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Tumour necrosis factor receptor distribution in human lymphoid tissue

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SUMMARY

The nature and location of cells responding to tumour necrosis factor-alpha (TNF-α) were investigated *in situ* by immunohistochemistry using monoclonal antibodies (mAb) directed against the p75 and p55 proteins of the TNF receptor. Receptor expression was found in the thymus and secondary lymphoid tissues. In the thymus the p75 receptor was confined to medullary lymphoblasts and dendritic cells, which co-stain with the Tac protein of the interleukin-2 (IL-2) receptor. In lymph nodes and other secondary lymphoid tissues, the p75 receptor was expressed on activated lymphocytes and interdigitating reticulum cells of the T-cell area, whereas the p55 receptor was confined to the germinal centre dendritic reticulum cells (DRC), which is the main site of TNF-α production. TNF receptor (TNFR) proteins were up-regulated in reactive hyperplasia together with increased TNF-α expression. Surprisingly, no TNFR was detectable on non-lymphoid tissues. The species specificity of these TNFR antibodies was high: whereas the antibodies cross-reacted with epitopes in non-human primates, no immunoreactivity was detected in lower animal species, e.g. dog, rabbit and rodents. The data presented suggest that TNF-α, which is produced by germinal centre DRC, might regulate an *in vivo* immune response through autocrine and paracrine pathways, e.g. through the p55 and p75 receptor proteins, which are expressed at different sites of the lymphoid tissue.

INTRODUCTION

Tumour necrosis factor (TNF) plays an important role in host defence to infection and in inflammation.[1 3] The biological significance of TNF and its role in various diseases are still areas of intensive research. TNF stands for two homologous peptide factors,[4] TNF-α, which is produced by activated monocytes, macrophages and activated T lymphocytes,[5,6] and TNF-β, which is derived from a subpopulation of activated T cells.[7] TNF exerts its biological activity by binding to two types of cellular TNF receptors.[8,9] Recent immunohistochemical investigations on the location of TNF-α production identified the germinal centre dendritic reticulum cells (DRC) as the main source of TNF-α in tissues.[10,11] *In vitro* studies suggest a ubiquitous occurrence of TNF receptors (TNFR), and TNF has been shown to exert important effects on lymphocytes,[12 14] monocytes,[15] fibroblasts and on the endothelium.[16,17]

In order to gain more insights into the immunoregulatory role of this locally produced cytokine, we investigated the nature of TNF-α-responsive cells *in situ* by examining the expression of the two TNFR proteins *in situ* in lymphoid tissues. It was a reasonable assumption that the TNF-α producing DRC are unlikely to be the only cells responding to this cytokine. Both types of TNFR have recently been characterized by molecular cloning and have been shown to differ by apparent molecular weight (75,000 and 55,000 MW) and by their relative expression on human cell lines.[18 21] Monoclonal antibodies (mAb) have been developed against the p75 TNFR (utr-series) and the p55 TNFR (htr-series) and shown to be specific and non-cross-reactive,[8] despite the high degree of sequence similarity in the extracellular region from both types of TNFR.[18]

Two mAb suitable for immunohistochemistry, utr-1, a TNF-blocking anti-p75 TNFR antibody, and htr-19, a non-blocking anti-p55 TNFR antibody, were used for this investigation. The present immunohistochemical study shows an exclusive expression of TNFR proteins in lymphoid tissues. The more abundant TNFR protein, p75, was expressed essentially in thymic medullary lymphoblasts and cells of the interfollicular area of lymph nodes, comprising the interdigitating reticulum cells and activated lymphocytes. The p55 protein was found in the germinal centre DRC, the site of TNF-α production. Thus, it is postulated that TNF-α regulates an immune response through autocrine and paracrine pathways which use different receptor proteins.

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MATERIALS AND METHODS

Tissue and cell preparation

Fresh human tissues removed at surgery for diagnostic purposes were snap-frozen in liquid nitrogen and stored at −70 until

used. Animal tissues from non-human primates (rhesus, marmoset) and from dog, rabbit, rat and mouse, obtained from Sandoz, Basel, Switzerland, were treated identically. Cryostat sections were mounted on gelatine- or polylysine-coated glass slides, air-dried for 3 hr, fixed in either 2% paraformaldehyde-lysine sodium periodate, or for anti-TNF-α antibody incubation in acetone for 10 min at room temperature, and were then rinsed twice in Tris buffer.

Fresh bone marrow cells and peripheral blood leucocytes (PBL) were obtained from healthy volunteers, isolated on a Ficoll gradient and suspended in RPMI-1640 medium containing 10% foetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Lymphocytes (10^6/ml) were stimulated with 10 μg/ml lipopolysaccharide (LPS) from *Escherichia coli* (Sigma Chemicals, St Louis, MO) for 20 hr at 37°. Thereafter, cells were washed and cytocentrifuged. Cytospin preparations were stored at −80° until immunostained.

Immunostaining

Serial cryostat sections were incubated sequentially for 30 min at room temperature with the following mAb: utr-1 (hybridoma supernatant, 1:50 dilution), htr-19 (hybridoma supernatant, undiluted) or an anti-TNFα mAb (1:200 dilution). After two 5-min washes in Tris buffer, the sections were incubated with rabbit anti-mouse immunoglobulin (Dakopatts, Glostrup, Denmark; 1:30) for 30 min, followed after rinsing by incubation with alkaline phosphatase–anti-alkaline phosphatase (APAAP) complex for 30 min, and then the addition of the substrate for 30 min according to the instructions of the manufacturer (Dakopatts). The immunostaining procedure for the cytospin preparation was identical; the smears were fixed in acetone for 10 min at room temperature.

Double staining

Cryostat sections from utr-1-positive tissues were stained serially for utr-1 and examined by a second staining to analyse the phenotype of TNFR-positive cells. Briefly, utr-1 staining was performed as above; sections were then stained for cell markers (Tac, T11 DRC1, T4, T8, T6, L26) and TNF-α using murine mAb and immunoperoxidase developed in 3,3-diaminobenzidine (DAB). As controls either the first or second specific antibody was replaced by a non-specific antibody, and the sequence of staining was reversed.

Controls

The following controls were included for the specificity of the immune reaction: omission of the primary antibody and/or substitution of the primary antibody by mouse serum or murine myeloma IgG (MOPC21, Sigma); for staining with utr-1 and TNF-α mAb, the immunoreactivity was blocked with excess recombinant human TNF-α.[8] Significant inhibition of staining was obtained at 1 μg/ml.

Reagents

The mAb against the p75 TNFR receptor protein, utr-1 has been described previously.[8] The antibody htr-19 (M. Brockhaus *et al.*, unpublished data) was derived from a mouse immunized with p55 TNFR in an analogous way, as described for other anti-p55 TNFR antibodies.[8] Monoclonal antibodies against TNF-α, tnf-14, were generated and characterized by standard procedures (M. Brockhaus, unpublished data). Monoclonal antibodies against resting and activated T and B lymphocytes (T11, T4, T8, L26, Tac) and dendritic reticulum cells and Langerhans' cells (DRC1, T6) were obtained from Dakopatts (Glostrup, Denmark). Rabbit antibodies against TNF-β were purchased from Genzyme (Boston, MA) and Endogen (Bioreba, Basel).

RESULTS

TNF receptor expression in thymus and bone marrow

The immunoreactivity of various mAb against TNFR from both the htr and utr series was tested. The best results were obtained with htr-19 (p55) and utr-1 (p75) on cryostat sections from frozen tissues, whereas no staining was obtained on paraffin sections. The immunoreactivity of both antibodies was confined to the lympho-histiocytic tissue, which includes the thymus, and the secondary lymphoid organs, such as spleen, tonsils, lymph nodes and mucosa-associated lymphoid tissue.

In the thymus, the immunoreactivity was mainly confined to the medulla (Fig. 1a). Medullary utr-1 (p75)-reactive cells comprised dendritic cells and lymphoblasts, which co-stained with Tac protein of the IL-2R (Fig. 1b). No utr-1 reactivity was found either in thymic epithelium or in cortical thymocytes. There was a faint staining of dendritic cells with the htr-19 antibody (Fig. 1c). Hasall's bodies gave no reaction with either antibody.

Human bone marrow cells expressed neither the p75 nor the p55 protein. On peripheral blood lymphocytes, a faint membrane staining was obtained with utr-1, but not with htr-19. After mitogenic activation utr-1 reactivity was increased (data not shown).

Secondary lymphoid tissues

In the spleen, utr-1-reactive cells, comprising dendritic cells and lymphoblasts, were mainly located in the T-cell area (periarterial lymphatic sheath); only a few utr-1-reactive cells were found in the mantle zone and follicles of the B-cell area (Fig. 1d).

Serial cryostat sections of lymph nodes were incubated with utr-1, htr-19, DRC1, Tac or T cell mAb. Incubation with the utr-1 mAb resulted in strong staining of interdigitating reticulum cells (IDC) and of activated T lymphocytes in the interfollicular T-cell area (Fig. 2a). The distribution of utr-1-reactive cells overlapped with that of cells expressing the IL-2 receptor (Tac) in the T-cell area (Fig. 2b). Based on sequential incubation with utr-1 and Tac on serial sections, it is concluded that utr-1-reactive lymphocytes and a subpopulation of IDC co-express the Tac protein. htr-19 immunoreactivity was confined to follicular dendritic cells (DRC) in germinal centres and was not found at other sites (Fig. 2c). The staining pattern with htr-19 was comparable with that obtained with DRC1 antibody (not shown). The specificity of the immunoreaction was supported by negative controls obtained by substitution or omission of the primary antibody, and by competition with excess TNF-α for utr-1 staining. In tonsils a similar staining pattern was observed as in lymph nodes (Table 1): few if any utr-1-reactive cells were found in germinal centres and the mantle zone of the follicle; the interfollicular T-cell area (paracortex) gave distinct utr-1 staining of the DRC and of T lymphocytes. Finally, the T6-positive Langerhans' cells in the epithelium stained only with the utr-1

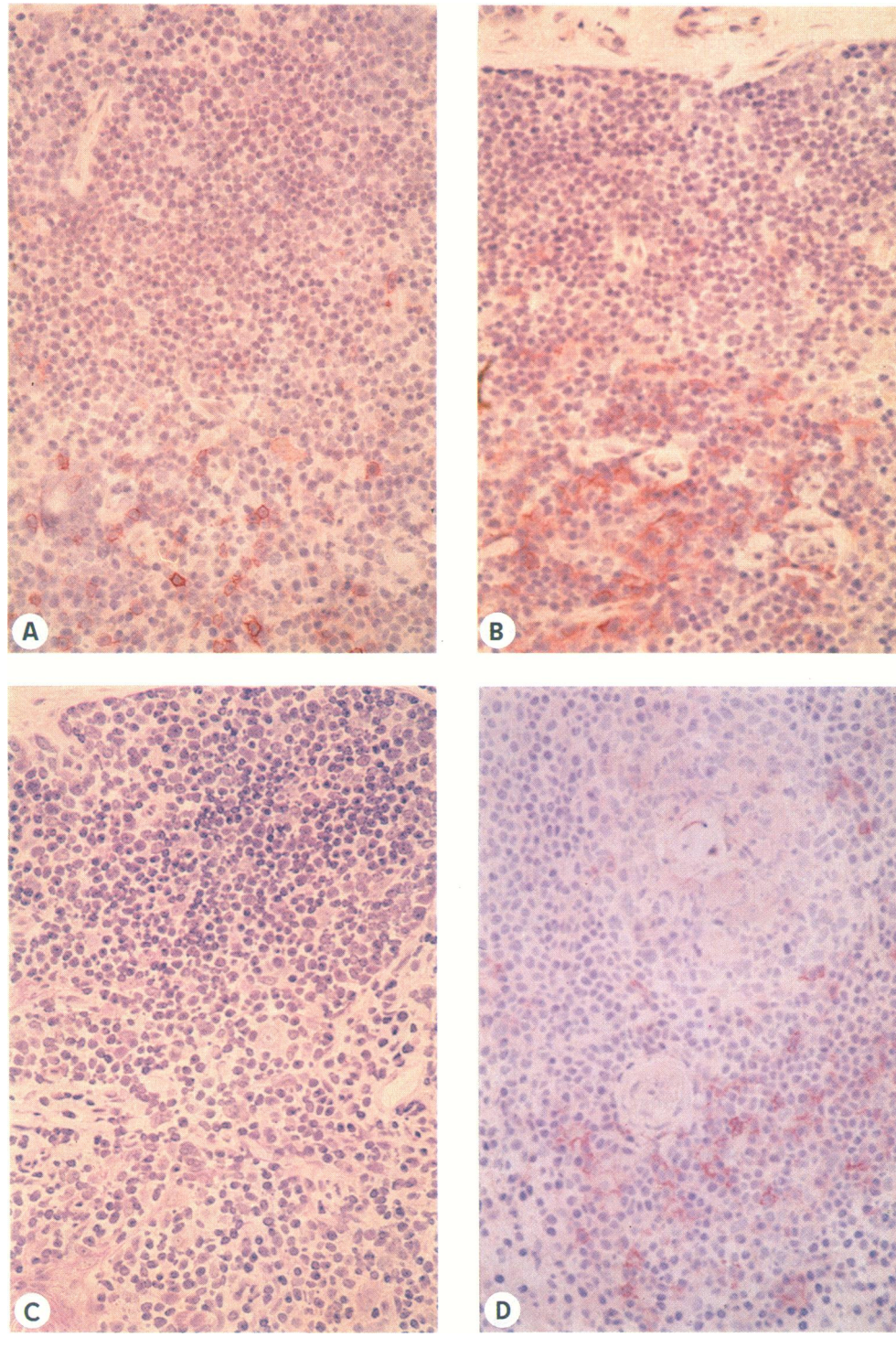

Figure 1. Immunostaining for TNF receptor on cryostat section of human thymus (a–c) and spleen (d), APAAP technique, magnification ×320. (a) utr-1-positive cells in the medulla. (b) Tac-positive cells at the same location. (c) Faintly htr-19-positive cells. (d) Spleen: utr-1-reactive cells in the periarterial lymphatic sheaths.

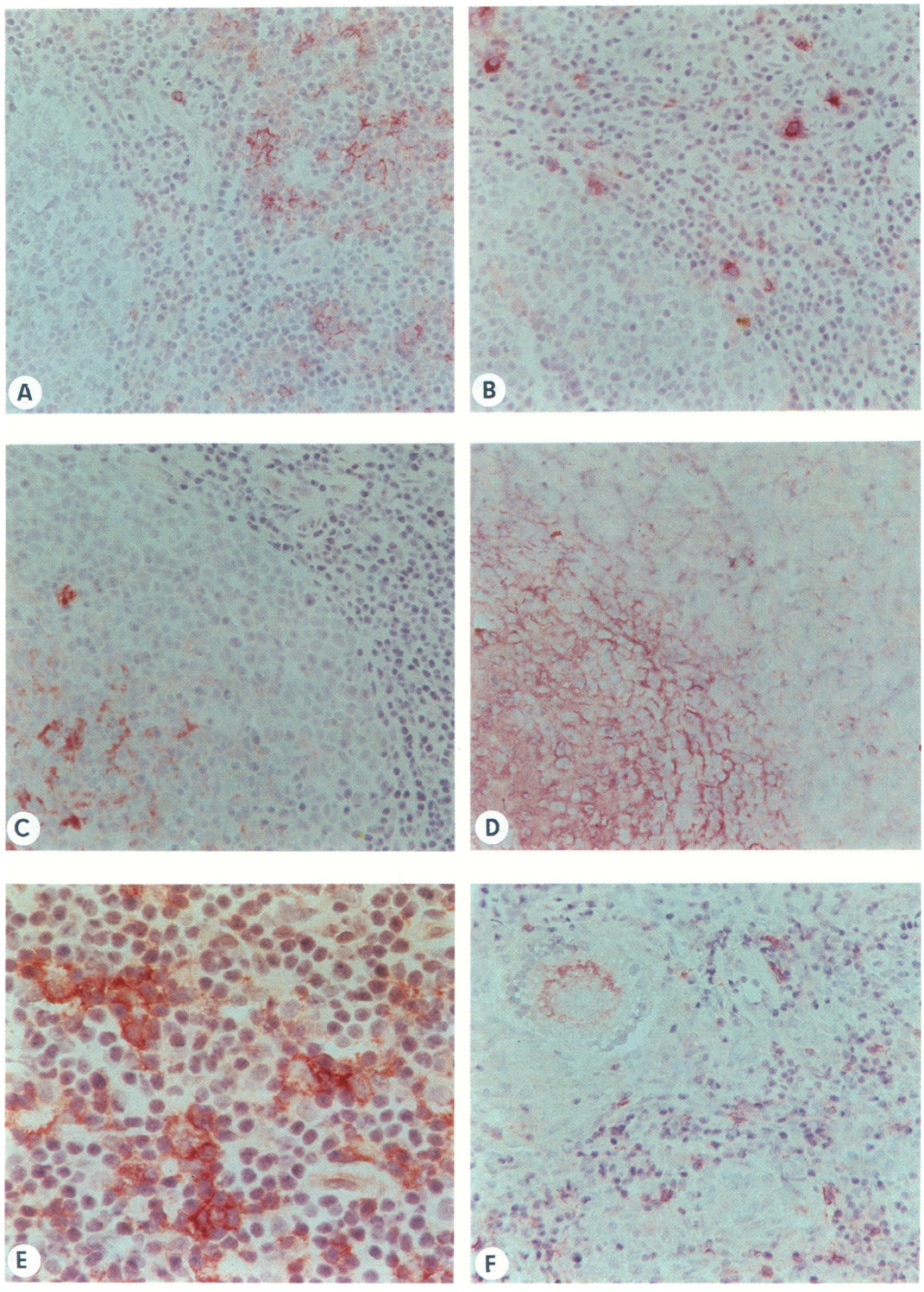

Figure 2. Immunostaining for TNF receptor on serial cryostat sections of human from normal (a–d) and inflammatory (e,f) lymph nodes, using utr-1 and htr-19 mAb (APAAP technique, magnification × 320). (a) utr-1-positive lymphocytes and IDC in T-cell area. (b) Tac-positive cells in the same location. (c) htr-19-positive DRC in germinal centres. (d) Immunostaining with TNF-α, essentially of cells in the germinal centre. (d) Acute lymphadenitis: utr-1. (f) Sarcoidosis with utr-1 showing positive multinucleated giant cells.

Table 1. Distribution of TNF-α receptor proteins in normal lymphoid tissues

Organ*	*n*	Area†	TNF-receptor protein			
			utr-1	htr-19	IL-2 receptor Tac	TNF-α
Thymus	2	Cortex	−	−	−	−
		Medulla	+++	+	++	−
Lymph node	6	TA	+++	−	+++	+
		MZ	+	−	+	+
		GC	+	+	+	++
Tonsil	6	TA	+++	−	+++	(+)
		MZ	+	−	+	+
		GC	(+)	+	+	++
Spleen	3	TA	+++	−	++	−
		MZ	+	−	++	−
		GC	−	+	−	+
		RP	+	−	+	−
Skin	2	Langerhan's cells	++	−	+	−
Mucosa-associated lymphoid tissue	4	TA	+++	−	++	−
		MZ	+	−	+	−
		GC	(+)	+	(+)	+
Kidney	12	Reticulum cells (interstitial)	++	−	+	

* Organs examined which were negative included: liver, heart, lungs, brain, adrenals, uterus, ovary, testes, prostate, stomach, intestines.

† Immunoreactivity within various areas (TA, T-cell area; MZ, mantle zone or marginal zone for the spleen; GC, germinal centre; RP, red pulp) was assessed by a semi-quantitative score (0−3+).

antibody (not shown). In the lymphoid tissues associated with the mucosae of the gut and the bronchus a similar pattern of utr-1-reactive cells was found in the T-cell areas.

The TNFR distribution was also investigated in non-lymphoid tissues (Table 1). Scattered utr-1-positive, mononuclear cells in the interstitial space of kidneys, the mucosae of various organs and at inflammatory sites were observed. In inflammatory lesions, utr-1-reactive mononuclear cells from chronic infiltrates gave variable staining with utr-1 antibody.

Endothelial cells and fibroblasts from various organs gave no detectable staining with either antibody.

TNF-α expression in tissues

In order to obtain information on the spatial relationship of TNFR expression and TNF production sites, TNF-α tissue expression was investigated by use of a mAb. TNF-α-reactive cells were confined to the lymph follicles (Fig. 2d); within this structure the follicular dendritic cells (DRC), with their cytoplasmic projections, stained distinctly. The immune reactivity was less in the mantle zone and was almost absent from the interfollicular T-cell area of lymphoid organs. The staining with TNF-α of DRC was superimposable with that of htr-9 mAb. In the thymus, only little immunoreactivity was found with TNF-α antibody, which was restricted to medullary dendritic cells. In the bone marrow, cells with monocytic morphology contained TNF-α.

For the specificity of the immunoreactivity, incubations with TNF-α antibody were performed in the presence of excess TNF-α, which reduced the TNF-α staining. Attempts to identify cells producing lymphotoxin (TNF-β) in the tissue with two different polyclonal antibodies failed.

TNF-α receptor expression in inflammation

The role of TNF and its receptor was investigated in lymph nodes from patients with various inflammatory conditions. In hyperplastic lymph nodes the number and intensity of utr-1 and htr-19-reactive cells were increased. Within the T-cell area a distinct increase of utr-1-positive activated lymphocytes and dendritic cells could be observed (Fig 2e). Epithelioid cells in non-specific granulomas and giant cells from two patients with sarcoidosis showed variable utr-1 positivity (Fig. 2f). Furthermore, cells co-expressing Tac and utr-1 were also increased in the mantle zone and occasionally in the centre of the follicle (Table 2). Concomitant with increased utr-1 expression, TNF-α immunoreactivity was also found in epithelioid cells and giant cells and in hyperplastic lymph follicles.

Species specificity of TNFR antibodies

Both antibodies utr-1 and htr-19 were tested for species specificity, using lymphoid tissues from various experimental animals. In one non-human primate, the marmoset, but not in rhesus monkeys, the immunohistochemical pattern with utr-1 and htr-19 was comparable to that obtained in humans (data not shown).

By contrast, no immunoreactivity of these antibodies was obtained in rodents (mouse, rat), rabbit and dog. The results were expected since the structures of the mouse and human TNF receptor, at least, show substantial differences.

Table 2. Expression of TNF receptor proteins in lymphadenitis (LA)*

Diagnosis	No. of cases	utr-1				htr-19	
		TA	MZ	GC	EC	GD	EC
Hyperplasia	4	+++	++	+	−	++	−
Non-specific LA	4	+++	+	+	−	+	−
Acute LA with abscess	1	+++	+	−	−	+	−
Epithelioid cell LA	3	+++	++	+	+	+	−
Sarcoidosis	2	++	+	−	+	+	−

* Immunoreactivity in T-cell (TA) and B-cell areas (MZ, mantle zone; GC, germinal centre; EC, epithelioid cells) was assessed by semi-quantitative score (0−3+).

DISCUSSION

A unique distribution of the two types of TNF receptors (TNFR) was found in human tissues by immunohistochemistry, using two specific mAb.[8] The p55 protein, reacting with the htr-19 antibody, constituted a minor population of TNFR, expressed in the dendritic reticulum cells (DRC) of germinal centres, while the p75 protein, reactive with the utr-1 antibody, constituted the major population. These comprised interdigitating reticulum cells (IDC) and activated lymphocytes in the thymic medulla and the interfollicular T-cell area of lymph nodes. Based on sequential staining of serial sections and double staining, a high percentage of utr-1-positive lymphocytes and IDC co-express the Tac protein of the IL-2R. The tissue expression of TNF-α has been reported recently by McCall *et al.*[11] Therefore, the TNF receptor distribution was compared with the immunostaining of the same tissues, using antibody directed against TNF-α and TNF-β. While the available anti-TNF-β antibodies gave no staining, the immunoreactivity of TNF-α antibodies was strong and essentially located in the germinal centre: DRC with projections of the cell bodies into the mantle zone were stained and a few dendritic cells stained in the T-cell areas. The latter results are in agreement with earlier publications.[11,12]

The present data suggest that the main source of TNF-α is the macrophage-derived DRC, which also expresses the p55 TNFR protein. In the absence of a suitable antibody, the nature of TNF-β-producing cells cannot be determined. By contrast, the p75 TNFR sites are anatomically separated and are located mainly in the T-cell areas.

Both DRC of germinal centres and IDC of the T-cell areas are known to function as antigen-presenting cells and are apparently responsive to the same lymphokines. It appears that co-operation with the respective antigen-reactive lymphocytes requires a different receptor structure, as recognized by the utr-1 and htr-19 mAb. The fact that activated lymphocytes co-express receptors for IL-2 and TNF is in accordance with previous findings of p75 TNFR expression on activated T cells[18] and supports *in vitro* observations that TNF-α plays a role in the activation of lymphocytes.[12,13,22]

These results demonstrate, for the first time, a distinct distribution of the two TNFR proteins in the lymphoid tissue. In view of the differential location of cells producing and responding to this cytokine, autocrine and paracrine signalling pathways of TNF-α, which use different receptor structures, are suggested. No information is presently available as to whether the two receptor proteins also have differing functions.

Investigations on pathological tissues might promote our understanding of the role of TNF-α in disease. In reactive lymph nodes, the number of p75 (utr-1)-positive cells was increased in T-cell areas; epithelioid cell granulomas and giant cells in sarcoidosis expressed p75 TNFR, suggesting a role of TNF in their formation. Experimental evidence in a parasite mouse model supports this assumption.[23]

Surprisingly, the present investigation with both TNF receptor antibodies did not give any staining of endothelial cells, and fibroblasts, smooth muscle cells, astrocytes and oligodendrocytes, cells which are known to respond to TNF *in vitro*.[16,17,24-27] Evidence for an important role of TNF and other cytokines in normal and pathological vascular endothelial responses[28] and in a astonishing variety of immunopathological disease conditions[29] has been reviewed recently. The absence of immunoreactivity may be due to a low expression of receptor molecules, low sensitivity of the immunohistochemical method or fundamental differences between cultured cells, cell lines and the *in vivo* situation. The only TNFR-positive cells found in non-lymphoid tissues were dendritic reticulum cells, which are located in the interstitial space of various organs such as kidneys, lung and mucosa.

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