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Immunocytochemical Characteristics of Small Cell Lung Carcinoma Associated with the Lambert-Eaton Myasthenic Syndrome

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The Lambert-Eaton myasthenic syndrome (LEMS) is characterized by the presence of IgG antibodies to motor nerve terminals, and associates with small cell lung carcinoma in more than 60% of cases. We have carried out a comparative immunocytochemical study on small cell lung carcinoma (SCLC) in five LEMS cases and six non-LEMS cases, using antibodies to tumor markers, MHC Class I and II, macrophages and lymphocytes. The authors found a reduced expression of the 200Kd neurofilament antigen and of MHC Class I antigens in the LEMS cases as well as a greater infiltration of activated macrophages. It is suggested that these findings are consistent with the view that SCLC antigenic determinants trigger the autoantibody response in SCLC-LEMS. (Am J Pathol 1992, 140:839–845)

Certain cancers can exert remote effects on the nervous system.[1] These paraneoplastic neurologic disorders include the Lambert-Eaton myasthenic syndrome (LEMS), subacute sensory neuropathy, encephalomyelitis, cerebellar ataxia and retinopathy, each of which can associate with small cell lung carcinoma (SCLC). Evidence is increasing that the immune system is implicated in their etiology. In all of them, for example, antibodies to the target neurologic tissue have been identified, and in many, these have been shown to crossreact with tumor determinants.[2–9] Only in LEMS, however, has the principal criterion of an antibody-mediated autoimmune disorder been met, namely transfer of the disorder to experimental animals by injection of patients' immunoglobulins.[2]

LEMS is characterized by a reduction in Ca^{2+}-dependent quantal release of acetylcholine from motor nerve terminals at the neuromuscular junction,[10] leading to muscle weakness and depressed tendon reflexes. About 60% of cases associate with SCLC,[11] the neurologic syndrome preceding radiologic evidence of the tumor by up to 5 years. In both the paraneoplastic and nonparaneoplastic forms, LEMS associates with particular immune response genes.[12] Freeze fracture, electron microscopy of LEMS muscle shows a reduction in the number of active zone particles, believed to represent voltage-gated calcium channels (VGCC) and a loss of their usual orderly arrangement in double parallel rows.[13] Patients' IgG injected into mice transfers the principal physiologic[14,15] and morphologic[16] changes. The findings are consistent with a 40% reduction in the number of nerve terminal VGCCs,[15] brought about by crosslinking of adjacent active zone particles by the IgG antibodies;[17] monovalent (papain-digested) IgG was without effect. Anti-VGCC antibodies can be detected in a proportion of patients using ^{125}I-ω-conotoxin labelled VGCCs,[18–20] and in longitudinal studies the titer appears to correlate inversely with disease severity.[20]

Several lines of evidence implicate SCLC in triggering the disorder. These tumors, which may be of neuroectodermal origin, express VGCCs.[21] K^+-stimulated (voltage-gated) $^{45}Ca^{2+}$ flux into cultured SCLC cells is significantly inhibited in the presence of LEMS IgG.[22] This inhibition correlates with disease severity,[23] and specific tumor therapy (resection and irradiation) may be followed by improvement or remission in the neurologic deficit,[24] suggesting that tumor VGCC determinants are driving the autoantibody response.[24] Moreover, they apparently initiate it at an early stage (up to 5 years before tumor

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diagnosis). At that time, the tumor is presumably small, suggesting either that it is immunogenic, or that the patients who have paraneoplastic/LEMS symptoms are high responders, or both. Evidence in favor of the latter is the increased frequency of particular immunoglobulin heavy chain gene markers, and perhaps of HLA-B8 and DR3, in LEMS cases with SCLC.[12]

In the light of these findings, it is of interest to investigate the characteristics of the tumor and of the cellular infiltrate in LEMS. In this immunocytochemical study, we compared the findings in five LEMS cases with those in six non-LEMS controls.

Materials and Methods

Clinical Details and Specimens

Tumor tissue was obtained from 11 patients. Five of these patients had typical clinical features of LEMS, which had been present for 0.8–5 years at the time of death (Table 1). The diagnosis of LEMS was confirmed by electrophysiologic studies that showed a reduced amplitude of the compound muscle action potential (range, 1.2–6.6; normal >8.5 mv), and an increment in amplitude following 15 seconds maximum voluntary contraction of muscle of 188–1233% (normal <25%). Non-LEMS tumor cases served as controls. One patient in the LEMS group and three in the control group had received tumor chemotherapy, and an additional patient in the LEMS group had been treated with prednisolone and azathioprine for 1 year.

The tissue specimens studied are given in Table 1. With the exception of two cases, for which a paraffin-embedded block was also available, all the work was done on frozen sections. Except for case 5 for which only lymph node metastatic tumor was available, only the blocks of the primary tumor were compared in detail but we were able to examine the metastatic tumors in one other LEMS case.

Table 1. *Clinical Details of Cases*

Case no.	Age/ Sex	Duration of LEMS (yr)	Specimen	Treatment
LEMS				
1	44 F	5	Biopsy	None
2	58 F	0.8	Biopsy	None
3	64 F	1	Autopsy	Chemotherapy
4	59 F	1.7	Autopsy	Pred/Aza* 1 yr
5	68 M	0.8	Autopsy	None
Non-LEMS				
6	60 M	—	Biopsy	None
7	64 F	—	Biopsy	None
8	41 M	—	Autopsy	Chemotherapy
9	48 M	—	Biopsy	None
10	64 M	—	Autopsy	Chemotherapy
11	70 F	—	Autopsy	Chemotherapy

* Pred = prednisolone; Aza = Azathioprine.
Case 6 was supplied by Dr. K. Gatter, Histopathology Dept., John Radcliffe Hospital, Oxford.

Immunocytochemistry

Frozen sections were cut at 6 μm, air-dried, and fixed in acetone for 10 minutes. The antibodies used in this study are detailed in Table 2. After a preliminary rinse in phosphate-buffered saline containing 0.05% Triton X-100 (PBS), the sections were placed in 3% aqueous H_2O_2 for 10 minutes to block endogenous peroxidase. The sections were briefly rinsed in distilled water and washed 3× in PBS. Nonspecific binding was blocked by 20-minute incubation in fetal calf serum diluted 1:20. The sections were incubated for 60 minutes at room temperature in the primary antibodies at their optimal dilution, which had been previously determined. After washing 3× in PBS, biotinylated sheep anti-mouse immunoglobulins 1:200 or biotinylated donkey anti-rabbit immunoglobulins 1:200 (both from Amersham International) were applied for 20 minutes. The sections were washed 3× in PBS before incubation with streptavidin-horseradish peroxidase for 20 minutes. After washing 3× in PBS the reaction product was visualized by incubating in 3-amino-9-ethylcarbazole (AEC) at 37°C for 20 minutes. The sections were lightly counterstained with Mayer's Haemalum

Table 2. *Antisera*

Antiserum	Specificity	Source
CAM5.2	Pan-cytokeratin	Becton Dickinson
NE14	200KD neurofilament	Boehringer
NSE*	Neurone specific enolase	Dako
W6/32	HLA Class I	Dako
TAL.1B5	HLA Class II (HLA.DR)	Dako
ED3†	Activated macrophages	Camon Inc.
KP1	Macrophages	Dr. K. Gatter
Mac387	Macrophages	Dako
2B11&PD7/26	LCA (lymphocytes)	Dako
T3-4B5	CD3 (pan T cell)	Dako
MT310	CD4 (helper/ inducer T)	Dako
DK25	CD8 (Cytotoxic/ suppressor T)	Dako
L26	B cells	Dako

* Polyclonal.
† ED3 is a monoclonal recognizing rat macrophages[25] which was found to crossreact with human "activated" macrophages [A. Marx, unpublished observations] occurring at places of alloreactivity (renal transplants), autoreactivity, and infection. ED3 immunoreactivity is absent from neonatal tissues and noninflammatory adult tissues. The antigen recognized by ED3 in humans has not been isolated so far (neither has the rat antigen) but most probably it is not homologous to the rat protein.

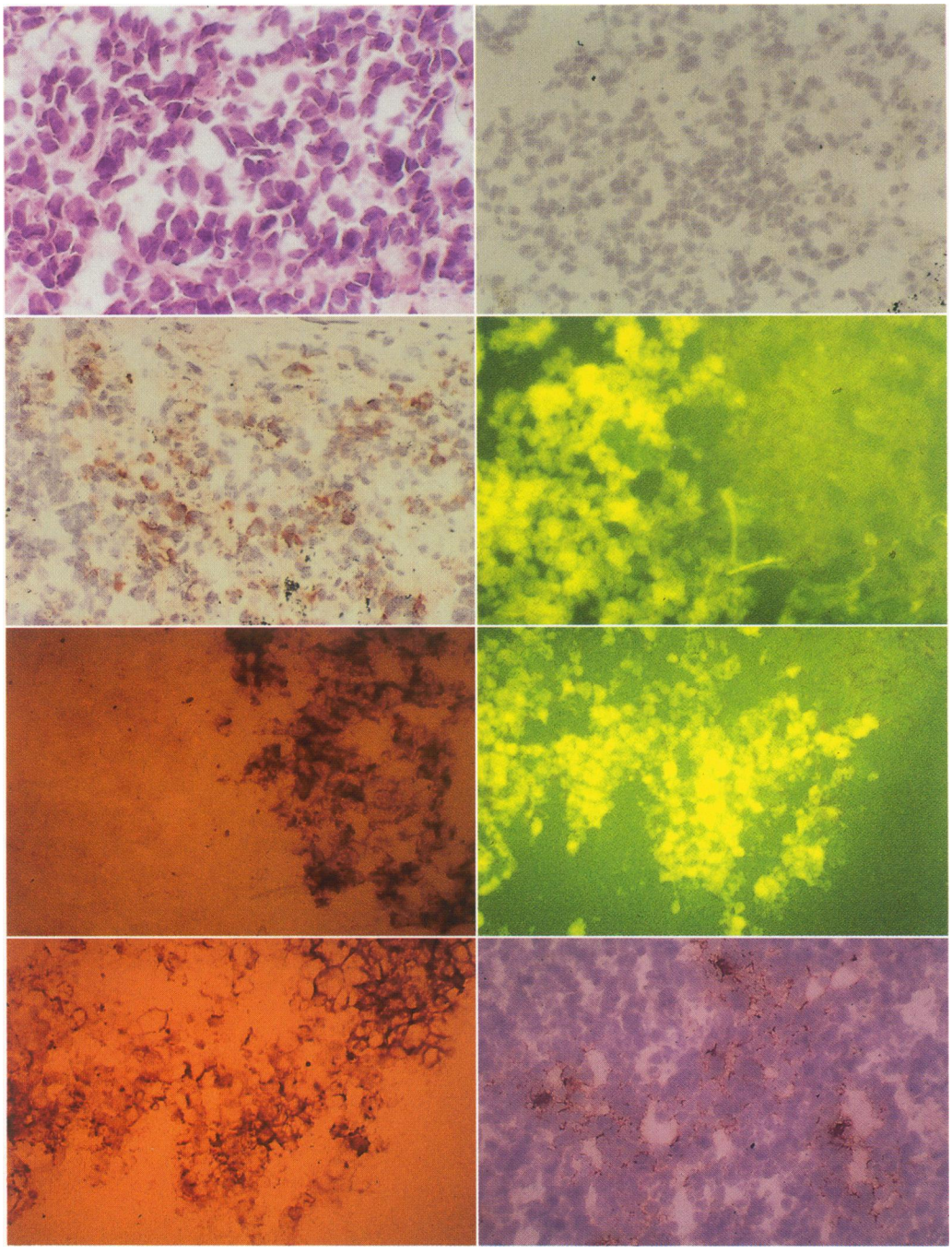

Figure 1 (first row, left). *Case 4: Frozen section to show the morphologic appearance of the tumor. Similar appearances were seen in all cases, H&E stain.*
Figure 2. *Reactions with the anti-neurofilament antibody (NE14) on tumor cells from case 4 (LEMS)* **(first row, right),** *and case 11 (non-LEMS)* **(second row, left).** *In LEMS, the reaction is negative and in non-LEMS it is positive.*
Figure 3. *Frozen section from case 4 (LEMS) double-labelled to show MHC Class 1* **(second row, right),** *and the tumor cell marker Cam 5.2* **(third row, left).** *MHC Class 1 is present on macrophages seen left but not on tumor cells reactive with CAM5.2, right.*
Figure 4. *Frozen section from case 8 (non-LEMS) double-labelled to show MHC Class 1* **(third row, right),** *and the tumor cell marker CAM 5.2* **(fourth row, left).** *Note colocalization of MHC Class I on CAM 5.2 positive tumor cells. The cells reactive for MHC Class I but not CAM 5.2 are macrophages.*
Figure 5 (fourth row, right). *Frozen section from case 3 (LEMS) reacted with Mac387 showing dendritic macrophages with processes interdigitating between and around tumor cells.* **Next page, top left:** *Frozen section of case 6 (non-LEMS) reacted with Mac387. In this case, no dendritic macrophages are present between tumor cells but there are two perivascular macrophages present at top right.*

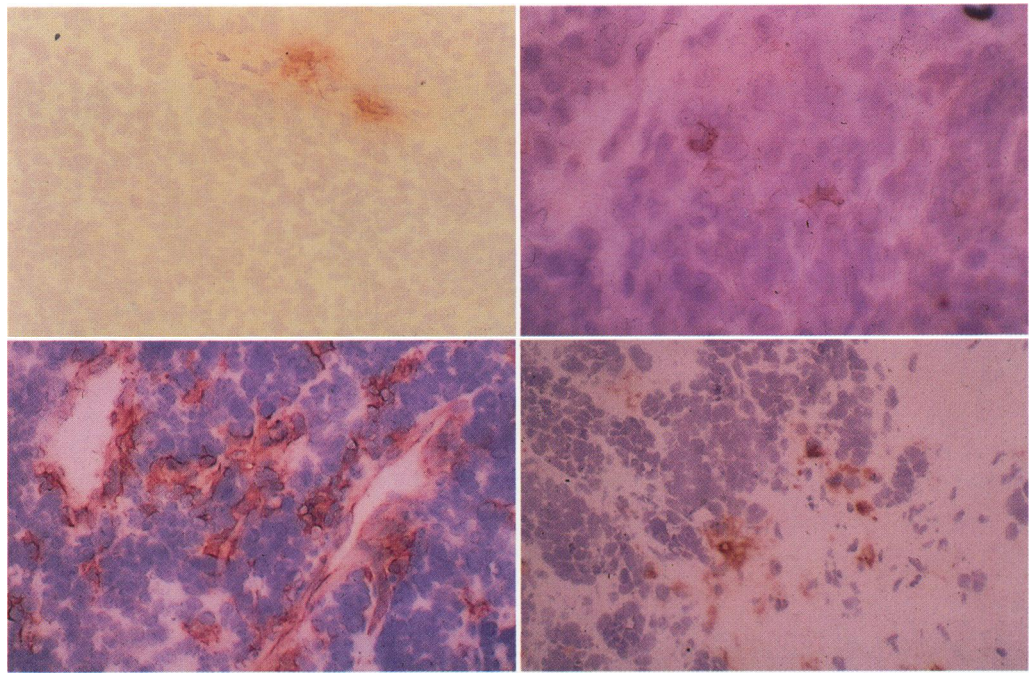

Figure 6 (top right). *Frozen section from case 1 (LEMS) showing reaction with macrophage activation marker ED3. Two reactive macrophages are distinguishable in this field.*
Figure 7 (bottom left). *Frozen section from case 1 (LEMS) showing reaction for MHC Class II. Note large numbers of reactive macrophages infiltrating between unreactive tumor cells.* **Bottom right:** *Frozen section from case 7 (non-LEMS) showing few peripherally situated macrophages reactive for MHC Class II.*

and mounted in glycerol gelatin (Sigma). Controls included omission of primary antibody or substitution with an irrelevant primary antibody matched for immunoglobulin class and protein concentration; omission of secondary antibody; and omission of tertiary reagent.

To clarify which cell type was expressing MHC class I and II, double-labelling was carried out by firstly labelling the tumor cells with CAM5.2 and the macrophages with KP1 and detecting these two monoclonals with peroxidase-conjugated goat anti-mouse immunoglobulins 1:50 (Dako) and the substrate AEC. MHC class I and II antibodies were then applied and detected by biotinylated sheep anti-mouse immunoglobulins (Amersham International) and avidin-FITC (Vector).

The immunocytochemical reactions were evaluated in detail by two observers and subsequently the slides were assessed blindly by a third observer. All three were in agreement.

Table 3. *Tumor Antigens in LEMS and Non-LEMS*

Case no.	CAM5.2	NE14	NSE	HLA I	HLA II
LEMS					
1	4	1	4	0	1
2	4	3	3	1	1
3	3	0	3	1	1
4	3	0	3	1	1
5	3	1	2	1	1
Scores*	85%	25%	75%	20%	25%
Non-LEMS					
6	3	3	3	2	1
7	4	4	4	3	1
8	2	0	3	2	1
9	3	3	3	1	0
10	1	0	4	2	1
11	3	2	4	3	1
Scores	67%	50%	87%	54%	21%

* Scores expressed as a percentage of the total score possible.
Scores for proportion of positive cells (0 = none: 4 = >80%).

Table 4. *Macrophage and Lymphocyte Infiltration in LEMS and Non-LEMS*

	Mφ					Lymphocytes
Case no.	KP1	Mac387	ED3	HLA I	HLA II	LCA
LEMS						
1	4	2	2	3	4	3
2	3	1	2	4	4	4
3	3	2	2	4	3	1
4	4	2	1	3	4	3
5	3	2	2	3	3	3
Scores*	85%	45%	45%	85%	90%	70%
Non-LEMS						
6	3	1	1	3	3	2
7	3	3	1	3	3	3
8	2	2	0	3	3	1
9	2	1	1	2	2	2
10	2	1	0	1	1	1
11	2	1	1	2	2	2
Scores	58%	38%	17%	58%	58%	46%

* Scores expressed as a percentage of total possible.
Scores for proportions of positive cells (0 = none; 4 = >80%).

Results

The results are presented in Tables 3 and 4. A simple method of quantitation was applied to the immunocytochemistry results. Numbers of positive cells were scored on a 0–4 scale.

All the tumors showed the characteristic morphology of SCLC, being composed of tightly packed clusters of anaplastic cells with hyperchromatic nuclei and scanty cytoplasm (Figure 1). There were numerous mitotic figures present in all the tumors.

Tumor Cell Antigen Expression

Comparison of the tumor markers in SCLC in LEMS and non-LEMS are shown in Table 3. Cytokeratin (CAM5.2) was strongly expressed on all but one of the tumors and acted as a good marker for distinguishing tumor cells from infiltrating inflammatory cells. Cytokeratin and NSE were similar in distribution and intensity of reaction in both LEMS- and non-LEMS–associated SCLC. The expression of 200 Kd neurofilament (NE14) in LEMS was restricted to only rare small groups of cells, in all but one case. This contrasted with the non-LEMS cases in which large groups of strongly positive cells were typically seen. Thus there was less expression of the 200 Kd neurofilament in LEMS-associated tumors (Figure 2).

There was a noticeable difference in the tumor cell expression of HLA Class I and II (DR), between LEMS and non-LEMS cases. In non-LEMS, there were more tumor cells positive for HLA Class I than in LEMS-associated SCLC. HLA Class I antigens were expressed on few tumor cells in LEMS, whereas they were readily detected on a greater proportion of cells in non-LEMS SCLC. HLA Class II antigens were only rarely detectable on tumor cells in either group. These differences were supported by the double-labelling reactions (Figures 3, 4).

Macrophage Infiltration

In LEMS-associated SCLC, there were numerous macrophages at the periphery of the tumor in the stroma and also many within the tumor mass that strongly expressed the macrophage marker KP1. This contrasted with the non-LEMS cases in which there were noticeably fewer macrophages, whether peripheral or within the tumor. Fewer macrophages were positive for Mac387 in both LEMS and non-LEMS. Most macrophages were typical round-bodied forms, but in LEMS especially, some that were Mac387$^+$ and a few that were KP1$^+$, had a dendritic appearance with fine processes interdigitating between and around the tumor cells (Figure 5). In both groups, few macrophages in the tumor expressed ED3, but most of those that were positive had the same dendritic appearance. ED3 reacted weakly with rare macrophages in the non-LEMS SCLC. In LEMS, there were more activated macrophages in the tumor as defined by their expression of ED3 (Figure 6) and HLA Class II (Figure 7). In the one case of LEMS in which metastatic tumor in a regional lymph node was studied, there were numerous ED3 macrophages in the lymphoid tissue surrounding the tumor.

In LEMS, there were more peripheral and tumor-infiltrating macrophages positive for both HLA Class I and Class II. This reflected the greater number of infiltrating macrophages in LEMS rather than a larger percentage of the population being positive.

Lymphocyte Infiltration

In LEMS, lymphocyte aggregates strongly positive for LCA were often seen adjacent to the tumor and there were scattered intratumoral lymphocytes usually sited near vessels. In non-LEMS lymphocyte aggregates were usually smaller and less intensely positive for LCA. Analysis of the lymphocyte populations (not tabulated) with subset-specific monoclonal antibodies revealed that the majority of lymphocytes were B cells. Few T cells were seen and there seemed little difference in proportions of helper/inducer and suppressor/cytotoxic cells in either LEMS or non-LEMS.

Endothelial Reactions

HLA Class I and II were expressed by endothelial cells of vessels within the tumor of both groups, but Class II antigens were more strongly expressed on endothelium in the LEMS-associated tumors.

Discussion

There were three distinguishing features between LEMS and non-LEMS–associated SCLC apparent in this study: reduced expression of 200 Kd neurofilament antigens and of MHC Class I antigens by tumor cells, and an increased number of infiltrating activated macrophages in LEMS. We suggest how our findings might be interpreted in light of the known clinical differences between the LEMS and non-LEMS subjects. The evidence of an enhanced immune response in LEMS, as reflected by increased numbers of infiltrating macrophages and enhanced expression of the activation marker ED3, is consistent with the ongoing occurrence of an aggressive autoimmune disease. However, the reduced number of neural antigens and of MHC Class I antigens by the tumor cells requires explanation.

One possible interpretation of these results regarding the tumor cells might be that the LEMS-associated tumors were less well-differentiated than the non-LEMS group. This is an interpretation suggested by the finding that in human tumors[26] and in lung tumors in particular[27] the poorest differentiated tumors show the least MHC Class I expression. Reduction of neurofilament expression might also reflect poor differentiation of neuroendocrine-derived cells. However, SCLC are all generally considered to be poorly differentiated and the prognosis in patients with LEMS-associated SCLC is apparently no worse. All the tumors we studied appeared comparable with regard to their mitotic activity and morphology. Thus, there is no independent evidence for regarding the tumor cells in LEMS as being less well-differentiated than those in other SCLC. Possibly in LEMS cases, the tumor loses its neuroendocrine differentiation or cells that are differentiated are preferentially destroyed resulting in fewer cells to detect. One LEMS case and two non-LEMS cases previously treated with chemotherapy had no detectable 200 Kd neurofilament suggesting that any neuronally differentiated cells may have been more sensitive and preferentially destroyed. Cytoskeletal and membrane disturbances may occur which result in the exposure of antigenic sites that provoke the autoimmune response to LEMS-associated tumors.

An alternative interpretation of our finding of fewer LEMS-associated tumor cells expressing MHC Class I and neurofilament antigens, is that there may be an enhanced immune response to these tumors that is responsible not only for the development of LEMS, through production of antibodies to VGCC on the tumor cells,[28] but also for cytotoxic T-cell killing of tumor cells expressing MHC Class I antigens. Such an immune response, capable of generating antibodies to a specialized membrane structure of nerve cells, the VGCC, would probably be more liable to eliminate the better differentiated, more neuron-like cells of the tumor, leaving behind more of the neurofilament negative cells. The finding of greater numbers of infiltrating macrophages, clearly activated as assessed from their expression of MHC Class II and ED3, in LEMS-associated tumors would be in agreement with this interpretation.

Although one LEMS case and two non-LEMS cases had received chemotherapy, there was no clinical or hematologic evidence of immunosuppression and the respective infiltrates of macrophages and lymphocytes were comparable to the other cases. There were more lymphocytes infiltrating the LEMS tumors. Most lymphocytes were B cells, few were T cells. This would correlate with antibody production in these patients, which is presumably composed of both anti-tumor activity as well as the autoimmune component. The paucity of T cells may reflect the stage at which the tumors were obtained for evaluation, which is usually late in tumor development and perhaps the T-cell response had largely died down. Alternatively it may only require a few T cells to maintain the ongoing response once the initial peak of immune activity is passed. The marked macrophage and lymphocyte infiltration in LEMS-associated SCLC and the state of activation of these cells, raises the possibility that it is the tumor in LEMS that is provoking the immune response. This is supported by the sustained improvement in LEMS patients after tumor excision and local radiotherapy[24] which presumably removes the stimulus maintaining the immune response.

Note Added in Proof

The findings of Faustman et al (Science 1991, 254:1756–1761) of reduced MHC class I in autoimmune diabetes are directly relevant to our observations of LEMS-associated SCLC.

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