar ratio in rat thymus during the various phases of EAE. The major changes are: (1) decrease in medullar zone; (2) increase in cortical zone; (3) decrease in thymocytes; (4) increase in reticular epithelial cells and (5) increase in floating cells.

Table 2 deals with intra-cellular and extra-cellular changes in rat thymus during the various phases of the EAE. The major changes are in intercellular spaces, intercellular contacts, intercellular digitations, apoptotic figures, cellular electron density, mitochondria, cellular endoplasmic reticulum, cellular vacuoles and thickness of nuclear membrane.

DISCUSSION

Our results show intra-cellular and extra-cellular alterations in thymus of rats induced by EAE. The first symptoms and the first morphological alterations begin around XII d.p.i. and run with the disease's course. In MS and in experimental autoimmune diseases related to nervous system degeneration (such as EAE) the correlation between hystopathological features and clinical signs is often impossible to make. This is due to early degenerative processes and to the following regenerative events in CNS (de-myelinization and re-myelinization)[19–21]. The thymus gland is the major site in which T-lymphocytes undergo differentiation. Thymocytes of varying stages of differentiation are found in close contact with the epithelial cells, by the action of many adhesion molecules and their relative cell surface receptors[22–24]. A close dialogue among the different

Table 1: Morphometric values of cellular elements of the thymus during the induction of EAE measured by QAI

Rat thymus	Normal	X days	XII days	XX days	XXX days
Cortical zone (%)	62	68	74	84	92
Medullar zone (%)	38	32	26	16	8
No. of thymocytes/mm^2	50	42	31	21	12
No. of reticular cells/mm^2	40	44	47	53	58
No. of floating cells/mm^2	10	14	22	26	30

Each value was obtained as CU (see Materials and methods) and was changed to a percentage according to the values reported in literature.

Table 2: Morphometric ultrastructural values of cellular constituents of the thymus during the induction of EAE measured by QAI

Rat thymus	Normal	X days	XII days	XX days	XXX days
Intercellular spaces	3.2±0.6	3.4±0.8	6.3±1.2	9.2±1.0	12.1±0.8
Apoptotic figures	0.3±0.3	0.5±0.3	1.4±0.6	3.6±1.6	7.3±1.2
Intercellular contacts	3.6±1.3	3.8±1.5	3.9±1.2	1.8±0.9	0.8±0.4
Electron density	18.1±2.1	19.3±2.4	29.1±3.2	34.5±2.9	43.2±3.3
Cellular inter-digitations	9.6±1.4	8.1±1.1	6.4±0.9	3.9±0.7	1.8±0.6
Rough endoplasm reticulum	8.9±1.1	11.4±1.3	15.6±1.7	19.3±1.6	21.2±1.9
Mitochondria	16.4±1.8	19.2±1.6	22.4±2.1	28.5±2.7	33.3±3.2
Cellular components	5.1±1.2	8.9±1.6	9.3±2.1	10.6±2.4	14.4±1.8
Intra-cytoplasmatic vacuoles	2.6±0.6	2.8±0.4	2.8±0.5	5.1±0.8	7.3±1.1

Each value was expressed as CU±SEM (see Materials and methods).

cellular components in the thymus is responsible for the thymic microenvironment required for thymocyte growth and differentiation[25,26].

The interaction between thymocytes and the epithelial cells, plays a fundamental role in the development and maintenance of the immune response[27]. Furthermore, the role of the thymus in the pathogenesis of some neurological autoimmune disorders has been suggested and the demyelinization process seems to be related to the quality and quantity of infiltrated inflammatory components (macrophages and lymphocytes).

Changes in thymocyte subpopulations have been described during MS and EAE development[28].The morphological modifications of the thymic gland corresponding to specific phases of the developing EAE were studied in our experiments. The clinical and pathological signs of cerebral injury started at 12 d.p.i. At the same time, ultra-structural findings show a depletion of thymocytes from the thymus and an increase of intercellular spaces due to a probable exit of lymphocytes from thymus into the blood, directed against the nervous tissue. The following ultra-structural features at 20 d.p.i. showed a thymic reorganization, together with a sporadic intrathymic death of thymocytes, related to a progressive alteration of epithelial cells. The death of thymocytes causes a progressive degeneration of thymus with an acme around 22 d.p.i. These observations suggest that thymic architecture needs to be well preserved in order to control the differentiation process of thymocytes, allowing a successful accomplishment of the disease.

The total thymus degeneration at 30 d.p.i. and the loss of tissue organization interfered on thymocytes development and their loss into the blood caused the progressive decrease of EAE and disappearance of clinical signs. Only 20% of rats survived while, on the contrary, about 80% of the treated animals died.

Progressive changes of thymic epithelial cells suggested the direct involvement of these cells in determining changes in thymus microenvironment and the development of EAE. The evidence of a thymic role in EAE and consequently also in MS may suggest the intriguing therapeutic concept of thymectomy in the management of these neurological disorders[29,30].

In conclusion, we observed that, in control animals, the structure of the thymus epithelium which regulates T-cell subsets was normal, but that there were some alterations to the morphology of the thymus in EAE rats.

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REFERENCES

1 Ortiz-Ortiz L, Weigle W. Cellular events in the induction of experimental allergic encephalomyelitis in rats. *J Exp Med* 1976; **144**: 604–616
2 Chow LH, Feurer C, Borel JF. Chronic relapsing experimental allergic encephalomyelitis in the Lewis rat: Studies on immune regulation. *J Neuroimminol* 1988; **19**: 329–338
3 Goverman J, Brabb T. Rodent models of experimental allergic encephalomylitis applied to the study of multiple sclerosis. *Lab Anim Sci* 1996; **46**: 482–488
4 Martin R. Immunological aspects of experimental allergic encephalomyelitis and multiple sclerosis and their application for new therapeutic strategies. *J Neural Transmission* 1997; **49**: 53–67
5 Aknin SB, Morel RF. The role of the thymus in myasthenia gravis: Immuno-histological and immunological studies in 115 cases. *Ann NY Acad Sci* 1987; **50**: 550–556
6 Martin R, Mcfarland H. Experimental immunotherapies for multiple sclerosis. *Springer Semin Immunopathol* 1996; **18**: 1–8
7 Armstrong RM. Immunologic mechanism in neurologic diseases. *Med Clin N Am* 1972; **56**: 515–527
8 Lynn Massman R, Edward AC, Sarka H, Ellsworth CA. Fluctuations of T- and B-cell subsets in basic protein induced experimental allergic encephalomyelitis in long tailed macaques. *Clin Immunol Immunopathol* 1987; **44**: 93–101
9 Keywsky BA. Thymic nurse cells: Possible sites of T-cell selection. *Immunol Today* 1986; **16**: 374–381
10 Metcalf D. The role of the thymus in auto-immunity. *Bibl Haematol* 1968; **29**: 446–453
11 Denman AM. Anti-lymphocytic antibody and autoimmune disease: A review. *Clin Exp Immunol* 1969; **5**: 217–249
12 Williams KC, Zhao W, Politopoulou G, *et al.* Inhibition of experimental allergic encephalomyelitis with an antibody that recognizes a novel antigen expressed on lymphocytes, endothelial cells, and microglia. *Lab Invest* 2000; **80**: 313–326
13 Kaye JF, Kerlero de Rosbo N, Mendel I, *et al.* The central nervous system-specific myelin oligodendrocytic basic protein (MOBP) is encephalitogenic and a potential target antigen in multiple sclerosis (MS). *J Neuroimmunol* 2000; **102**: 189–198
14 Weiner HL. The fine line between autoimmune and allergic encephalomyelitis. *Nat Immunol* 2001; **2**: 193–194
15 Liu H, MacKenzie-Graham AJ, Kim S, *et al.* Mice resistant to experimental autoimmune encephalomyelitis have increased thymic expression of myelin basic protein and increased MBP specific T cell tolerance. *J Neuroimmunol* 2001; **115**: 118–126
16 Matthaei I, Polman CH, De Groot CJA. Observer agreement in the assessment of clinical signs in experimental allergic encephalomyelitis. *J Neuroimmunol* 1989; **103**: 215–222
17 *Manuale dei Metodi Quantimet 500* Leica Microsystems Imaging Solutions, Clifton Road Cambridge, UK, 1997: pp. 1–102
18 Serio A. *Appunti dalle lezioni di statistica sanitaria.* Kappa, E. F. ed., Rome, 1997: pp. 1–186
19 Dal Canto MC, Wismiewski HN, Johnson AB, *et al.* Vesicular disruption of myelin in autoimmune demyelination. *J Neurol Sci* 1975; **24**: 313–319
20 Lampert PW. Electron microscopic studies on ordinary and hyperacute experimental allergic encephalomyelitis. *Acta Neuropathol* 1967; **9**: 99–106
21 Lampert PW. Demylination and remyelination in experimental allergic encephalomyelitis. *J Neuropathol Exp Neurol* 1965; **24**: 371–377
22 Couture C, Patel PC, Potworowski EF. A novel thymic epithelial adhesion molecule. *Eur J Immunol* 1990; **20**: 276–284
23 Imhof BA, Ruiz P, Hesse B, Palacio R, Dunon D. EA-1 a novel adhesion molecule involved in the homing of progenitor T lymphocytes to the thymus. *J Cell Biol* 1991; **95**: 1069–1076
24 Singer KH, Denning SM, Whichard LP, *et al.* Thymocyte LFA-1 and thymic epithelial cell ICM-1 molecules mediate binding of activated human thymocytes to thymic epithelial cells. *J Immunol* 1990; **144**: 2931–2933
25 Jonossy G, Thomas JA, Bollum FJ. The human thymic microenvironment: An immunohistologic study. *J Immunol* 1980; **134**: 202–208
26 King PD, Katz DR. Mechanism of dendritic cell function. *Immunol Today* 1990; **11**: 206–210

27 Ramson J, Fisher M, Mercer L, *et al.* Lymphokine mediated induction of antigen-presenting ability in thymic stromal cells. *J Immunol* 1987; **139**: 2620–2625
28 Zaffaroni M, Caputo D, Ghezzia A, *et al.* Monoclonal antibody analysis of blood T-cell subsets in multiple sclerosis. *Ital J Neurol Sci* 1984; **5**: 45–49
29 Rubin JW, Ellison RG, Moore HV, *et al.* Thymectomy in myasthenia gravis: The timing of surgery and significance of thymic pathology. *Am Surg* 1981; **47**: 152–158
30 D'Andrea V, Biancari F, Cavallotti D, *et al.* Thymectomy and multiple sclerosis: Ultrastructural study of an experimental model. *G Chir* 1999; **20**: 119–124