

Focal lymphocytic infiltration in the adrenal cortex of the elderly: immunohistological analysis of infiltrating lymphocytes

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SUMMARY

The incidence of mononuclear cell infiltration in the adrenal cortex was examined in autopsy cases of young and old subjects, and the infiltrating mononuclear cells were immunohistologically characterized by monoclonal antibodies. Histologically, 110 of 174 autopsy cases of persons > 60 years (63.2%) were shown to have mononuclear cell infiltration of varying degree within the adrenal cortex, whereas such a lesion was observed in lesser incidence (7.4%) in the 54 younger, control subjects aged < 49 years. In addition, severely infiltrating lesions in the adrenal cortex were found frequently in the elderly > 70 years. Immunohistochemical study revealed that the infiltrating mononuclear cells were mainly composed of CD3⁺ T cells. The major proportion of CD3⁺ T cells expressed CD4, whereas CD8⁺ T cells were less in number. Moreover, a considerable proportion of CD4⁺ T cells was activated as judged by interleukin 2 receptor expression. These findings indicate that T lymphocytes infiltration in aged human adrenal cortex may represent a pre-clinical manifestation of organ-specific autoimmune adrenalitis which is based on autoimmunity associated with ageing process.

Keywords immunohistochemistry adrenal cortex autoimmunity ageing T lymphocytes

INTRODUCTION

It has been well known that T-cell dependent immune responses decrease with advancing age in humans and in rodents, and the decline in immunologic activities appears to be responsible for the increase in the incidence of autoimmune disorders as well as cancer or infectious diseases (Walford, 1969; Makinodan & Kay, 1980; Hirokawa, 1985). In previous communications, we have demonstrated that the incidence of T lymphocytes infiltration in aged human salivary and thyroid glands probably referred to as an autoimmune disorder showed an apparent trend to increase with advancing age (Kurashima & Hirokawa, 1985, 1986). In addition, we have recently reported a murine experimental system in which autoimmune sialadenitis spontaneously develops in aged mice of non-autoimmune-prone BDF1 strain (Hayashi *et al.*, 1988).

Idiopathic Addison's disease is an organ-specific autoimmune disease of the adrenal cortex in humans characterized by the destruction of adreno-cortical cells by the prominent mononuclear cell infiltration and the production of circulating autoantibodies specific for the cytoplasm of the adrenocortical

cells (Anderson *et al.*, 1968; Petrei & Nerup, 1971). Recently, we have found mononuclear cell infiltration rather frequently in the adrenal cortex of the aged people, although without apparent clinical manifestation. Thus, we were interested to find out whether any pathological states of autoimmune nature can be observed spontaneously in the human adrenal cortex associated with ageing process.

The aim of this study was to determine the incidence of lymphocytic infiltration in aged human adrenal cortex, and to analyse the histologic features and phenotypic characteristics of these infiltrating mononuclear cells. Data obtained from this study provide critical evidence for spontaneously occurring autoimmune phenomena in the adrenal cortex associated with ageing process in humans.

MATERIALS AND METHODS

Subjects

Adrenal glands were obtained from 174 autopsy cases > 60 years of age (range 60–98; 84 men, 90 women) performed at the Tokyo Metropolitan Geriatric Hospital. For comparison, younger adrenal glands obtained from 54 autopsy cases < 49 years of age (range 28–49; 28 men, 26 women) performed at the Japan Red Cross Medical Center. The necropsy specimens were removed within 10 h of death. Cases with autoimmune disorders

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such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), Hashimoto's thyroiditis, etc., with documented endocrine abnormalities, malignant lesions or infectious diseases were carefully excluded. In addition, cases with mononuclear cell infiltration within the adrenal medulla were omitted from the present study to avoid possible confusion with respect to different morphogenesis, although its incidence is quite uncommon.

Histology

The adrenal glands were fixed with 10% phosphate-buffered formalin (pH 7.2), and prepared for histologic examination. The sections were stained with haematoxylin and eosin. Histological grading in this study was divided into four groups as follows; grade 0, absence of small focus with mononuclear cell infiltration. In this case, 'focus' means mononuclear cell infiltration including lymphocytes, plasma cells, and macrophages composed of >50 mononuclear cells; grade 1, one focus per standard representative longitudinal section; grade 2, two to five foci showing in both separate and continuous feature per standard representative sections without significant degree of cell loss; and grade 3, over six foci with mononuclear cell infiltration and/or diffuse infiltrating pattern of mononuclear cells with a considerable degree of cell loss.

Immunohistochemistry

A portion of the adrenal glands obtained from 63 autopsy cases was freshly frozen in liquid nitrogen. Frozen sections, approximately 4 μ m thick were fixed in acetone for 5 min and rinsed in cold phosphate-buffered saline (PBS, pH 7.2), and then incubated with appropriate blocking agent (Vector Laboratories, Burlingame, CA) for 30 min. Sections were serially stained with the following monoclonal antibodies: a CD3⁺ pan-T-lymphocyte marker, Leu 4; a CD4⁺ helper/inducer T-cell marker, Leu 3a; a CD8⁺ suppressor/cytotoxic T-cell marker, Leu 2a; a monocyte/granulocyte marker, Leu M1; a tissue-macrophage marker, Leu M5; a marker of activated T lymphocytes, anti-IL-2-receptor; and a CD22⁺ pan-B-cell marker, Leu 14 (all from Becton Dickinson, Sunnyvale, CA). Controls consisted of omission of the initial antibodies or use of irrelevant antibodies. They were then washed three times with PBS, and incubated with biotinylated anti-mouse IgG (Vector Laboratories) for 30 min. They were washed with cold PBS for 30 min and incubated with horseradish peroxidase avidin (Vector Laboratories) for 30 min. After being washed with PBS, the sections were reacted with fresh mixture of 0.05% 3,3-diaminobenzidine and 0.005% H₂O₂ in Tris-HCl buffer (0.05 M, pH 7.6) for 5 min, washed with distilled water, and were lightly counterstained with haematoxylin. Positively stained cells were enumerated as the percentage to the total number of the infiltrating mononuclear cells under $\times 100$ magnification in each serially stained tissue sections.

RESULTS

Histologic study

Focal and/or diffuse mononuclear cell infiltration in the adrenal cortex were observed in 110 of 174 autopsy cases (63.2%) > 60 years of age. The lesions were usually located in the zona reticularis of adrenal cortex and/or in the corticomedullary border. In younger autopsy cases (< 49 years of age) however, the infiltrating foci in the adrenal cortex were observed in only

Table 1. Incidence and degree of mononuclear cell infiltrate in aged human adrenal cortex

Age (years)	Sex	n	Grade*				Percent incidence†
			0	1	2	3	
29-39		19	18	1	0	0	5.3
	Male	11	10	1	0	0	9.1
	Female	8	8	0	0	0	—
40-49		35	32	3	0	0	8.6
	Male	17	16	1	0	0	5.9
	Female	18	16	2	0	0	11.1
60-69		38	18	12	7	1	52.6
	Male	21	10	7	3	1	52.3
	Female	17	8	5	4	0	52.9
70-79		68	23	32	8	5	66.2
	Male	32	12	14	4	2	62.5
	Female	36	11	18	4	3	69.4
80-89		49	16	18	8	7	67.3
	Male	21	7	8	3	3	66.7
	Female	28	9	10	5	4	67.9
90-98		19	7	8	3	1	63.2
	Male	10	4	4	2	0	60.0
	Female	9	3	4	1	1	66.7

* Infiltrating foci were divided into four grades as described in Materials and Methods.

† Proportion of number of cases with mononuclear cell infiltration of \geq grade 1 versus total number of cases examined.

four of 54 cases (7.4%) and the lesions were all grade 1. Table 1 summarizes the incidence and degree of the infiltrating foci developing spontaneously in the adrenal cortex in aged human. Among 110 infiltrating foci in aged adrenal cortex, the number of cases with each grade 1, 2, 3 was 70, 26, and 14, respectively. Mean age of respective grades was 78.5 ± 8.3 , 75.1 ± 9.6 , and 79.7 ± 5.9 . Of 14 severe lesions of grade 3, 13 (97.1%) were observed in the adrenal cortex of autopsy cases of ≥ 70 years of age. Sex difference in incidence of the infiltrating foci was not found in any age group. Representative histological features of the infiltrating patterns with grade 1 to grade 3 are shown in Fig. 1a-c. In grade 3 lesions, residual swollen adrenocortical cells were occasionally observed singly or in groups within the infiltrating cells, which often looked similar to megakaryocyte-like cells (Fig. 1d). [Additional immunohistochemical study revealed that these cells were all negative for factor VIII-related antigen (Dakopatts, Denmark).]

Immunohistochemical study

Phenotypes of infiltrating mononuclear cell population were analysed using a panel of monoclonal antibodies. CD3⁺ lymphocytes were found to be a major population of the infiltration foci in the tissue sections, comprising $81.4 \pm 10.9\%$ of total mononuclear cells. The majority of these cells were of the CD4⁺ lymphocytes ($70.3 \pm 9.8\%$), and CD8⁺ lymphocytes comprised $12.5 \pm 6.8\%$ of cells. The proportion of Leu M5⁺ lymphocytes was found to be less in percentage ($5.2 \pm 2.7\%$), implying that the confusion of stainability between CD4⁺ helper/inducer T cells and CD4⁺ tissue-macrophages could be disregarded. Moreover, a minor proportion of CD22⁺ lympho-

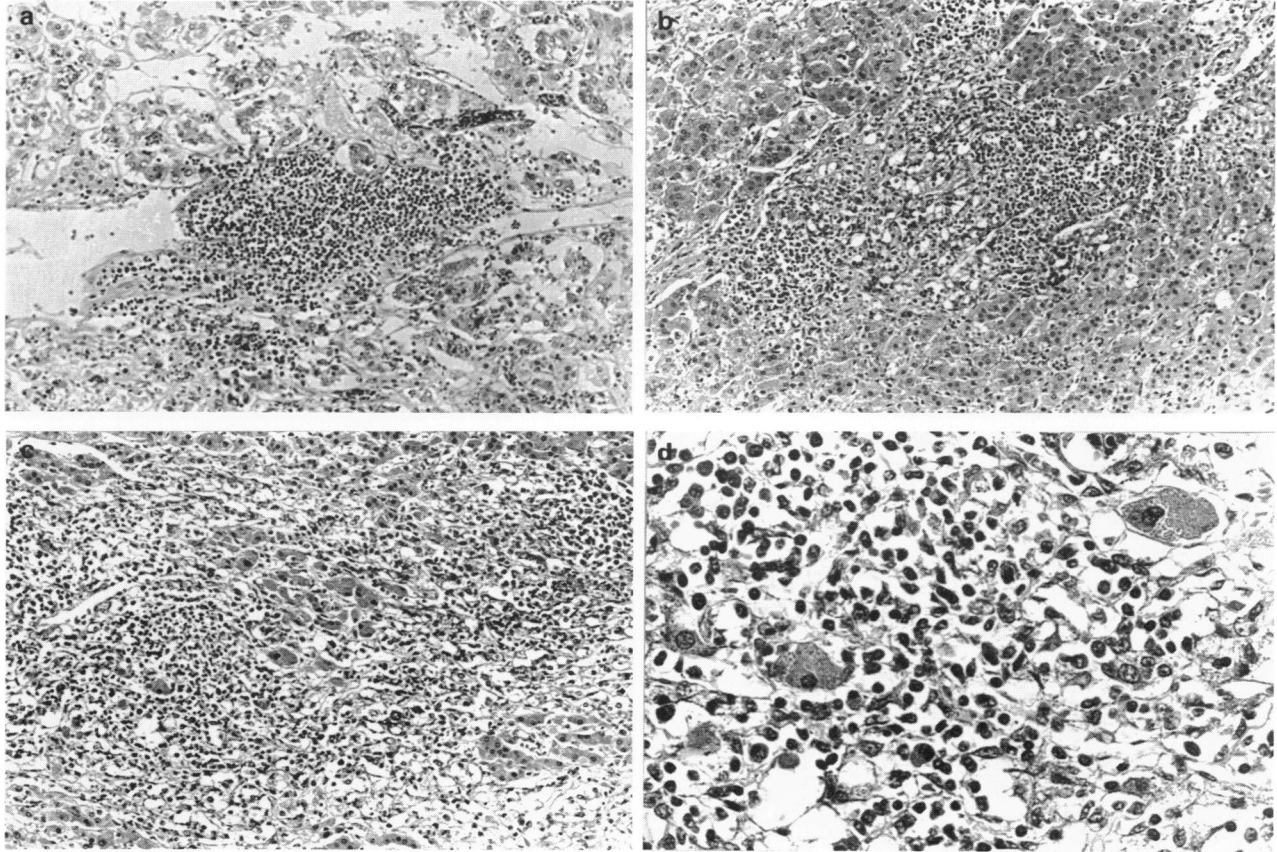


Fig. 1. Histologic appearance of mononuclear cell infiltration in the aged human adrenal cortex; (a) Photomicrograph demonstrating a mild focus of infiltrating mononuclear cells (grade 1); (b) Photomicrograph demonstrating contiguous foci of infiltrating mononuclear cells (grade 2); (c) Photomicrograph demonstrating a severe lesion of infiltrating mononuclear cells with a considerable degree of cell loss (grade 3); (d) High-power figure of (c), demonstrating residual adrenocortical cells within mononuclear cell infiltration. Haematoxylin and eosin staining; a-c, $\times 110$; d, $\times 410$.

cytes, and Leu-M1⁺ cells was observed sporadically within the infiltrating lesions ($10.8 \pm 4.2\%$ and 7.9 ± 3.1). Figure 2 shows representative features of phenotype expression of CD3, CD4, CD8 and CD22, in serially stained tissue sections.

To determine whether these infiltrating T lymphocytes in the aged organs were activated or not, serial frozen sections were studied by IL-2 receptor expression. Consequentially, it was demonstrated that a significant proportion of CD4⁺ lymphocytes was activated as judged by IL-2 receptor expression ($23.8 \pm 8.2\%$) (Fig. 3).

DISCUSSION

We have observed the infiltration of mononuclear cells at a higher incidence in samples of aged human adrenal cortex than in younger ones, both obtained from autopsy cases: the infiltrating mononuclear cells were mainly composed of T lymphocytes. In previous communications (Kurashima & Hirokawa, 1985; 1986), it was reported that focal lymphocytic infiltration in the human salivary and thyroid glands increased in incidence with advancing age, and it was postulated that the lesions of focal lymphocytic infiltration in these organs could be referred to as an organ-specific autoimmune phenomenon without overt clinical manifestation and could be based upon an autoimmunity associated with ageing process (Walford, 1969;

Makinodan & Kay, 1980; Hirokawa, 1985). This assumption was again strongly supported by the present study showing that T lymphocyte infiltration in the adrenal cortex was more frequently found in the elderly than in younger people.

In view of the histological features in this study, the affected glands with grade 3 mononuclear cell infiltration in the aged showed a considerable degree of cell loss, that was quite similar to the adrenal glands in Addison's disease (Anderson *et al.*, 1968; Petrei & Nerup, 1971). However, the patients with grade 3 mononuclear cell infiltration had no relevant, biochemical abnormalities such as low Na⁺ levels from clinical records. Immunohistochemical data obtained from aged human materials as well as adrenal cortex indicated that most infiltrating lymphocytes within these organs express CD4⁺ helper/inducer phenotype. Phenotypic analysis of the lymphocytes infiltrating salivary glands with primary Sjögren's syndrome has defined a predominance of CD4⁺ T cells in both peripheral blood and the damaged tissues (Fox *et al.*, 1982; Adamson *et al.*, 1983). Furthermore, immunohistochemical characterizations in the various affected tissues with organ specific autoimmune diseases in autoimmune-prone rodents have demonstrated that the majority of infiltrating lymphocytes in lachrymal glands, salivary glands, renal vascular lesions, pulmonary vascular lesions, and pancreatic islets express CD4⁺ phenotype (Hoffman *et al.*, 1984; Jabs & Prendergast, 1987; Harbeck, Launder & Staszak,

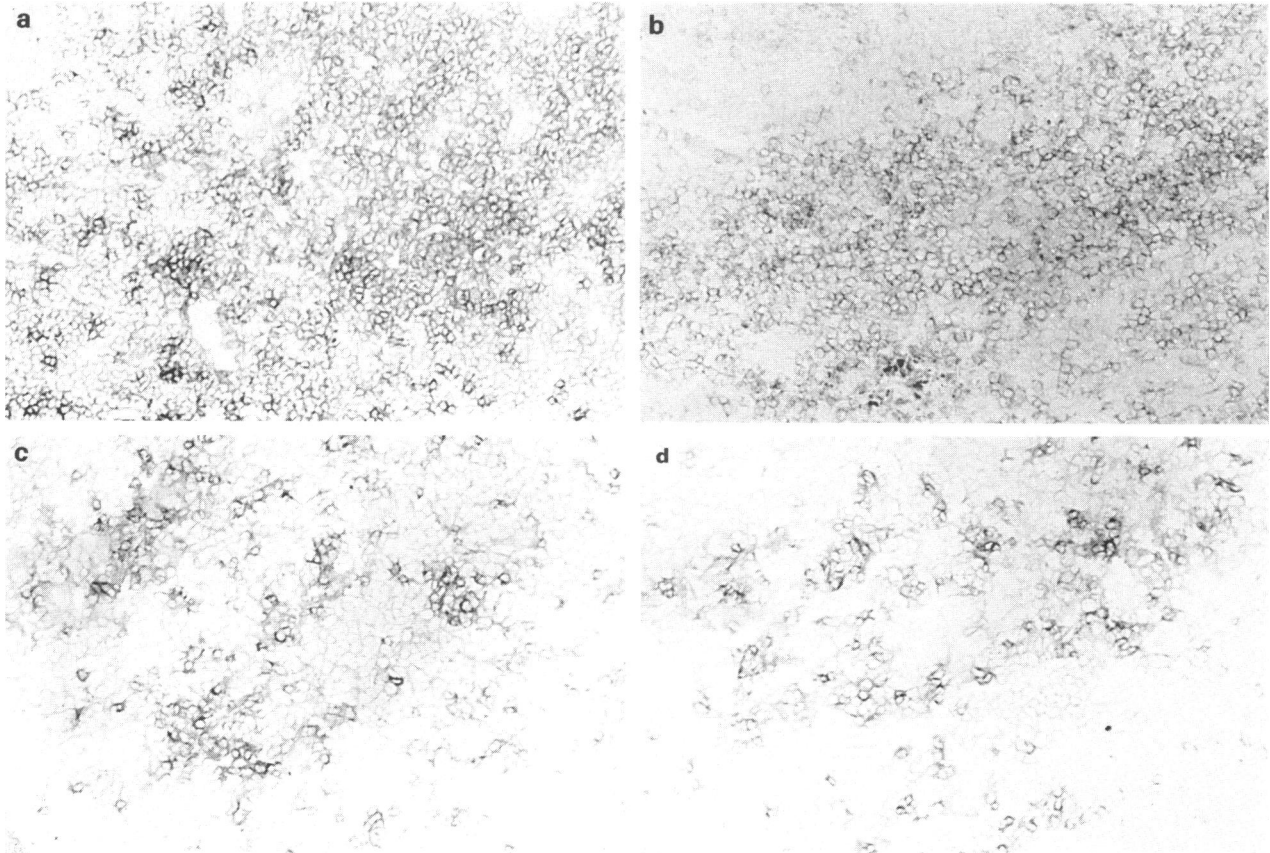


Fig. 2. Immunohistochemical detection of infiltrating mononuclear cells in aged human adrenal cortex. Positive staining of CD3 (a); CD4 (b); CD8 (c); and CD22 (d) in subserial tissue sections. Most recognized as T cells (a), that were composed mainly of CD4⁺ cells (b), and a lesser number of CD8⁺ (c), and CD22⁺ cells. Immunoperoxidase staining $\times 170$.

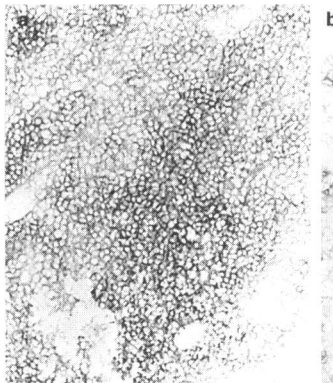


Fig. 3. Immunohistochemical detection of activated T lymphocytes as judged by IL-2 receptor expression. Positive staining of CD4 (a) and IL-2 receptor (b) in serial tissue sections. A considerable proportion of CD4⁺ cells ($\sim 20\%$) was activated, judged by IL-2 receptor expression. Immunoperoxidase staining, $\times 120$.

1986; Weringer & Like, 1986, 1988; Koike *et al.*, 1987). In addition, *in vivo* administration of anti-L3T4(CD4) monoclonal antibody has been effective in preventing autoimmune diabetes in NOD mice (Koike *et al.*, 1987). Recently we reported that the

major phenotype of infiltrating lymphocytes in spontaneously occurring sialadenitis in aged non-autoimmune BDF1 mice is also CD4 (Hayashi *et al.*, 1988). From these data, including ours, it can be considered that CD4⁺ lymphocytes plays a central role in the induction of organ-specific autoimmune diseases both in humans and in rodents; it can also be strongly suggested that most autoreactive T cells in these affected organs bear the CD4⁺ phenotype. However, it cannot be denied that CD8⁺ lymphocytes within these inflammatory lesions, even though its proportion is lower, may contribute in part to the development of the autoimmune disorders. With regard to this, recent evidences indicate that the induction of autoimmune diabetes in the adoptive transfer systems in NOD mice depends on both the CD4⁺ and CD8⁺ lymphocytes (Miller *et al.*, 1988; Bendelac *et al.*, 1987; Bach, 1988).

Immunohistochemical analysis of infiltrating lymphocytes within the adrenal cortex expressing CD4⁺ helper/inducer phenotype is well in keeping with the role played by these cells in activating process as judged by IL-2 receptor expression, although its proportion is relatively small. It has been reported that an increased prevalence of activation antigens (Ia; OIK10) was found to be present on salivary gland infiltrating lymphocytes in patients with Sjögren's syndrome ($> 50\%$ positive) (Adamson *et al.*, 1983). The expression of MHC-specific

antigens may be induced by factors released from activated T cells or macrophages during the course of inflammatory process. Thus, it was also interesting to confirm the presence of a considerable proportion of infiltrating cells within the adrenal cortex expressing activation antigens.

This is the first report strongly suggesting that some sort of autoimmune mechanisms associated with the ageing process functions for the formation of T lymphocytes infiltrating foci within the adrenal cortex in aged humans. In this context it may be possible to reject the classical concept that lymphoid infiltrates in the human adrenal glands are hormonally induced transformations into myeloid tissue, shown by the histological examination alone (Selye & Stone, 1950). Data obtained from this study demonstrate a significant insight to search for the pathogenesis of autoimmune Addison's disease as well as the pathogenic role of autoimmune phenomena in the adrenal cortex associated with age-related decline in normal immune functions.

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