SOMATOSTATIN RECEPTORS IN THE IMMUNE SYSTEM AND IMMUNE-MEDIATED DISEASE

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SOMATOSTATINE RECEPTOREN
IN HET IMMUUNSYSTEEM EN
IMMUUN-GEMEDIEERDE ZIEKTEN

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Het proefschrift werd gedrukt door Ponsen & Looijen B.V. te Wageningen

We shall not cease from exploration
And the end of all our exploring
Will be to arrive where we started
And to know the place for the first time

T.S. Elliot

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SOMATOSTATIN AND SOMATOSTATIN RECEPTORS IN THE IMMUNE SYSTEM

The vertebrate body has at its disposal three different systems, which together help it to maintain homeostasis and to respond to environmental signals: the nervous system, the endocrine system and the immune system. Traditionally, these systems have been studied as separate entities. However, in the interest of stability of the organism, it is essential that these different systems be able to communicate and reciprocally regulate each other's activities [1]. Communication can take place via direct cell-cell contact or through soluble signalling molecules and their specific receptors on target cells. It is hypothesised that the signalling molecules, which are shared between the three systems, are evolutionarily ancient [2]. Among others, endorphins [3, 4], cytokines such as interleukin-1 (IL-1) and IL-6 [5-7], hormones such as corticotrophin-releasing factor (CRF) [8, 9] and adrenocorticotrophic hormone (ACTH) [3, 4] and neuropeptides such as substance P and somatostatin [2, 10] have been identified as signalling molecules with effects on many different cell types and effector systems.

Through its effects on vasodilatation and blood flow the nervous system can modulate the local immune microenvironment in any tissue. All specialised lymphoid tissues are densely innervated by neurons of the sympathetic nervous system and by sensory neurons containing peptide neurotransmitters. Hormones with an endocrine or paracrine mode of action also modulate the tissue microenvironment [11, 12].

This chapter will further focus on the role of the ubiquitous neuropeptide somatostatin as an intersystem signalling molecule, with emphasis on the immune system.

Somatostatin, a ubiquitous neuropeptide

Somatostatin is a neuropeptide that is widely distributed throughout the body. It was first identified as a growth hormone release-inhibiting factor synthesised in the hypothalamus. Outside the central nervous system (CNS), the peptide is present in a variety of endocrine and non-endocrine tissues, and it appears to have many functions. In the CNS it can act as a neurotransmitter and neurohormone, while in peripheral tissues it regulates endocrine and exocrine secretion and acts as a modulator of motor activity in the gastrointestinal tract (for reviews see [13-15]).

Somatostatin functions as a neurotransmitter with generally inhibitory action [16] in many regions of the central nervous system (cerebral cortex, limbic system, brain stem, and spinal cord) [17]. In the peripheral nervous system (PNS) it is found in sympathetic and sensory neurons [13] and may have a down-modulatory effect on nociception, i.e. the perception of harmful and/or painful stimuli [18].

Somatostatin produced in the hypothalamus travels through the portal circulation to the anterior pituitary, where it inhibits the secretion of growth hormone (GH) but also of other pituitary hormones such as thyroid-stimulating hormone (TSH) and prolactin (PRL). Somatostatin is produced in specialised cells (D cells) in the gastrointestinal tract and the pancreas [19, 20]. In peripheral tissues the effect of somatostatin is also mainly inhibitory, regulating among others the secretion of calcitonin, gastrin, insulin, glucagon and vasoactive intestinal peptide (VIP). Furthermore, it down-regulates exocrine secretion of pancreatic

enzymes and gastric acid and is an inhibitor of motor activity of the stomach, small intestine and gall bladder [14, 21]. Somatostatin has also been shown to have anti-proliferative effects *in vitro* [22]. The actions of somatostatin are summarised in Figure 1.

Somatostatin is secreted in two biologically active forms: a 14 amino acid form (somatostatin-14) and an amino-terminally extended 28 amino acid form (somatostatin-28). In mammals, these two products are generated by proteolytic processing of a 92 amino acid precursor, prosomatostatin, which in turn is derived from a 116 amino acid form, preprosomatostatin; in lower vertebrates somatostatin-14 and somatostatin-28 are encoded by two separate genes [23, 24]. A disulphide bridge between two cysteine residues stabilises the β-turn that forms the receptor-binding domain of the peptide.

Somatostatin analogues

Native somatostatin has a plasma half-life of less than 3 minutes. Therefore, synthetic, metabolically stable analogues have been developed for clinical use. Structure-function analysis of native somatostatin and peptide analogues has shown that the amino acid residues Phe^7, Trp^8, Lys^9 and Thr^{10} are necessary for receptor binding. These residues are at the apex of the loop formed by the disulphide bridge connecting the two cysteine residues in native somatostatin and have been shown to form a β-turn [25]. Trp^8 and Lys^9 are essential for biological activity whereas the flanking residues tolerate minor substitutions [26].

Many of the synthetic analogues are shortened versions of native somatostatin, keeping the disulphide-bridged loop with the receptor-binding domain intact but modifying the N-terminal and sometimes the C-terminal amino acid residues to make them more resistant to proteolytic degradation [27]. The structures of the somatostatin analogues that are used clinically are shown in Figure 2. Most of these analogues are cyclic octapeptide structures. The smallest somatostatin analogues that are capable of receptor binding are hexapeptides [26].

Molecular aspects of somatostatin receptors

Somatostatin receptors were first identified in membrane preparations of the rat pituitary tumour cell line GH4C1, and subsequently in many other cell types [29, 30].

Somatostatin receptor structure

Structurally, somatostatin receptors are so-called seven transmembrane domain (TMD) glycoproteins, comprised of 7 membrane-spanning α-helical domains connected by short loops, an N-terminal extracellular domain and a C-terminal intracellular domain. The 7 transmembrane domains are thought to be arranged in a barrel-like configuration within the membrane (see Figure 3), forming a ligand-binding pocket. Hydrophobic and charged amino acids within TMD 3, 6 and 7 [31, 32] are thought to be important for the interaction with somatostatin, but extracellular loop 2 (between TMD 4 and 5) may also be involved [33-35].

Somatostatin receptor genes

The genes for a family of five somatostatin receptors ($sst_{1\text{-}5}$ by IUPHAR convention; see [36]) have been cloned in recent years. The sequences of human [37-40] and rat $sst_{1\text{-}5}$ [41-45] and of mouse $sst_{1\text{-}3}$ [37, 38, 46] were reported in 1991-1993; the mouse sst_4 and sst_5 genes were not cloned until 1996 and 1997, respectively [47, 48]. There is evidence for the

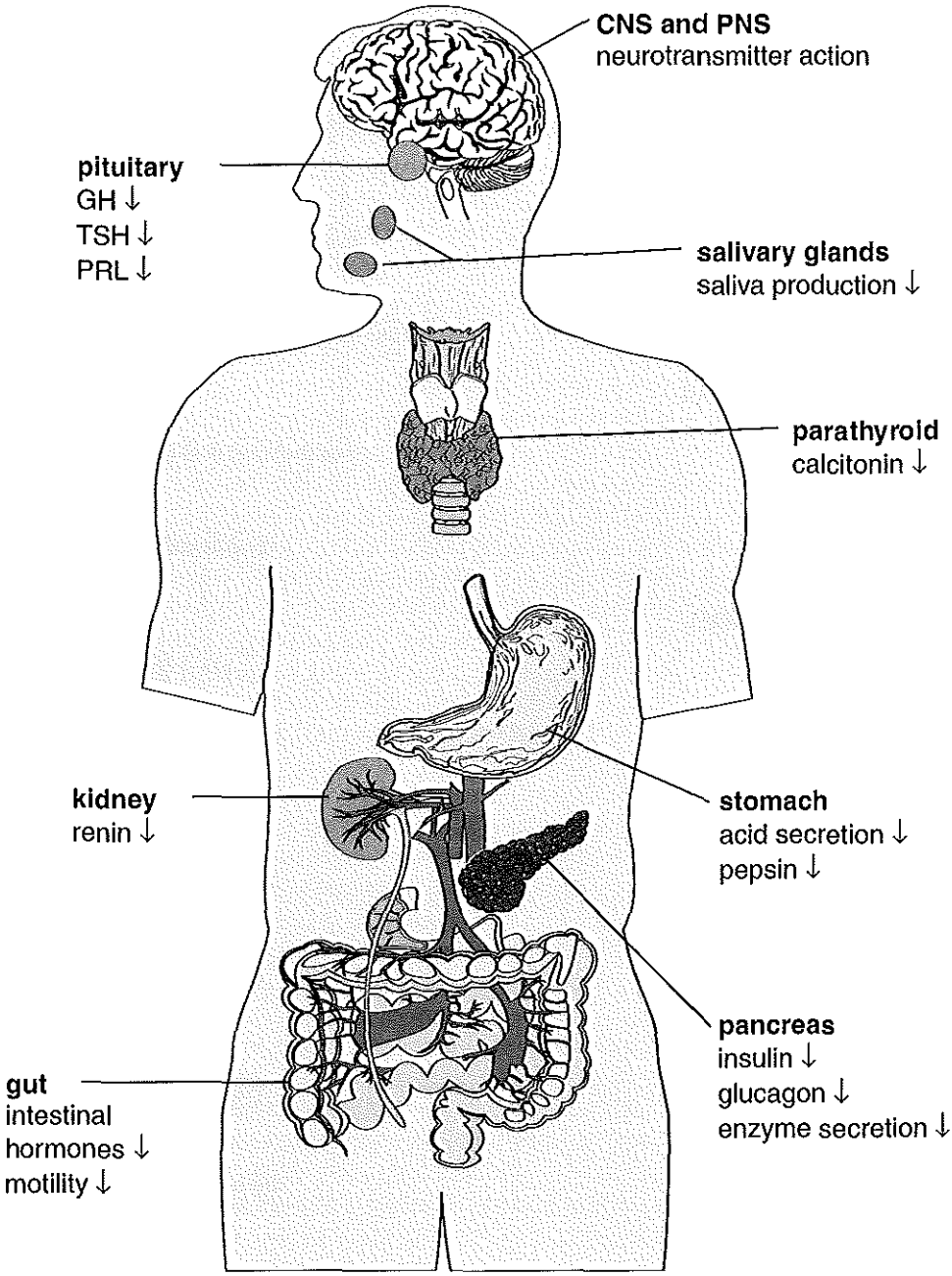

Figure 1. The actions of somatostatin in different organ systems (after [13]). CNS: central nervous system; PNS: peripheral nervous system; GH: growth hormone; TSH: thyroid-stimulating hormone; PRL: prolactin.

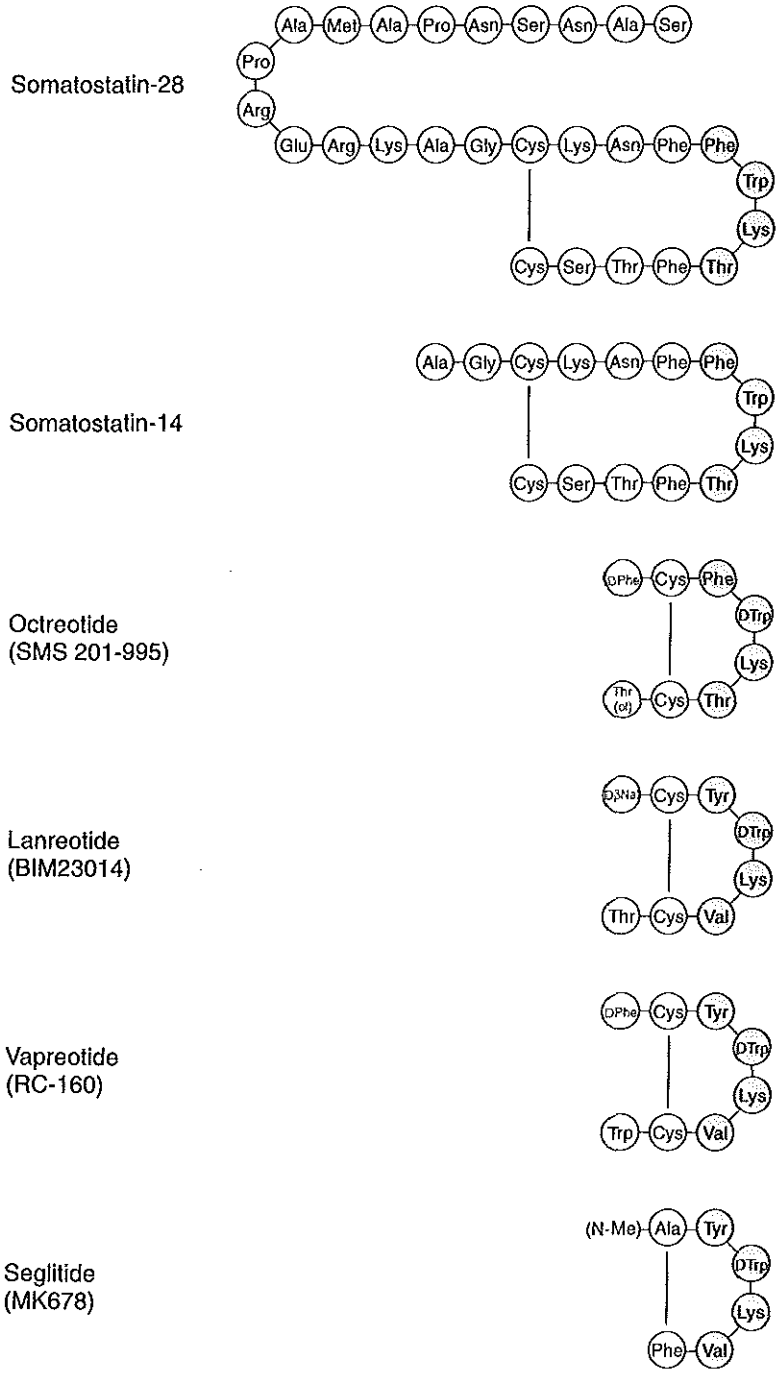

Figure 2. The amino acid sequences of native somatostatin (somatostatin-14 and somatostatin-28) and synthetic, metabolically stable somatostatin analogues used in the clinic (after [28]).

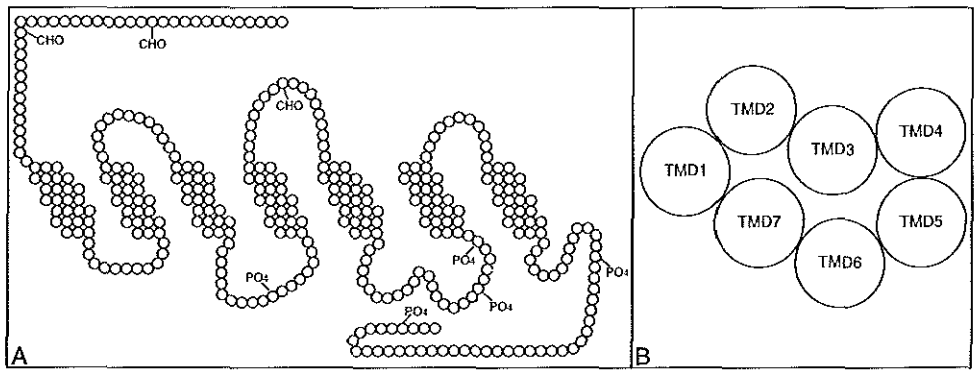

Figure 3. The putative configuration of somatostatin receptors within the membrane. A. Side view showing the seven membrane-spanning α-helices. B. Top view showing the possible configuration of the seven transmembrane domains (TMD1-TMD7) within the membrane.

existence of additional sst subtypes [49]. Although the sst genes do not have introns, there is a cryptic intron in the sst_2 gene, and alternatively spliced forms of sst_2 (named sst_{2A} for the unspliced form and sst_{2B} for the spliced variant) have been identified in humans, mice and rats [18, 50, 51]. The sst genes are all located on different chromosomes, but have a high degree of sequence homology [52].

Somatostatin receptor gene expression

Different sst genes can be expressed in the same tissues in overlapping patterns. The tissue distribution of sst messenger RNA (mRNA) has been studied most extensively in the rat; in other species the study of the distribution of mRNA for the different sst subtypes has focussed mainly on the brain and on tumour tissue. In the rat, mRNA for all five subtypes was found in brain and pituitary. Sst_2 mRNA is the major isoform in the pancreas and the adrenal glands, sst_3 mRNA is found in rat spleen and liver, sst_4 mRNA can be detected in the lung and sst_5 mRNA is found among others in the small intestine and the adrenal glands [53-55]. Currently, little is known about the tissue-specific regulation of sst subtype expression, but studies of the promoter regions of sst genes should begin to shed some light on this issue [28, 56].

Receptor binding of somatostatin analogues

On the basis of binding studies using synthetic somatostatin analogues, sst have been found to cluster into two different subgroups: sst_2, sst_3 and sst_5 are able to bind octapeptide analogues (sst_3 with lower affinity than sst_2 and sst_5), whereas sst_1 and sst_4 have negligible affinity for these compounds and only bind somatostatin-14 and -28 [57]. Only one synthetic compound, CGP23996, has been shown to bind all five sst with comparable affinity [58]. This compound is closely related to native somatostatin-14, lacking only the N-terminal residues outside the ring. However, it is degraded much faster than smaller somatostatin analogues and is therefore of little use clinically [59]. Recently, non-peptide subtype-selective agonists were described. These compounds bind with high affinity and have been shown to inhibit the release of growth hormone, glucagon, and insulin [60].

Biochemical aspects of somatostatin receptor activation

G-protein coupling

The signal transduction pathways coupled to activation of sst have been studied in transfected cell systems. All five subtypes of sst have been found to be linked to adenylyl cyclase via pertussis toxin sensitive, i.e. inhibitory, guanine nucleotide-binding proteins (G-proteins). Coupling to other intracellular signalling pathways is also generally mediated by G-proteins (for a review see [61]). Using mutational analysis, transfection studies, immunoprecipitation and affinity purification techniques, a number of the G-proteins associated with the different sst have been identified [62-65]. The presently known spectrum of G proteins associated with sst is summarised in Table 1, with the caveat that the G-proteins identified may differ depending on the cell system used for transfection and the signal transduction pathway studied.

Signal transduction pathways

Neuromodulation by somatostatin can be mediated through G-protein-dependent coupling to Ca^{2+} and K^+ ion channels [66, 67]. Inhibition of endocrine and exocrine secretion may depend on a reduced intracellular Ca^{2+} concentration, but may also be mediated through a receptor-mediated effect on the exocytotic machinery of the target cell [68]. The anti-proliferative effects of somatostatin are thought to be due to activation of a subclass of protein tyrosine phosphatase enzymes [69-72]. The major intracellular signalling pathways coupled to the different sst are summarised in Table 1.

Table 1. The major intracellular signalling pathways coupled to somatostatin receptors (sst).

Effector	sst_1	sst_2	sst_3	sst_4	sst_5
Inhibition of adenylyl cyclase	+	+	+	+	+
Inhibition of Ca^{2+} channels		+			
Activation of K^+ channels		+			
Activation of protein tyrosine phosphatase	+	+	+	+	
Inhibition of Na^+/H^+ exchange	+				
G-protein coupling	$G_i\alpha2$, $G_i\alpha3$	$G_i\alpha1$, $G_i\alpha3$, $G_0\alpha2$	$G_i\alpha1$		

after [61, 64, 66]

Regulation of somatostatin receptor activity: desensitisation and internalisation

The activity and availability of sst has been shown to be regulated by the availability of ligand. Prolonged treatment with somatostatin can cause receptor desensitisation [73]. This desensitisation could be due either to ligand-induced internalisation of the receptor and/or to desensitisation mechanisms such as phosphorylation of the receptor or uncoupling from G-proteins. Receptor desensitisation has been shown to be dependent on G-protein coupling in mouse lymphoma cells, which provides evidence against this last mechanism [74]. Receptor desensitisation has been linked to internalisation [75], but may also be mediated by receptor phosphorylation [76]. Evidence for agonist-induced internalisation of sst in

naturally sst-expressing and stably transfected cells has been provided at the electron and confocal microscopic level at least for sst_{2A} [77-79]. Receptor-mediated endocytosis of radioisotope-labelled somatostatin or somatostatin analogues has also been observed for sst_{2-5}, mainly in stably transfected cells [52, 80-82]. The cytoplasmic C-terminal domain of sst has been shown to contain sequence motifs which are important for the regulation of ligand-induced receptor internalisation [82-84]. Ligand-induced receptor internalisation has been shown to be only partially dependent on coupling to G-proteins [80, 81].

Ligand-induced up-regulation of membrane-expression of sst has also been observed [85, 86]. Using cell lines stably expressing human sst, Hukovic *et al.* [87] showed that prolonged agonist treatment led to up-regulation of the membrane expression of sst_1, sst_2 and sst_4. These authors showed that subtype selectivity for internalisation and up-regulation was inversely related, e.g. membrane expression of sst_1 was shown to be rapidly up-regulated in the presence of ligand, whereas this subtype showed only negligible ligand-induced internalisation. In the rat, the sst_4 subtype has been shown to be resistant to ligand-induced internalisation and desensitisation [84].

None of the peptide analogues bind exclusively to only one of the sst subtypes [55, 58, 88, 89], although new approaches might yield subtype-selective agonists or antagonists [60, 90-95].

Somatostatin receptors in the immune system

Somatostatin receptor expression can be detected by *in vivo* somatostatin receptor scintigraphy after injection of ^{111}In-labelled octreotide, a somatostatin analogue. This technique is used extensively for the localisation of neuroendocrine tumours and other malignancies that express high levels of sst [96]. Among the non-neuroendocrine tumours that can be visualised by octreotide scintigraphy are malignant lymphomas: both T and B non-Hodgkin lymphomas and Hodgkin disease lymphomas [97].

In a number of infectious diseases (e.g. tuberculosis), autoimmune diseases (e.g. Graves' ophthalmopathy) and other immune-mediated diseases (e.g. sarcoidosis and rheumatoid arthritis), the sites of inflammation can also be visualised [98-100]. In addition, octreotide scintigraphy labels the spleen and this labelling is reduced in patients pre-treated with unlabelled octreotide, indicating that the observed binding of the radioligand is receptor-mediated [101, 102].

Somatostatin receptor autoradiography, in which tissue sections are incubated with isotope-labelled somatostatin or somatostatin analogues, showed the presence of somatostatin binding sites in biopsies of malignant lymphomas [103]. Sst could also be localised by autoradiography in biopsies of inflamed tissue from patients with sarcoidosis, rheumatoid arthritis and inflammatory bowel disease. In sarcoidosis the granulomas were specifically labelled, whereas in rheumatoid arthritis and inflammatory bowel disease mainly the venules were labelled [99, 104, 105].

Additionally, binding sites were also identified by autoradiography in lymphoid organs of humans and mice. In the human thymus, the medulla was strongly labelled, whereas in the spleen the red pulp was diffusely labelled. In lymph nodes, tonsils, Peyer's patches and lymphoid follicles in the gut, the germinal centres of B cell follicles were preferentially labelled [102, 103, 106]. However, the resolution of this technique is too low to allow the identification of the specific cell types expressing sst in these tissues. Therefore, somatostatin binding studies on isolated immune cells were necessary.

Sst were identified on normal and activated leukocytes and on several lymphoid cell lines and leukaemic cells by classical receptor binding studies using fluorescence- or isotope-labelled somatostatin. These early studies have been reviewed by Van Hagen *et al.* [107]. In summary, it was found that resting human peripheral blood monocytes and lymphocytes expressed low-affinity receptors for somatostatin, whereas mitogen-activated cells and transformed and leukaemic cells expressed either low and high affinity receptors or high affinity receptors only. In our hands, we were unable to detect somatostatin receptors on human lymphocytes by flow cytometry measurements using fluorescently labelled somatostatin-28 (S. Oomen, unpublished observations). Murine B and T lymphocytes from spleen and Peyer's patches were also shown to express sst, as were lymphocytes isolated from the granulomas of mice infected with the parasitic helminth *Schistosoma mansoni*. Most of these studies were carried out using native somatostatin, thus giving no additional information about which of the five sst subtypes were expressed. Only recently a number of these early studies was revisited using molecular biological techniques in order to answer this question. Human lymphocytic leukaemia cells, lymphoid cell lines and peripheral blood mononuclear cells (PBMC) were found to express mainly sst_2 mRNA as analysed by RT-PCR. Sst_3 mRNA was also detected in all of the cell lines in this study. Sst_2 mRNA expression in PBMC, which was barely detectable in unstimulated cells, was found to be up-regulated upon stimulation with the lectin phytohaemagglutin (PHA) and after transformation with Epstein Barr virus [108]. The human Jurkat T cell line was shown to express only sst_3 mRNA [109]. Murine splenocytes, thymocytes, $CD4^+$ T cell lines, and $CD4^+$ T lymphocytes isolated from schistosome granulomas were also shown by RT-PCR to express sst_2 mRNA, although expression of sst_4 and sst_5 was not investigated in this study [110]. We ourselves recently showed that sst_3 and sst_4 are the main subtypes to be expressed at the mRNA level in rat splenocytes, thymocytes and lymph node cells [111], whereas Sedqi *et al.* [112] showed that cultured rat thymocytes expressed mRNA for sst_1 and sst_2. Sst_1 mRNA expression was up-regulated after stimulation with PHA and IL-1 in that study. Rat microglial cells, the resident macrophages of the central nervous system, were shown to express mRNA for sst_2, sst_3 and sst_4 [113].

Expression of sst mRNA does not necessarily mean that sst proteins are expressed on the surface of these cells. Therefore it is still not certain which sst subtypes are responsible for the binding of somatostatin to immune cells. New antisera able to subtype-specifically detect sst in tissue sections have recently been developed [65, 114]. To our knowledge, these antisera have not yet been used to study the distribution of the different sst subtypes in the immune system.

Sources of somatostatin within the immune microenvironment

The presence of sst on cells of the immune system implies that these cells are capable of showing a functional response to somatostatin. As somatostatin is rapidly degraded in the circulation, it is expected that the somatostatin to which these cells respond is produced locally, in the vicinity of sst-bearing cells.

Central and peripheral lymphoid organs have been shown to be innervated by nerves of the sympathetic nervous system and by sensory nerves containing neuropeptides such as somatostatin (for a review see [115]). Many other tissues are also innervated by peptidergic sensory nerves (unmyelinated C fibres and small myelinated Aδ fibres). Neuropeptides

have been shown to be released from the peripheral endings of such nerves during a local inflammatory response in these tissues [116, 117].

In endocrine organs and the gastrointestinal tract, somatostatin is produced by specialised neuroendocrine cells, where it may reach high concentrations locally [19, 20]. Neuropeptides which function as hormones in the gastrointestinal tract may regulate in a paracrine fashion the function of cells of the gut-associated lymphoid tissue and other immune cells present in this compartment [118, 119]. In addition, immune cells themselves have been shown to contain somatostatin [120-124], although the presence of mRNA for somatostatin within lymphoid organs and isolated immune cells has been shown in only a few cases [125, 126]. Among others, synovial cells isolated from the inflamed synovium of patients with rheumatoid arthritis [127], macrophages isolated from the murine thymus [128] and cells from murine schistosome granulomas [129] were shown to produce mRNA for somatostatin. Production of mRNA for somatostatin could be induced in splenocytes from normal and severe combined immunodeficient (SCID) mice by stimulation with cytokines [129]. In murine schistosome granulomas, which are not innervated, granuloma cells are believed to be the only local source of somatostatin [130]. Somatostatin produced by immune cells may act as an autocrine or paracrine regulator within the local immune microenvironment.

Effects of somatostatin on immune cell function and local inflammation

Numerous effects of somatostatin on immune cell functions have been described *in vitro* as well as *in vivo*. It is difficult to compare these studies, as experiments were carried out with cells from different animal species and under different experimental conditions. Often a so-called biphasic response was observed, i.e. inhibition of a certain response at low somatostatin concentrations (nM range) and stimulation of that same response at higher concentrations (μM range). This biphasic response appears to be characteristic for the interaction of neuropeptides with immune cells [131], and may have a physiological correlate: the lower concentrations (10^{-12}-10^{-10} M) are characteristic of levels of neuropeptides found in blood, whereas the higher concentrations (10^{-7}-10^{-6} M) may be found at nerve endings [132]. The differential effects may be due to activation of different receptor classes with high and low affinities for the ligand or to modulation of receptor number or activity.

Inhibition of proliferation

Inhibition of lymphoid cell proliferation is the best-documented effect of somatostatin on immune function. Van Hagen *et al.* [107] summarised the results of *in vitro* studies on the effect of somatostatin on the spontaneous or mitogen-induced proliferation of lymphoid cell lines and on cells isolated from lymphoid tissues of several animal species. Most of these studies involved mixed cell populations under T cell-stimulating conditions. The clearest inhibitory or biphasic effects of somatostatin were observed when purified T lymphocytes or T cell lines were used [133] or when sub-optimal stimulation conditions were used, e.g. allogeneic stimulation versus stimulation using concanavalin A (Con A) or lipopolysaccharide (LPS) [134, 135]. Most of the studies were carried out using native somatostatin, but in one study the octapeptide analogue BIM23014c was shown to inhibit murine lymphocyte proliferation to the same extent as native somatostatin [136], indicating that the effect was mediated by sst_2, sst_5 or possibly sst_3. A recent study showed that PHA-stimulated proliferation of human PBMC was inhibited by the octapeptide analogue octreotide and also by an analogue with enhanced selectivity for sst_5 [137]. Inhibition of proliferation

of cells other than T cells also possibly has a role in the modulation of inflammation by somatostatin [127].

The importance of endogenously produced somatostatin in regulating the proliferation of immune cells was shown in a very elegant set of experiments by Aguila *et al.* [138]. These authors showed that an antisense oligonucleotide designed to block translation of somatostatin mRNA stimulated the spontaneous proliferation of rat splenocytes *in vitro*.

Sst_2 is the sst subtype that is most often implicated in inhibition of cell proliferation by somatostatin, although inhibition of proliferation of human Jurkat T lymphocytes was shown to be mediated by sst_3 [109]. Activation of the sst_2 receptor has been shown to increase transcription of the somatostatin gene, thereby inducing an anti-proliferative autocrine feedback loop [139, 140], although this mechanism has not yet been shown to operate in cells of the immune system. The induction of somatostatin gene transcription was believed to be due primarily to activation of a protein tyrosine phosphatase (see Table 1). Induction of somatostatin mRNA by proinflammatory stimuli has been demonstrated in murine splenocytes [141]. Together these studies indicate that somatostatin has an important role in maintaining immune homeostasis.

Effects of somatostatin on T lymphocyte functions

Besides the inhibitory effect on proliferation, somatostatin has been shown to affect the production of cytokines by T lymphocytes. Somatostatin reduced the secretion of interferon-γ (IFN-γ) from human peripheral blood mononuclear cells (PBMC) [142]. Production of IFN-γ by splenocytes and T lymphocytes isolated from murine schistosome granulomas was decreased by somatostatin and octreotide *in vivo* and *in vitro* [143]. This effect was probably mediated by sst_2 as mRNA for this receptor subtype was shown to be expressed by granuloma T lymphocytes [110] and the effect was blocked by an antiserum directed against sst_2 [144]. In contrast, somatostatin enhanced the production of IL-2 by an ovalbumin-specific mouse T cell line [145] and the production of IL-2, IL-4, IL-10 and IFN-γ by murine T lymphocytes with distinct T helper phenotypes [146]. IL-2 secretion from mononuclear cells isolated from the human intestine was also enhanced by somatostatin in a dose-dependent manner [145]. The enhancement of IL-2 secretion from human Jurkat T lymphocytes has been shown to be mediated by sst_3 [109]. Production of IL-1β and tumour necrosis factor-α (TNF-α) by human whole blood was also stimulated by somatostatin [147]. However, T lymphocytes are not the only possible source of these cytokines.

The expression of activation markers on T lymphocytes can be modulated by somatostatin: spontaneous IL-2 receptor expression was shown to be increased after *in vivo* administration of octreotide in humans [148]. There is, however, some conflict with the results of Casnici *et al.* [135] who reported that the mitogen-induced expression of the IL-2 receptor α-chain (CD25) and the activation marker CD69 by human PBMC was decreased by somatostatin *in vitro*.

Controversial reports have been published concerning the possible enhancement of lymphocyte cytotoxicity by somatostatin, but it is not thought that this is an important mechanism in immune regulation by somatostatin [107]. An exciting new finding is that somatostatin stimulates adhesion of resting human T lymphocytes to fibronectin [149]. This may indicate a role for neuropeptides in the regulation of lymphocyte migration and recirculation.

In summary, somatostatin at physiological concentrations inhibits T lymphocyte

proliferation but may enhance a number of effector functions such as cytokine secretion and receptor expression.

Effects of somatostatin on B lymphocyte functions

Somatostatin has been shown to inhibit the production of immunoglobulins by B lymphocytes in a number of experimental settings. Somatostatin dose-dependently reduced the secretion of IgA from a murine plasmacytoma cell line [150] and from murine Con A-stimulated lymphocytes from spleen, Peyer's patches and mesenteric lymph nodes. The secretion of IgM and IgG was less affected [132]. The secretion of IgG2a but not IgG1 or IgM by splenocytes from *Schistosoma mansoni*-infected mice was also inhibited *in vitro* by somatostatin. *In vivo* treatment with octreotide completely abrogated IgG2a secretion but not IgG1 or IgM secretion from granuloma cells. These effects were thought to be due in part to the inhibitory effect of somatostatin on the secretion of IFN-γ from T lymphocytes, which was hypothesised to have an effect mainly on B cell activation, proliferation and differentiation [151]. *In vivo* treatment of rats diminished the number of antigen-specific plasma cells formed during a primary immune response [152], again pointing to an effect on B cell activation, proliferation and/or differentiation. *In vitro* differentiation of human peripheral blood B lymphocytes to plasma cells was shown to be reduced by octreotide, indicating the involvement of sst_2, sst_3 or sst_5 [153]. These findings correlate with the presence of binding sites for octreotide in the germinal centres of secondary lymphoid follicles [102, 103, 106], as they are sites of the generation of B memory cells.

Effects of somatostatin on the functions of monocytes and macrophages

Somatostatin has been shown to influence a number of activities of cells of the monocyte-macrophage lineage. Chemotaxis of human peripheral blood monocytes and of a murine macrophage cell line were blocked by somatostatin, although a strong dose-dependency of the effect was not observed [154, 155]. However, enhancement of chemotaxis by the somatostatin analogue octreotide has also been described [156]. In our hands, we found no significant inhibition of chemotaxis of human monocytes by either somatostatin or octreotide (unpublished observations).

There are also contradictory claims about the modulation by somatostatin of cytokine release from monocytes and macrophages. Komorowski and Stepien [157] showed that LPS-induced secretion of IL-1β and IL-6 from human peripheral blood monocytes was potentiated by somatostatin in nM concentrations, whereas Peluso *et al.* [158] showed that somatostatin decreased LPS-induced secretion of IL-1β, IL-6 and TNF-α from human monocytes. Enhancement of TNF-α production by rat peritoneal macrophages was shown at low concentrations (10^{-11} M) of somatostatin or octreotide, whereas production was reduced in the presence of higher concentrations (10^{-9}-10^{-5} M) of these peptides [159]. Somatostatin antagonised the substance P-induced enhancement of the secretion of IL-1, IL-6 and TNF-α from LPS-stimulated murine peritoneal macrophages [160].

Somatostatin and octreotide dose-dependently inhibited the respiratory burst in human monocytes [161]. Somatostatin also suppressed mediator release from rat Kupffer cells, i.e. hepatic macrophages [162]. So despite some contradictory results, somatostatin in general appears to suppress monocyte and macrophage effector functions.

Other effects of somatostatin on the immune microenvironment

Octreotide has been shown to be capable of inducing apoptosis in mouse pituitary cells and rat enterocytes [163, 164]. This effect has been linked to sst_3 in transfected cells [165]. Induction or facilitation of apoptosis may be an alternative mechanism for the observed anti-proliferative effects of octreotide and somatostatin, although this has not yet been demonstrated to occur in immune cells.

Sst are present on venules in inflamed tissues [104, 105]. Through effects on vasodilatation, somatostatin might influence the local blood flow and the migration of leukocytes into the inflamed tissue. Somatostatin has also been shown to inhibit angiogenesis and thus may have an influence on tissue remodelling [166, 167]. In addition, there is anecdotal evidence that octreotide may have an effect on the circulation of lymph fluid [168, 169].

In vivo effects of somatostatin in immune-mediated diseases

Many of the studies described above were performed *in vitro* using isolated cell populations. Therefore it is difficult to judge the relevance of these findings for the *in vivo* modulation of a local inflammatory response. In this section a number of studies investigating the effects of somatostatin on the modulation of autoimmune disease and chronic inflammation *in vivo* will be discussed.

The effect of somatostatin on local inflammatory processes has been studied in a subcutaneous air pouch in experimental animals. Aseptic inflammation in a rat air pouch was shown to be modulated by local or systemic administration of octreotide and BIM23014. Both somatostatin analogues dose-dependently reduced the volume of the exudate, the number of leukocytes and the levels of inflammatory mediators such as TNF-α and the neuropeptide substance P in the inflamed air pouch via either route of administration [170].

Murine schistosomiasis has been used as a model to study the role of somatostatin in the regulation of granulomatous inflammation. The hepatic schistosome granulomas were shown to contain a somatostatin immunoregulatory circuit [130]. Somatostatin is produced by granuloma macrophages. Granuloma $CD4^+$ T lymphocytes express sst_2. Somatostatin and octreotide markedly reduce the production of IFN-γ by granuloma T lymphocytes and this in turn decreases the secretion of IgG2a from B lymphocytes. Somatostatin antagonises the effects of substance P, another neuropeptide produced within the granuloma. Somatostatin has also been shown to antagonise the effects of substance P in a murine model of intestinal inflammation [171].

The role of somatostatin as an anti-inflammatory agent in rheumatoid arthritis has been studied extensively. Neurogenic inflammation, which is induced by the release of neuropeptides such as substance P from sensory nerves within the joint, is thought to play an important role in the induction and maintenance of joint inflammation. Somatostatin can be produced by synovial cells [127] or can be secreted from sensory nerve endings in the joint capsule. These data point to the existence of a somatostatin immunoregulatory circuit within the rheumatoid synovium. Neurogenic plasma extravasation can be inhibited by somatostatin, and this effect is possibly systemic [172, 173]. Intra-articular injection of somatostatin in patients with rheumatoid arthritis has been shown to reduce pain and synovial swelling [174, 175]. The analgesic effect of somatostatin may have been mediated by reduction of the hypersensitivity of afferent sensory nerves in the joint [176]. Sst have been identified associated with blood vessels in human rheumatoid synovium [104]. Somatostatin decreases the extravasation of plasma and leukocytes [170] and may have reduced synovial

swelling through this mechanism. Somatostatin may also have inhibited synovial vasculoneogenesis [166, 167] in these patients. Reduction of joint swelling by intra-articular injection of somatostatin was also observed in a rabbit model of chronic arthritis. In addition, histological studies in this model revealed reduction of lymphocyte infiltration of the synovium and reduced vasculitis in somatostatin-treated animals [177]. BIM23014 was shown to reduce the severity of adjuvant-induced arthritis in rats [178]. Recently, Takeba *et al.* [127] showed that somatostatin at physiological concentrations inhibited the *in vitro* proliferation of fibroblast-like synovial cells from rheumatoid arthritis patients. The stimulation-induced production of IL-6, IL-8 and matrix metalloproteinases by these cells was also reduced by somatostatin treatment *in vitro*. mRNA for sst_1 and sst_2 was expressed by cultured fibroblast-like synovial cells and sst_2 mRNA was sensitive to up-regulation by proinflammatory stimuli.

Systemic somatostatin or octreotide delayed the onset of autoimmune disease in rat experimental autoimmune encephalomyelitis and in murine autoimmune diabetes [179, 180], although it is not known whether these effects were due to an immunomodulatory mode of action of octreotide.

In conclusion, systemic or local treatment with somatostatin or somatostatin analogues has been shown to be beneficial in a number of models for autoimmune disease and chronic inflammation, but the mode of action is not clear in most cases.

Aim of the studies

We are interested in the potential for treatment of autoimmune diseases and chronic inflammatory diseases using the synthetic, metabolically stable somatostatin analogue octreotide. For the purpose of preclinical studies it is necessary to establish animal models for such diseases which mirror the human diseases as closely as possible. Because octreotide interacts only with the sst subtypes sst_2 and sst_5, and to a lesser extent sst_3 [89], it is important to establish that these sst subtypes are expressed in the immune system of the animal model of choice in a similar expression pattern to that found in the human immune system. Studies into the sst subtype expression in the immune system of rats and mice are described in chapters 2 and 3 respectively. As somatostatin has already been shown to be useful in the treatment of rheumatoid arthritis, and because receptors with high affinity for octreotide have been shown to be present in the inflamed joints of rheumatoid arthritis patients, we studied the effects of systemic administration of octreotide in rat and mouse models for arthritis. These studies are described in chapters 2 and 4.1. We also studied the effects of systemic octreotide on the development and progression of autoimmune disease in murine models for systemic lupus erythematosus (SLE) and multiple sclerosis (MS), as described in chapters 4.1 and 4.2.

Binding sites for octreotide have been demonstrated in the inflammatory lesions in human rheumatoid arthritis, human granulomatous diseases such as sarcoidosis and Wegener's granulomatosis, and in inflammatory bowel disease (IBD) by *in vitro* receptor autoradiography [99, 104, 105]. This indicates the presence of sst subtypes sst_2, sst_3 and/or sst_5. No subtype-specific analogues are yet available to narrow this down to one subtype. Furthermore, the resolution of the autoradiography technique is too low to identify the specific cell types expressing the receptors. In chapter 5 we describe studies in which we used a polyclonal rabbit antiserum directed against a peptide epitope in the intracellular C-terminal domain of sst_{2A} [65], to determine which cell types in the inflammatory lesions in

rheumatoid arthritis, sarcoidosis, vasculitis and inflammatory bowel disease express this receptor subtype.

The implications of the findings from these studies are discussed in chapter 6 together with suggestions for further research. An extension of the somatostatin-substance P immunoregulatory circuit as proposed by Elliott & Weinstock [130] is also presented, along with a hypothesis about the regulation of the default class of immune response in different organ systems.

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Chapter 2

SOMATOSTATIN RECEPTOR SUBTYPE EXPRESSION IN CELLS OF THE RAT IMMUNE SYSTEM DURING ADJUVANT ARTHRITIS

Somatostatin receptor subtype expression in cells of the rat immune system during adjuvant arthritis
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Chapter 2

SOMATOSTATIN RECEPTOR SUBTYPE EXPRESSION IN CELLS OF THE RAT IMMUNE SYSTEM DURING ADJUVANT ARTHRITIS

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Abstract

Somatostatin is a neuropeptide that is widely distributed throughout the body. It acts as a neurohormone and a neurotransmitter and may also have an immunomodulatory role. The genes for five subtypes of somatostatin receptors (sst) have been cloned, suggesting that the diverse effects of the peptide might be mediated by different receptors.

We are interested in studying the role of sst in inflammation, using an animal model. Because of the up-regulation of sst expression in inflamed joints in human rheumatoid arthritis, we chose rat adjuvant arthritis as an experimental model. In order to determine which of the sst might be important in immune modulation, subtype expression in leukocytes isolated from different lymphoid tissues of the rat was studied. Also, the expression levels of the most abundantly expressed sst mRNAs in leukocytes from spleen and blood were compared in rats with adjuvant arthritis and controls, using a semi-quantitative approach. Furthermore, the effect of systemic administration of a long-acting somatostatin analogue, octreotide, which binds selectively to sst subtypes 2 and 5 (sst_2 and sst_5), on the incidence and the severity of rat adjuvant arthritis was studied.

The main sst expressed in cells of the rat immune system, both resting and activated, were found to be sst_3 and sst_4. This contrasts with the human and murine situations, in which sst_2 appears to be the main subtype expressed in the immune system. No quantitative differences in sst subtype mRNA levels in leukocytes from spleen and blood were found between rats with adjuvant arthritis and controls. Finally, no effect of systemic administration of octreotide on either the incidence or severity of adjuvant arthritis in Lewis rats was found. As octreotide binds selectively to sst_2 and sst_5, the absence of an immunomodulatory effect of this analogue in rat adjuvant arthritis corroborates our finding that these sst subtypes are not expressed in cells of the rat immune system. In conclusion, cells of the rat immune system appear to express a spectrum of sst (sst_3 and sst_4) different from that found in human granulomatous and autoimmune disease (mainly sst_2). Therefore, the rat adjuvant arthritis model appears to be suitable only for studying the immunomodulatory effects of somatostatin analogues, which have a high affinity for sst_3 and sst_4, but not for studying the immunomodulatory effects of octreotide, which has a high affinity only for sst_2 and sst_5.

Introduction

Somatostatin is a neuropeptide that is widely distributed throughout the body. It was first identified as a growth hormone release-inhibiting factor synthesised in the hypothalamus. Outside the central nervous system, the peptide is present in a variety of endocrine and non-endocrine tissues, and it appears to have many functions. In the central nervous system it can act as a neurotransmitter and neurohormone, while in peripheral tissues it regulates endocrine and exocrine secretion and acts as a modulator of motor activity in the gastrointestinal tract [1, 2]. There is also increasing evidence that somatostatin can have immunomodulatory actions (for a review see [3]). Specific receptors (sst) for somatostatin have been identified in lymphoid tissues and on the surface of various white blood cells and cell lines by classic ligand binding studies [3]. In addition it was found, in a number of granulomatous (e.g. sarcoidosis) and autoimmune diseases (e.g. Graves' ophthalmopathy and rheumatoid arthritis), that sites of active inflammation could be visualised after the administration of an isotope-coupled somatostatin analogue [4, 5].

Since we are interested in studying the role of sst in such inflammatory diseases, we decided to use an animal model. Because of the up-regulation of sst expression in inflamed joints in human rheumatoid arthritis [5, 6], we specifically chose rat adjuvant arthritis as an experimental model (for a review, see [7]). The low numbers of leukocytes that can be obtained from the joints of rats with adjuvant arthritis, prohibit analysis of the sst subtypes expressed by these cells. However, as adjuvant arthritis is considered to be a systemic disease in which the causative agent disseminates throughout the tissues of the body [8-10], an experimental approach was set up to study the sst subtype expression in leukocytes isolated from lymphoid organs and peripheral blood of rats with adjuvant arthritis and controls. We also made a comparison of the expression levels of the most abundantly expressed sst mRNAs in leukocytes from spleen and blood of rats with adjuvant arthritis and controls, using a semi-quantitative approach. Furthermore, we studied the effect of systemic administration of a long-acting somatostatin analogue, octreotide, which binds specifically to sst subtypes 2 and 5 (sst_2 and sst_5), on the incidence and the severity of rat adjuvant arthritis, as somatostatin has been shown to have beneficial effects in human rheumatoid arthritis [11].

Materials and methods

Animals

Female Lewis rats (Harlan Sprague Dawley, Bicester, UK), 7-8 weeks of age, were used. The animals were kept under clean conventional conditions with free access to pelleted food and water in the animal facilities of the Department of Immunology. The experimental protocol was approved by the Animal Welfare Committee of the Erasmus University.

Induction of adjuvant arthritis

In order to induce adjuvant arthritis, rats were injected intradermally in the base of the tail with 1 mg of a suspension of heat-killed *Mycobacterium tuberculosis* strain H37 RA (Difco Laboratories, Detroit, MI, USA) in incomplete Freund's adjuvant (IFA) (Difco), prepared by grinding in a mortar, as described by Wauben *et al.* [7]. This preparation will be further referred to as complete Freund's adjuvant (CFA). Control rats were injected with IFA only.

Preparation of cell suspensions

In order to study the sst subtype expression in rats in an inflammatory versus a non-inflammatory state, adjuvant arthritis was induced in ten rats, as described above. Ten control rats were injected with IFA only. The rats were killed by decapitation 3 weeks p.i., at peak inflammation. Cell suspensions were prepared as described below for analysis by RT-PCR.

Peripheral blood

Peripheral blood was collected in polypropylene tubes containing 100 I.E. heparin (Leo Pharmaceutical Products BV, Weesp, The Netherlands). Peripheral blood mononuclear cells (PBMC) were purified by density centrifugation on Ficoll-Paque d=1.077 kg/l (Pharmacia, Uppsala, Sweden). PBMC from rats within one experimental group were pooled.

Lymphoid organ cell suspensions

Spleen, thymus and peripheral lymph nodes (popliteal, inguinal and axillary nodes) were removed from the animals and cell suspensions were prepared in RPMI 1640 medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% foetal calf serum (FCS) by pressing the organs through nylon gauze (mesh size 100 µm).

Erythrocytes were depleted from the spleen cell suspension by hypotonic lysis in a 10 mM $KHCO_3$ buffer (pH=7.4) supplemented with 155 mM NH_4Cl and 100 mM EDTA (3 ml per spleen). After 3 min at 4°C, the cells were washed twice in RPMI 1640 medium with 10% FCS. Half of the total lymph node suspension was used to prepare a non-adherent cell fraction by passage over nylon wool (Leuko-Pak, Fenwall Laboratories, Deerfield, IL, USA).

The different cell populations were pooled per experimental group. The cells were washed in ice-cold PBS, pelleted and stored as dry pellets at -80°C.

RNA isolation and cDNA reaction

PolyA$^+$ mRNA was isolated from the cells using paramagnetic oligo(dT) beads (Dynabeads Oligo$(dT)_{25}$, Dynal AS, Oslo, Norway) according to the manufacturers' instructions. The polyA$^+$ mRNA was eluted in 30 µl diethyl pyrocarbonate-treated water. All mRNA preparations were treated with deoxyribonuclease I (Amplification Grade, Gibco BRL, Grand Island, NY) for 15 min at room temperature in the buffer provided with the enzyme. The reaction was stopped by the addition of 2.5 mM EDTA followed by heating at 65°C for 10 min.

mRNA was extracted from 10^7 cells in the case of mixed cell populations (spleen, thymus, total lymph node cells) and $2x10^6$ cells in the case of more purified cell populations (PBMC and lymph node non-adherent cells).

Complementary DNA (cDNA) synthesis was performed starting with one third of the isolated mRNA. Random hexamer primers were used to ensure that all RNA was represented equally in the cDNA pool [12]. The reaction mixture contained 50 mM Tris-HCl buffer, pH=8.3, 10 mM $MgCl_2$, 50 mM KCl, 1 mM dithiotreitol, 1 mM EDTA, 10 µg/ml bovine serum albumin, 1 mM salmon spermine HCl (Sigma, St. Louis, MO, USA), 1 mM of each of the four deoxynucleotide triphosphates (DNA Polymerisation Mix, Pharmacia), 20 U of RNA Guard (Pharmacia) 0.02 OD $(dN)_6$ (Pharmacia), 0.2 µg oligo$(dT)_{12-18}$ (Boehringer Mannheim, Mannheim, Germany) and 5 U reverse transcriptase (from avian myeloblastoma virus, Promega, Madison, WI, USA) in a total reaction volume of 20 µl. The reaction mixture was incubated at 41°C for 1 hour and the reaction was stopped by heating at 75°C for 10 min. Each cDNA reaction was carried out in duplicate.

Controls

To ascertain that no genomic DNA was present in the polyA$^+$ preparations (which would give false-positive signals upon amplification, as sst genes do not contain introns), the cDNA reactions were also performed once without reverse transcriptase (negative control samples). These negative control samples never yielded positive signals on amplification.

cDNA from the GH3 rat pituitary cell line was used as a positive control for sst_1 mRNA expression and cDNA from the transplantable prolactin-secreting rat pituitary tumour 7315b as a positive control for sst_2 mRNA expression [13]. Rat genomic DNA, isolated by lysis of rat splenocytes in PCR buffer, was used as a positive control in the PCR reactions for sst_{3-5}.

The PCR reactions were optimised to detect at least 10^4 copies of the sequence of interest (corresponding to 0.1 µg genomic DNA) in ethidium bromide-stained gels. If in a particular series of PCR reactions, 10^4 copies of genomic DNA after amplification did not give a clear signal in ethidium bromide-stained gel, the series was discarded.

PCR

One tenth of the synthesised cDNA was used for each PCR reaction. The reaction mixture consisted of 10 mM Tris-HCl buffer, pH=9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatine, 0.1% (v/v) Triton X-100, 0.05 mM of each of the four deoxynucleotide triphosphates (DNA Polymerisation Mix; Pharmacia), 5 pmol each of the forward and reverse primers specific for one of the five rat sst subtypes or for the β-actin gene, and 1 U AmpliTaq DNA polymerase (Perkin-Elmer-Cetus, Norwalk, CT, USA) in a final reaction volume of 50 µl. PCR reactions for all primer pairs were performed with each of the duplicate cDNA samples and positive and negative control samples.

The primers specific for the human sst_1 and sst_2 as described by Wulfsen *et al.* [14] and for sst_{3-5} as described by Kubota *et al.* [15] were modified by us to match the rat sequences [16-21]. The rat β-actin primers were as described by Vidal *et al.* [22].

sst_1 forward 5'-ATGGTGGCCCTCAAGGCCGG-3'
sst_1 reverse 5'-GGCAGTGGCGTAGTAGTCAA-3'
(product size 318 bp);
sst_2 forward 5'-TCCTCTGGGATCCGAGTGGG-3'
sst_2 reverse 5'-TTGTCCTGCTTACTGTCGCT-3'
(product size 332 bp);
sst_3 forward 5'-TGCCAGTGGGTACAGGCACC-3'
sst_3 reverse 5'-CTGGAGGGCCAGACCCTGGC-3'
(product size 328 bp);
sst_4 forward 5'-TGCGGGCTGGCTGGCAACAA-3'
sst_4 reverse 5'-GTAGTCCAGGGGCTCTTCCT-3'
(product size 311 bp);
sst_5 forward 5'-AGCCTTCATCACCTACACGT-3'
sst_5 reverse 5'-GGCCAGGTTGACGATGTTGA-3'
(product size 226 bp);
β-actin forward 5'-TCATGCCATCCTGCGTCTGGACCT-3'
β-actin reverse 5'-CCGGACTCATCGTACTCCTGCTTG-3'
(product size 582 bp).

The reactions were carried out in a DNA thermal cycler (Perkin-Elmer-Cetus). After an initial denaturation for 5 min at 94°C, the samples were subjected to 35 cycles (β-actin) or 40 cycles (sst subtypes) of denaturation at 94°C for 1 min, annealing at 59°C (β-actin and sst_{1-4}) or 55°C (sst_5) for 2 min and extension at 72°C for 1 minute. These numbers of cycles had previously been shown to lie within the linear range of the reaction. After a final extension phase of 10 min at 72°C, 40 µl aliquots of the amplified products were analysed on 2% agarose gels stained with ethidium bromide. Weak bands were confirmed by hybridising Southern blots of the PCR products with subtype-specific oligonucleotide probes.

For semi-quantitative comparison of the amount of mRNA, a limiting dilution RT-PCR method was used [23]. Briefly, 2-fold serial dilutions of the cDNA preparations were normalised to give approximately equal β-actin signals, before performing PCR for the sst subtypes. The linearity of the dilution series was assessed by scanning the photographs of the ethidium bromide-stained gels and determining the intensity of the bands [24]. For the most accurate quantification, care was taken to include a dilution of which the β-actin signal was no longer visible when analysed on ethidium bromide-stained gel. Comparisons were based on this dilution and the two preceding it.

Receptor saturation curves

The radioligands used in the binding studies were [^{125}I-Tyr^3]-octreotide and [^{125}I-Tyr^0]-somatostatin-28. [Tyr^3]-octreotide was iodinated and HPLC-purified as described previously [25]. [^{125}I-Tyr^0]-somatostatin-28 was purchased from ARC-Biotrend (Cologne, Germany). Reaction conditions were the same as described by Reubi [26]. Briefly, cell membrane preparations (corresponding to 15-30 µg protein) were incubated at room temperature for 60 min with increasing concentrations of radioligand in a total volume of 100 µl HEPES buffer (10 mM HEPES, 5 mM $MgCl_2$ and 0.2 g/l Bacitracin, pH=7.6) containing 0.2% (w/v) bovine serum albumin (BSA). After the incubation, 1 ml of ice cold HEPES buffer was added to the assay mixture and membrane-bound radioactivity was separated from unbound activity by centrifugation (2 min 10000x*g* in an Eppendorf centrifuge). The remaining pellet was washed twice with ice-cold HEPES buffer and radioactivity remaining in the final pellet was counted in a gamma counter (LKB Wallac, Finland). Specific binding was taken to be the total bound activity minus the activity bound in the presence of 1 µM unlabelled octreotide (Sandostatin; Novartis Pharma, Basel, Switzerland) or somatostatin-28 (Bachem, Bubendorf, Switzerland), respectively. As a positive control for binding, cell membranes from the mouse pituitary cell line AtT20 were included in each test.

Systemic administration of octreotide and monitoring of arthritis

The effect of systemic administration of octreotide, a long-acting somatostatin analogue, on adjuvant arthritis in the rat was studied. Sandostatin-LAR, a slow-release formulation of octreotide, was used. This formulation gives a continuous release of octreotide for a period of six weeks after subcutaneous injection.

One day prior to the induction of arthritis, ten rats were injected subcutaneously in the dorsal neck region with a suspension of Sandostatin-LAR (a gift from Novartis Pharma) in standard injection vehicle (buffered sodium carboxyl-methylcellulose), corresponding to 10 (experiments 1 and 2) or 30 mg/kg octreotide (experiment 3). Ten control rats were injected with standard injection vehicle only. The next day adjuvant arthritis was induced in all the rats as described above. Disease course was monitored twice weekly for six weeks

by measuring the diameter of all four paws using industrial callipers. The animals were weighed once a week and their general appearance and behaviour were monitored to assess their condition. Blood was collected by orbital puncture on days 21 and 42 of the experiment. The rats were killed by CO_2-asphyxiation on day 42.

Results

With a view to using rat adjuvant arthritis as an animal model in which to study the role of sst in inflammation, we determined the expression pattern of the five known sst subtypes in the major lymphoid organs of the rat, using RT-PCR.

In order to study mainly leukocytes, stromal components were removed from the lymphoid organs under study by passing them through nylon gauze. Other authors have studied only whole organs of the rat [27, 28].

Table 1. Sst subtype mRNAs in cells of the rat immune system: summary of RT-PCR study.

	sst_1	sst_2	sst_3	sst_4	sst_5
Source of leukocytes					
Spleen	±	-	+	+	-
Peripheral blood	-	-	+	+	-
Thymus	-	-	+	±	-
Lymph nodes	-	-	+	±	-
Lymph nodes (non-adherent cells)	-	-	+	±	-

+, visible on ethidium bromide-stained agarose gel; ±, not visible, but visualised by Southern blotting.

The results of the RT-PCR studies are shown in Table 1. The major sst subtypes expressed in rat leukocytes were found to be sst_3 and sst_4. A very weak sst_1 signal was observed in spleen cells in some but not all experiments. No expression of sst_2 or sst_5 was observed in any of the cell populations, even after a repeat amplification. A ladder pattern of amplification products was consistently observed in the sst_4 PCR of thymocytes, but hybridisation of a Southern blot of the PCR-gel with a sst_4-specific probe yielded only a single band of the expected size. The sst4-specific band for the total lymph node and lymph node non-adherent cell preparations was barely visible in the ethidium bromide-stained gel, but could be visualised by Southern blot hybridisation with a sst_4-specific probe. The pattern of expression of sst in rat leukocytes was confirmed in splenocytes from other strains of rats (females: Wistar, Brown Norway, Wag/Rij, Sprague-Dawley, Buffalo and Fisher F344). Again only sst_3 and sst_4 were expressed, with occasionally a very weak sst_1 signal (not shown).

Receptor saturation curves

Receptor saturation experiments were carried out on membrane preparations of splenocytes and thymocytes of control Lewis rats using the radioligands [^{125}I-Tyr^0]-somatostatin-28 and [^{125}I-Tyr^3]-octreotide. K_d values and receptor densities were determined by Scatchard analysis of the saturation curves.

No membrane binding of [^{125}I-Tyr$_3$]-octreotide was found (not shown), but [^{125}I-Tyr^0]-somatostatin-28 bound with a K_d of 0.5 and 0.3 nM for splenocytes and thymocytes, respectively. As a control for binding, sst positive AtT20 mouse pituitary tumour cells were used (K_d 0.9 nM). The calculated numbers of binding sites were 755, 492 and 2679 fmol/mg protein for splenocytes, thymocytes and AtT20 cells, respectively. Figure 1 shows the Scatchard plots for the binding of [^{125}I-Tyr^0]-somatostatin-28.

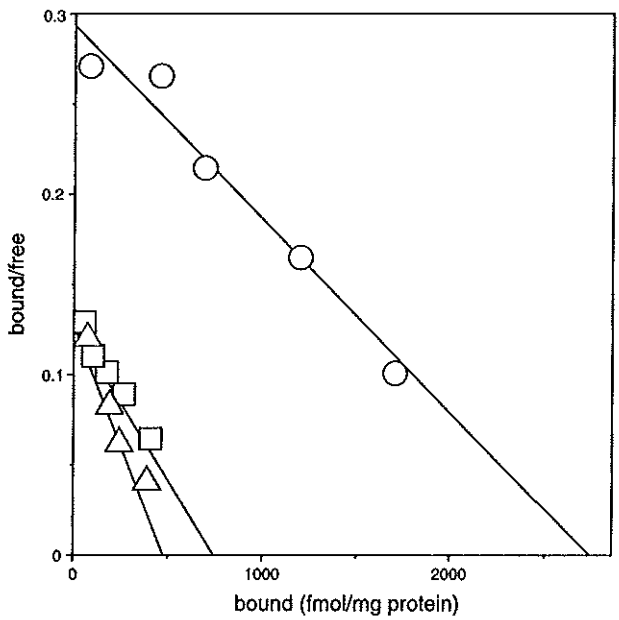

Figure 1. Sst expression at the protein level in rat splenocytes and thymocytes. Scatchard analysis of [^{125}I-Tyr^0]-somatostatin-28 binding to Lewis rat splenocytes and thymocytes and the sst-positive mouse pituitary tumour cell line AtT20. O AtT20 cells (K_d=0.9nM, n=2679 fmol/mg), □ Lewis splenocytes (Kd=0.5nM, n=755 fmol/mg), Δ Lewis thymocytes (K_d=0.3 nM, n=492 fmol/mg).

Semi-quantitative comparison of sst$_3$ expression

Limiting dilution RT-PCR was performed to semi-quantitatively compare sst$_3$ mRNA levels in splenocytes from CFA-injected rats with adjuvant arthritis (further referred to as CFA-injected rats) and controls. Two-fold serial dilutions of cDNA were titrated to give approximately equal β-actin signals and were then subjected to PCR for sst$_3$. The results are shown in Figure 2. No substantial differences in the amount of sst$_3$ mRNA in CFA-injected versus control rats could be demonstrated.

Differential expression of sst$_4$ mRNA in leukocyte sub-populations

Sst$_4$ was expressed at levels readily detectable on ethidium bromide-stained gel only in splenocytes and peripheral blood, whereas expression in the other lymphoid cell preparations was only detectable after Southern blotting. This was true both for CFA-injected rats and control rats. Peripheral blood and spleen, as well as peripheral lymph nodes harbour large numbers of activated cells during systemic inflammation. However, spleen and peripheral blood contain a relatively larger proportion of monocytes in addition to their lymphocytes. The difference observed in sst$_4$ expression levels between peripheral blood

and splenocytes on the one hand and lymph nodes on the other hand, suggested that sst_4 might be expressed by monocytes and cells of the monocyte lineage such as dendritic cells.

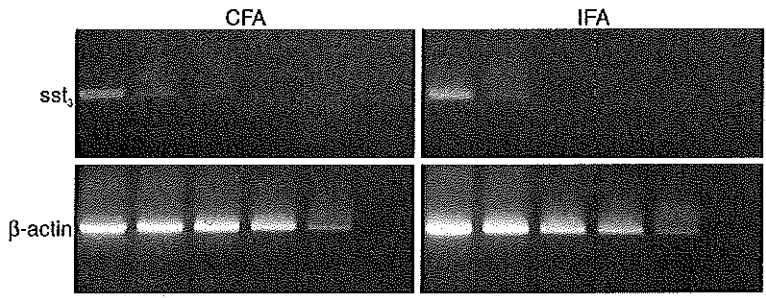

Figure 2. Sst_3 mRNA expression levels in rats with adjuvant arthritis and controls. For semi-quantitative comparison of sst_3 mRNA expression in splenocytes of Lewis rats with adjuvant arthritis (CFA-injected) versus controls (IFA-injected), serial two-fold dilutions of cDNA were subjected to PCR for β-actin and sst_3.

A cell population enriched for dendritic cells, isolated from the spleen of a normal Lewis rat was found to express sst_4 mRNA (not shown). We therefore set out to prepare, from peripheral blood, a cell population enriched for monocytes, using density centrifugation over Percoll. Sst_3 and sst_4 were expressed in the unseparated population and also in both populations obtained after density centrifugation (not shown). The cellular composition of the different populations is summarised in Table 2.

Table 2. Cellular composition of the peripheral blood cell populations before and after Percoll density centrifugation (as determined by nuclear morphology).

	CFA			IFA		
	PMN	Lymphocyte	Monocyte	PMN	Lymphocyte	Monocyte
PBMC	7	91	2	12	80	8
Percoll pellet	9	85	5	2	90	8
Percoll interphase	4	78	18	4	80	16

PMN: polymorphonuclear.

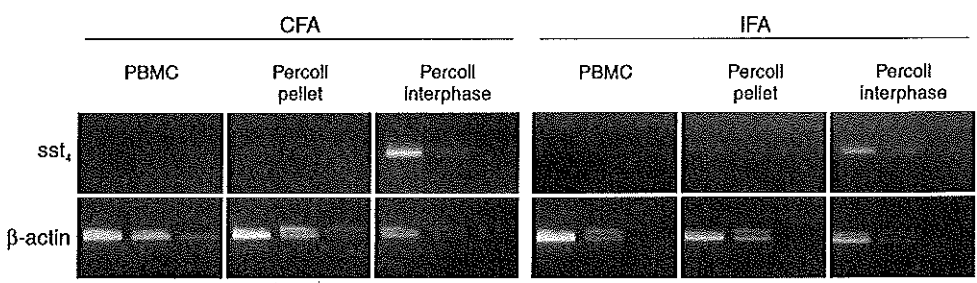

Figure 3. Increased expression of sst_4 mRNA in a population of rat peripheral blood cells enriched for monocytes. Sub-populations of peripheral blood mononuclear cells (PBMC) from Lewis rats with adjuvant arthritis (CFA-injected) and controls (IFA-injected) were prepared by density centrifugation over Percoll. For semi-quantitative comparison of sst_4 mRNA expression levels, serial two-fold dilutions of cDNA were subjected to PCR for β-actin and sst_4. The cDNA samples in the β-actin panel of the figure are diluted 8-fold as compared to the corresponding samples in the sst_4 panel.

Limiting dilution RT-PCR was carried out as described above to semi-quantitatively compare the sst_4 mRNA expression in the different populations. The results are shown in Figure 3. It was shown that the interphase cell population had a relatively higher sst_4 expression than either the pellet population or total PBMCs. This was true for both CFA-injected and control rats.

The effect of systemic administration of octreotide on adjuvant arthritis

We studied the effect of systemic administration of a pharmacological somatostatin analogue, octreotide, on the incidence and the severity of rat adjuvant arthritis, as somatostatin has been shown to have beneficial effects in human rheumatoid arthritis [11]. Sandostatin-LAR was used in three separate experiments (see Table 3). The data show that neither a low dose (10 mg/kg) nor a higher dose (30 mg/kg) of Sandostatin-LAR had an effect on incidence and severity of the arthritis.

Table 3. Effect of systemic administration of octreotide (as Sandostatin-LAR) on the incidence and severity of adjuvant arthritis in Lewis rats

	Incidence			Severity*		
	Sandostatin-LAR	Controls	P-value**	Sandostatin-LAR	Controls	P-value**
Octreotide dose (mg/kg)						
10 (experiment 1)	7/9	10/10	0.21	38.1 ± 10.9	59.2 ± 14.1	0.29
10 (experiment 2)	8/10	7/10	0.50	58.3 ± 12.4	57.4 ± 12.4	0.97
30 (experiment 3)	8/9	10/10	0.47	105.0 ± 18.3	116.3 ± 10.2	0.81

* Severity expressed as mean maximal increase in total paw diameter (in 0.1 mm) ± SEM

** Statistics: Incidence was compared using a Fisher exact test. Severity was compared using a Mann-Whitney test (two-sided).

Arthritis was confirmed histologically. Marginal erosion of the bone and hyperplastic synovium extending into the joint cavity were observed with an infiltrate consisting mainly of lymphocytes, polymorphonuclear cells and histiocytes.

In the first experiment the octreotide levels in the serum were determined on days 21 and 42. The mean concentrations were 2.353 ± 0.641 ng/ml and 1.524 ± 0.744 ng/ml, respectively for the octreotide-treated group, and undetectable (< 0.1 ng/ml) for the control group. Nine rats out of ten still had detectable serum levels of octreotide on day 42. These data show that the octreotide was released from the subcutaneous depot continuously throughout the course of the experiment.

Discussion

Somatostatin is a ubiquitous neuropeptide with diverse physiological functions. The recent cloning of a family of sst and the demonstration that these receptors differ in their affinities for synthetic somatostatin analogues [29], suggests that the diverse actions of somatostatin might be mediated by different receptor subtypes.

We are interested in the immunomodulatory effects of somatostatin and somatostatin analogues. Because of the up-regulation of sst expression in inflamed joints in human rheumatoid arthritis, we chose rat adjuvant arthritis as an experimental model. In order to determine which of the sst subtypes might be important in immune modulation, we studied the sst subtype expression in lymphoid tissues and cells of the rat, using RT-PCR.

The main sst expressed in cells of the rat immune system, both resting and activated, were found to be sst_3 and sst_4. This contrasts with the situation in humans and mice, in

which sst_2 appears to be the main subtype expressed in the immune system ([3, 30] and unpublished observations).

Furthermore, our results indicate that sst_4 is expressed by distinct sub-populations of rat leukocytes. A peripheral blood cell fraction enriched for monocytes showed an increased sst_4 expression. However, we were not able to obtain a highly purified rat monocyte population using density centrifugation. Flowcytometric analysis suggests that, in contrast to what is seen in humans, rat peripheral blood monocytes exhibit a wide range of sizes and densities, and cannot be identified as a separate population in forward-sideward-scatter plots ([31] and unpublished observations). The low-density cell population also contains activated lymphocytes (lymphoblasts), leaving open the possibility that sst_4 mRNA is up-regulated upon activation. However, we found no quantitative differences in sst subtype mRNA levels between CFA-injected rats with adjuvant arthritis and control rats. This observation again seems in contrast with the situation in the human immune system in which binding sites for somatostatin seem to be preferentially expressed on activated leukocytes [3].

Our results on the sst subtype expression in rat splenocytes contrast with the expression patterns found by others in intact rat spleen. Raulf *et al.* [28] detected sst_3 mRNA but not sst_4 mRNA in rat spleen and lymph nodes. These authors also found a weak sst_5 signal in the spleen. However, this expression might be due to the presence of stromal cells, e.g. nerves, which are largely eliminated in our procedure of making cell suspensions. Using a highly sensitive nuclease protection assay, Bruno *et al.* [27] found mRNA for all five sst subtypes in rat spleen. It was clear from their results also that sst_3 was the main subtype in spleen, whereas the other four subtypes showed much lower expression. Our results on the sst subtype expression in rat thymocytes contrast with the findings of Sedqi *et al.* [32], who showed transcripts for sst_2 in resting and activated thymocytes and sst_1 in activated thymocytes, but who did not detect mRNA for sst_3. A contributing factor to the major discrepancy in sst_2 expression reported in the two papers, could be the fact that Sedqi *et al.* analysed cells from male rats, whereas we used female rats on account of their susceptibility to adjuvant arthritis. It is known that the expression of sst_2 can be influenced by estrogens; e.g. the transplantable rat prolactin-secreting pituitary tumour 7315b expresses sst_2 when grown *in vitro*, but loses this expression when grown *in vivo* under the influence of circulating estrogens [13]. However, the difference in expression of sst_3 as determined in the study of Sedqi *et al.* and in this study needs further investigation.

At the protein level, no binding of the sst_2- and sst_5-selective radioligand [^{125}I-Tyr^3]-octreotide was found. This result was in accordance with the lack of sst_2 and sst_5 expression, as found by RT-PCR. Other studies have generally found a good correlation between the presence of octreotide-binding sites and the presence of sst_2 and/or sst_5 mRNA [33]. The radioligand [^{125}I-Tyr^9]-somatostatin-28 was bound, demonstrating the presence of other subtypes of sst. We therefore conclude that the mRNA for sst_3 and sst_4, which was detected by RT-PCR, is functional, and can give rise to membrane expression of sst proteins in rat leukocytes.

Finally, no therapeutic effect of systemic administration of octreotide, in the form of Sandostatin-LAR, was found on the incidence and severity of adjuvant arthritis in Lewis rats. This may be due to the absence in the immune system of the rat of the sst subtypes sst_2 and sst_5, which have a high affinity for this octapeptide somatostatin analogue. The absence of octreotide-binding sites *in vitro* is correlated with the absence of *in vivo* biochemical response to octreotide, at least in the case of hormone-secreting tumours [34]. Using a different somatostatin analogue, BIM23014 (1 μg/hour; the doses we used correspond to

2.5 and 7.5 µg octreotide/hour), Rees *et al.* [35] did find a statistically significant decrease in arthritis severity, although a different measure of severity was used. The effect cannot be explained by differences in binding profiles of octreotide and BIM23014 [29] on the distinct sst subtypes (although to our knowledge no comparisons of the binding profiles of these compounds on the rat sst subtypes are available). Alternatively, the somatostatin analogues might not have acted directly on the immune cells in these studies, but might have had a more generalised effect through neuroendocrine mechanisms. However, no differences were found in the efficacies of octreotide and BIM23014 in inhibiting the release of several hormones [29, 36].

In human rheumatoid arthritis the inflamed joints may be visualised by *in vivo* scintigraphy using the sst_2- and sst_5-selective somatostatin analogue [^{111}In-DTPA-D-Phe^1]-octreotide [5]. In rat adjuvant arthritis the affected joints could not be visualised using this analogue [37], indicating that the cells of the inflammatory infiltrate in the joint also lack the sst subtypes (sst_2 and sst_5) which have a high affinity for octreotide. We recently showed the presence of sst_{2A} in endothelial cells and cells of the monocyte/macrophage lineage in human rheumatoid synovium (ten Bokum *et al.*, submitted).

In conclusion, cells of the rat immune system appear to express a spectrum of sst (sst_3 and sst_4) different from that found in human granulomatous and autoimmune diseases [4, 5]. Therefore, the rat adjuvant arthritis model appears to be suitable only for studying the immunomodulatory effects of somatostatin analogues, which have a high affinity for sst_3 and sst_4, but not for studying the immunomodulatory effects of octreotide, which has a high affinity only for sst_2 and sst_5.

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Chapter 3

SOMATOSTATIN RECEPTOR SUBTYPE EXPRESSION IN THE IMMUNE SYSTEM OF NORMAL MICE

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SOMATOSTATIN RECEPTOR SUBTYPE EXPRESSION IN THE IMMUNE SYSTEM OF NORMAL MICE

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Abstract

Somatostatin is a 14 amino acid neuropeptide with diverse functions throughout the body. Somatostatin and synthetic, metabolically stable analogues (i.e. the octapeptide analogue octreotide) have been shown to have immunomodulatory effects. In a number of granulomatous and autoimmune diseases, sites of active inflammation can be visualised by the administration of isotope-coupled octreotide. The functional significance of the presence of somatostatin receptors in inflammatory lesions is unknown. We want to establish an animal model to investigate this.

Most of the animal models for immunological diseases are murine. Therefore we determined the presence and tissue distribution of somatostatin receptors in the mouse, with emphasis on the immune system, using four different methods: *in vivo* tissue distribution of radioisotope-labelled octreotide, *in vitro* somatostatin receptor autoradiography, RT-PCR and immunohistochemistry.

By *in vivo* and *in vitro* receptor binding we found receptors for octreotide in the thymus but not in the spleen or lymph nodes of normal mice. By RT-PCR we detected mRNA for sst_4 in peripheral blood mononuclear cells and splenocytes, but not in lymph node cells or thymocytes. mRNA for sst_2 was detected in thymocytes only.

These results differ from what has been found for humans. Studies using sorted subpopulations of human and murine immune cells will be necessary to establish whether the human and murine immune systems are comparable with respect to sst subtype expression and modulation of function by somatostatin and somatostatin analogues. This may have important consequences for future animal studies of modulation of immune function by somatostatin.

Introduction

Somatostatin is a 14 amino acid neuropeptide that is widely distributed throughout the body. It was first identified as a growth hormone release-inhibiting factor synthesised in the hypothalamus [1]. The genes for five G-protein-coupled somatostatin receptors (sst_{1-5}) have been cloned (for a review see [2]). Outside the central nervous system, somatostatin is present in a variety of endocrine and non-endocrine tissues. In the central nervous system it can act as a neurotransmitter and neurohormone, while in peripheral tissues it regulates endocrine and exocrine secretion and acts as a modulator of motor activity in the gastrointestinal tract [3, 4].

Somatostatin and synthetic, metabolically stable analogues (e.g. the octapeptide analogue octreotide) have been shown to have a suppressive effect on a number of immune functions (for a review see [5]). Specific receptors for somatostatin have been identified in lymphoid tissues and on the surface of various white blood cells and cell lines by classic ligand binding studies [5]. In addition it was found, in a number of granulomatous (e.g. sarcoidosis) and autoimmune diseases (e.g. Graves' ophthalmopathy and rheumatoid arthritis), that sites of active inflammation could be visualised by the administration of an isotope-coupled somatostatin analogue [6, 7].

The functional significance of the presence of somatostatin receptors in inflammatory lesions is unknown. In order to further explore this issue and to determine the potential of somatostatin and metabolically stable analogues in the treatment of immune-mediated diseases, we are interested in establishing an animal model in which such studies could be performed. Most of the animal models for immunological diseases are murine. However, not much is known about the presence and tissue distribution of somatostatin receptors in the mouse, as the majority of animal studies on somatostatin and somatostatin receptors have focussed on the rat (the model of choice for endocrinological studies). Therefore, in this study we wished to determine the presence and tissue distribution of somatostatin receptors in the mouse, with emphasis on the immune system, using four different methods: *in vivo* tissue distribution of radioisotope-labelled octreotide, somatostatin receptor autoradiography on tissue sections, RT-PCR and immunohistochemistry.

Materials and methods

Animals

Female and male BALB/c mice, 10 weeks of age, were used. The animals were bred and maintained under SPF-conditions with free access to gamma-irradiated pelleted food (Hope Farms, Woerden, The Netherlands) and water acidified to pH 2.2 in the animal facilities of the Department of Immunology. In some experiments female and male non-obese diabetic-severe combined immunodeficient (NOD/*scid*) mice were used as well. These mice were bred and maintained in cages with filter tops. The experimental protocol was approved by the Animal Welfare Committee of the Erasmus University.

Radiolabelling of [DTPA-D-Phe^1]octreotide

[DTPA-D-Phe^1]octreotide and $^{111}InCl_3$ (DRN 4901, 370 MBq/ml in HCl, pH 1.5-1.9) were obtained from Mallinckrodt (Petten, The Netherlands). [DTPA-D-Phe^1]octreotide was labelled as described previously [8]. Thirty min after the start of radiolabelling the labelling efficiency was higher than 98%. Quality control was performed as described previously [8].

In vivo tissue distribution of radioactively labelled octreotide

Mice were injected intravenously (i.v., into the tail vein) with 1 MBq (0.1 μg) of [^{111}In-DTPA-D-Phe^1]octreotide. This had been previously determined to be the optimal mass for receptor binding in the mouse (unpublished observations). To determine the specificity of binding, mice were injected with 1 MBq (0.1 μg) [^{111}In-DTPA-D-Phe^1]octreotide and 100 μg unlabelled octreotide (Novartis Pharma, Basel, Switzerland). The volume of injection was kept constant at 0.2 ml per mouse. The mice were sacrificed 24 h after injection by CO_2-asphyxiation. Peripheral blood was collected and the organs of interest were removed, weighed using a digital analytical balance and collected into separate polyurethane tubes.

Tissue and blood radioactivity were determined using an LKB-1282-Compu-gammasystem. Specific binding was defined as the difference between the tissue uptake of radioactivity in mice injected only with [^{111}In-DTPA-D-Phe^1]octreotide (total binding) and that in animals coinjected with excess unlabelled peptide (non-specific binding), and expressed as % of the injected radioactivity per gram of tissue as described previously [8]. Statistical analysis was performed using Student's *t*-test. A P value of ≤ 0.05 was considered statistically significant.

Autoradiography

The presence of somatostatin receptors in lymphoid organs of female and male BALB/c mice was investigated by autoradiography on unfixed cryosections using [^{125}I-Tyr^3]octreotide, which was prepared as described previously [9]. Briefly, mice were sacrificed by CO_2-asphyxiation and the organs of interest were removed as quickly as possible. The organs were embedded in TissueTek (Miles Inc., Elkhart, IN, USA) and processed for cryosectioning. Ten µm thick tissue sections were mounted on gelatine-coated glass slides and stored at -20 °C for at least 3 days to improve adhesion of the tissue to the slide. Sections were air-dried, preincubated in 170 mM Tris-HCl buffer, pH 7.6 for 10 min at room temperature (RT) and then incubated for 60 min at RT with the iodinated ligand (3.0 MBq/ml, about 250 pM). The incubation solution was 170 mM Tris-HCl buffer, pH 7.6 containing 1% (w/v) bovine serum albumin (BSA), 40 µg/ml bacitracin (as a peptidase inhibitor; Merck, Darmstadt, Germany) and 5 mM $MgCl_2$. Non-specific binding was determined by incubating a sequential section in the presence of 1 µM of unlabeled octreotide (Novartis Pharma). After incubation, the sections were washed twice for 5 min in cold incubation buffer containing 0.25 % BSA, then in buffer alone and dried quickly. The sections were exposed to ^{3}H-Ultrafilms (Amersham, Little Chalfont, UK) for 3 weeks in X-ray cassettes. Mouse brain cortex was used as a positive control tissue.

Preparation of cell suspensions: peripheral blood

Female mice were killed by CO_2-asphyxiation and peripheral blood was collected by cardiac punction into polypropylene tubes containing 100 I.E. heparin (Leo Pharmaceutical Products BV, Weesp, The Netherlands). Peripheral blood mononuclear cells (PBMC) were purified by density centrifugation over Histopaque 1115, density = 1.115 kg/l (Sigma Diagnostics, St. Louis, MO, USA).

PBMC from 5 mice were pooled and aliquotted into portions of $5x10^6$ cells. Each aliquot of cells was lysed in 500 µl lysis-binding buffer (100 mM Tris-HCl pH 8.0, 500 mM LiCl, 10 mM EDTA, 1% (w/v) LiDS, 5 mM dithiotreitol (DTT)) and stored at -80°C.

Lymphoid organ cell suspensions

Spleen, thymus and peripheral lymph nodes (popliteal, inguinal, axillary and mesenteric nodes) were removed from the mice and cell suspensions were prepared in RPMI 1640 medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% foetal calf serum (FCS) by pressing the organs through nylon gauze (mesh size 100 µm).

Erythrocytes were depleted from the spleen cell suspension by hypotonic lysis in a 10 mM $KHCO_3$ buffer (pH 7.4) supplemented with 155 mM NH_4Cl and 100 mM EDTA. After 3 min on ice, the cells were washed twice in RPMI 1640 medium with 10% FCS.

Cells from 5 mice were pooled and aliquotted into portions of $5x10^6$ cells. Each aliquot of cells was lysed in 500 µl lysis-binding buffer and stored at -80°C.

Preparation of whole organs for RNA isolation
Organs of interest were collected into polypropylene tubes and snap-frozen in liquid nitrogen. Organs from 5 mice were pooled and ground to powder while frozen using a mortar and pestle. Tissue powder was stored in RNase-free containers at –80 °C. Approximately 10 mg of tissue powder was used for RNA isolation.

RNA isolation and cDNA reaction
Ten mg of frozen tissue powder or $5x10^6$ cells were lysed in 500 µl lysis-binding buffer and $polyA^+$ mRNA was isolated using paramagnetic oligo(dT) beads (Dynabeads $Oligo(dT)_{25}$, Dynal AS, Oslo, Norway) according to the manufacturer's instructions. In order to eliminate contaminating genomic DNA, the mRNA was eluted from the beads at 65°C into 100 µl 2 mM EDTA pH 8.0, mixed with an equal volume of 2x binding buffer (20 mM Tris-HCl pH 8.0, 1 M LiCl, 2 mM EDTA, 0.4% (w/v) LiDS) and reapplied to fresh Dynabeads.

After washing, an anchored complementary DNA (cDNA) synthesis reaction was carried out on the mRNA attached to the beads. The reaction mixture contained 25 mM Tris-HCl pH 8.3 at 42°C, 50 mM KCl, 2 mM DTT, 5 mM $MgCl_2$, 1 mM of each of the four deoxynucleotide triphosphates (DNA Polymerisation Mix, Pharmacia, Uppsala, Sweden), 3 U RNA Guard (Pharmacia) and 4 U SUPER RT reverse transcriptase (HT Biotechnology Ltd, Cambridge, UK), in a total reaction volume of 40 µl. For half of the mixture (negative control sample), the reverse transcriptase enzyme was replaced by enzyme storage buffer (HT Biotechnology).

PCR
One-twentieth of the synthesised cDNA was used for each PCR reaction. The reaction mixture consisted of PCR buffer (PCR buffer II, PE Biosystems, Foster City, CT, USA), 1.5 mM $MgCl_2$, 0.05 mM of each of the four deoxynucleotide triphosphates (DNA Polymerisation Mix, Pharmacia), 5 pmol each of the forward and reverse primers specific for one of the five mouse sst subtypes and 1 U Taq Gold DNA polymerase (PE Biosystems) in a final reaction volume of 50 µl. Negative control samples (cDNA reactions performed without reverse transcriptase and water controls) and mouse genomic DNA as product size control were always included in the same PCR series alongside the samples of interest.

The primers were based on the mouse sst cDNA sequences as published by Yamada *et al.* (sst_1 and sst_2) [10], Yasuda *et al.* (sst_3) [11], Schwabe *et al.* (sst_4) [12] and Lublin *et al.* (sst_5) [13]. The β-actin primers were as described by Vidal *et al.* [14].

sst_1 forward 5'-ATGGTGGCCCTCAAGGCTGG-3'
sst_1 reverse 5'-GGCAGTG GCATAGTAGTCGA-3'
(product size 318 bp);
sst_2 forward 5'-TCCTCTGGAATCC GAGTGGG-3'
sst_2 reverse 5'-TTGTCCTGCTTACTGTCGCT-3'
(product size 332 bp)
sst_3 forward 5'-TGTCAGTGGGTACAGGCACC-3'
sst_3 reverse 5'-CTGGAGGTCCCGACCCT GGC-3'
(product size 327 bp);
sst_4 forward 5'-TGCGC GGTGGCTGGCAACAA-3'
sst_4 reverse 5'-GTAGTCCAGGGGCTCTTCCT-3'
(product size 311 bp);

sst_5 forward 5'-CA GCCTTCATCACTTACACAT-3'
sst_5 reverse 5'-GGCCAGGTTGACGATG TTGA-3'
(product size 226 bp);
β-actin forward 5'-TCATGCCATCCTGCGTCTGGACCT-3'
β-actin reverse 5'-CCGGACTCATCGTACTCCTGCTTG-3'
(product size 582 bp).

The reactions were carried out in a DNA thermal cycler (Perkin Elmer Cetus, Norwalk, CT). After an initial denaturation for 10 min at 94°C, the samples were subjected to 30 cycles (β-actin), 35 cycles (sst_{1-4}) or 37 cycles (sst_5) of denaturation at 94°C for 1 min, annealing at 59°C (β-actin and sst_{1-4}) or 55°C (sst_5) for 2 min and extension at 72°C for 1 minute. These numbers of cycles had previously been shown to lie within the linear range of the reaction. After a final extension phase of 10 min at 72°C, 40 µl aliquots of the amplified products were analysed on 2% agarose gels stained with ethidium bromide.

Immunohistochemistry
Three female mice were killed by CO_2 asphyxiation and the organs of interest were removed. The organs were fixed in 4% buffered formaldehyde and embedded in paraffin. Four µm thick sections were mounted on glass slides coated with 3-amino propyl tri-ethoxy silane (APES; Sigma, St. Louis, MO). The sections were deparaffinised, rehydrated and boiled for 5 min in citric acid buffer (100 mM) using a microwave oven. Immunohistochemistry was performed according to standard methods using a polyclonal rabbit antiserum directed against a peptide in the intracellular C-terminal domain of sst_{2A} (Biotrend Chemicals GmbH, Cologne, Germany) [15] with horseradish peroxidase (HRP)-conjugate swine-anti-rabbit immunoglobulin (Dakopatts, Glostrup, Denmark) as the secondary antibody and HRP-conjugated rabbit-anti-swine immunoglobulin (Dakopatts) as the tertiary antibody. HRP activity was revealed with H_2O_2 with nickel-diamino benzidine, which gives a black stain, as the chromogen. The sections were lightly counterstained using Nuclear Fast Red, dehydrated and embedded in Entellan (Merck). Controls for immunohistochemistry included: 1) omission of the primary antiserum, 2) pre-absorption of the sst_{2A} antiserum with the immunising peptide (3 µg/ml).

Results

In vivo tissue distribution of radioactively labelled octreotide
Octreotide is a metabolically stable cyclic octapeptide analogue of somatostatin with high affinity for sst subtypes sst_2, sst_5, and to a lesser extent sst_3 [16]. In order to determine *in vivo* tissue distribution of these sst subtypes in normal mice, we injected healthy BALB/c mice with [^{111}In-DTPA-D-Phe^1]octreotide, an analogue that is used clinically for *in vivo* somatostatin receptor scintigraphy. We determined uptake of radioactivity in control tissues (blood, skeletal muscle), in the pituitary as a classical sst-positive tissue, and in spleen and thymus. Lymph nodes were not included for technical reasons (not enough tissue to perform accurate measurements of uptake of radioactivity). Uptake of radioactivity was determined 24 h after injection. Total binding (expressed in % of the injected dose (% ID) per g tissue) is summarised in Table 1; specific binding, i.e. after subtraction of binding in the presence of excess unlabelled octreotide, is summarised in Table 2.

Table 1. Tissue distribution of octreotide binding in BALB/c mice (% injected dose per g tissue, mean ± S.D.).

Tissue	Males (n=4)	Females (n=5)
Blood	0.01 ± 0.002	0.02 ± 0.003
Skeletal muscle	0.03 ± 0.02	0.01 ± 0.00
Pituitary	1.6 ± 0.5	2.1 ± 0.6
Spleen	0.1 ± 0.02	0.1 ± 0.01
Thymus	1.4 ± 0.4	1.0 ± 0.1

Total uptake of radioactivity in the absence of unlabelled octreotide 24 h after intravenous administration of 1 MBq (0.1 μg) [^{111}In-DTPA-D-Phe^{1}]octreotide.

Table 2. Specific octreotide binding in tissues of BALB/c mice (% injected dose per g tissue, mean ± S.D.).

Tissue	Males (n=4)	Females (n=5)
Blood	-0.0003 ± 0.002	0.0003 ± 0.005
Skeletal muscle	0.01 ± 0.02	-0.008 ± 0.003
Pituitary	1.4 ± 0.5 *	1.6 ± 0.6 *
Spleen	-0.01 ± 0.03	0.02 ± 0.02
Thymus	1.2 ± 0.5 *	1.0 ± 0.09 *

* $P \leq 0.05$; *specific binding significantly larger than zero*

Specific binding 24 h after intravenous administration of 1 MBq (0.1 μg) [^{111}In-DTPA-D-Phe^{1}]octreotide.

Specific uptake of radioactivity was observed in the pituitary and the thymus, but not in the spleen. No statistically significant differences were found between male and female mice. A repeat experiment confirmed these results. Specific uptake of radioactivity was also observed in the thymus of NOD/*scid* mice (0.3 ± 0.1 % ID/ g, not shown).

Autoradiography

In order to determine the distribution of octreotide binding sites within lymphoid tissues of the mouse, we performed autoradiography on tissue sections from male and female mice (both n=4) using [^{125}I-Tyr^{3}]octreotide. Mouse brain was used as a positive control tissue. Specific binding was found in the tissues that also showed specific uptake of [^{111}In-DTPA-D-Phe^{1}]octreotide in the *in vivo* study described above, i.e. the thymus and the pituitary, and in mouse brain. Specific binding was also observed in the thymus of NOD/*scid* mice. Autoradiograms showing the pattern of binding are shown in Figure 1. Specific binding to pituitary was homogeneous over the entire tissue, but of low intensity (not shown). No specific binding was observed in lymph nodes (not shown) and spleen. In the thymus of normal BALB/c mice, high affinity specific binding sites were seen at the border of cortex and medulla, as shown in Figure 2. No differences in binding patterns were seen between male and female mice (not shown). Therefore in subsequent experiments only female mice were used.

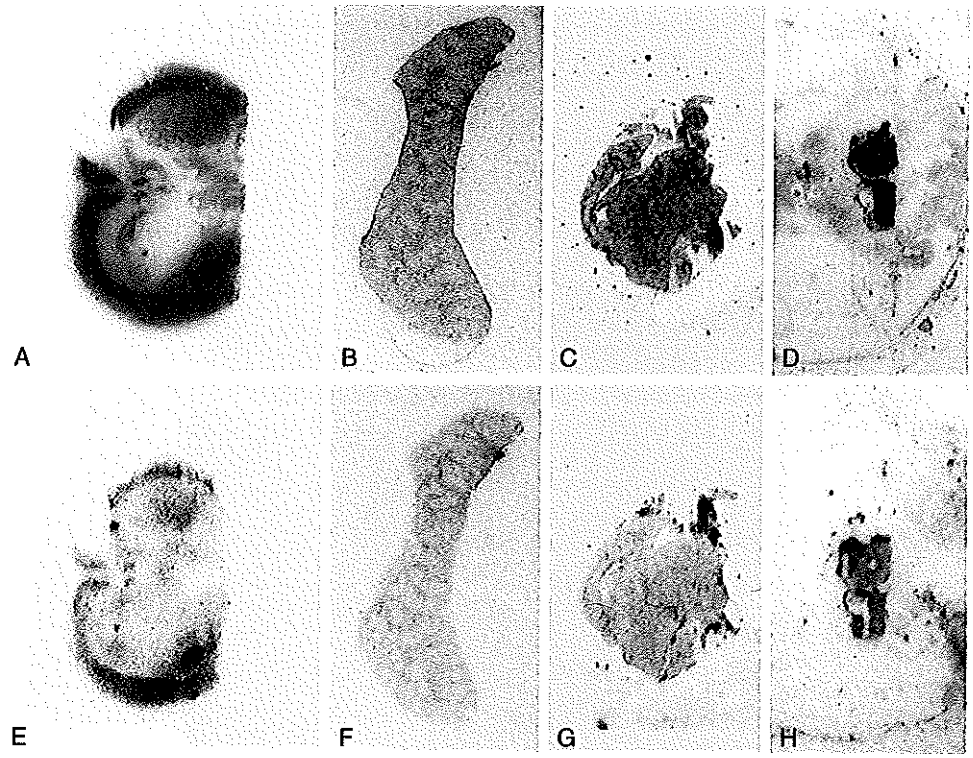

Figure 1. Somatostatin binding sites in murine tissues. A-D Autoradiograms showing total binding of [^{125}I-Tyr^3]-octreotide. E-H Autoradiograms showing non-specific binding of [^{125}I-Tyr^3]-octreotide in the presence of 1 μM octreotide. A, E Brain; B, F Spleen; C, G Thymus; D, H Thymus of an NOD/*scid* mouse.

RT-PCR

In order to determine which sst subtypes are expressed in the different tissues of the mice, we performed RT-PCR on mRNA extracted from tissue homogenates of mouse brain cortex and lymphoid organs and suspensions of cells from lymphoid organs, which consisted mainly of leukocytes.

The PCR reactions were optimised using mouse genomic DNA, to detect 10^2 copies of sst cDNA(corresponding to 1 ng genomic DNA). This was deemed an appropriate detection limit, as at a combined efficiency of mRNA isolation and cDNA reaction of 10%, each PCR reaction would contain cDNA equivalent to mRNA of approximately 10^4 cells (starting from $5x10^6$ cells). Therefore, a detection limit of 10^2 copies would enable the detection of 1 copy of sst mRNA in 1% of the cells under study.

To ascertain that no genomic DNA was present in the polyA$^+$ preparations (which would give false-positive signals upon amplification, as sst genes do not contain introns), the cDNA reactions were also performed without reverse transcriptase. These negative control samples never yielded positive signals on amplification.

The results of the RT-PCR are summarised in Table 3 and Figure 3. Pituitary (not shown) and brain cortex expressed mRNA for all 5 sst subtypes. No expression of sst_3 or sst_5 mRNA was found in any of the lymphoid organs studied. Sst_1 mRNA was detected in lymph node tissue,

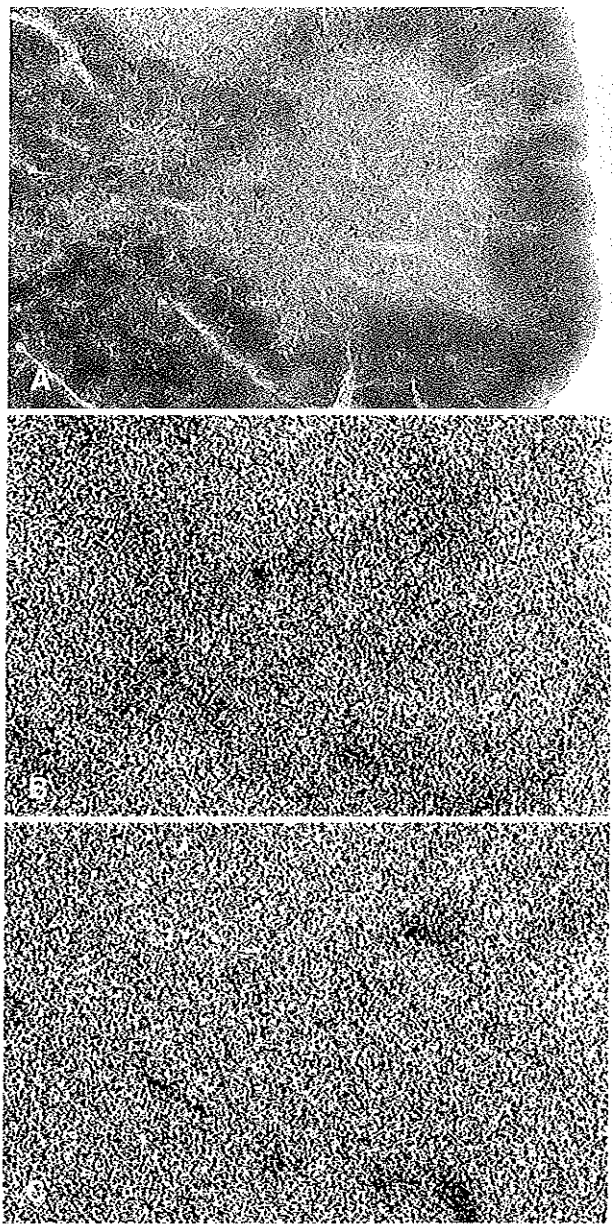

Figure 2. Somatostatin binding sites in the murine thymus. Enlargement to show the pattern of binding. A. Haematoxylin-eosin stained section. B. Autoradiogram showing total binding of [^{125}I-Tyr^3]-octreotide. C. Autoradiogram showing non-specific binding of [^{125}I-Tyr^3]-octreotide in the presence of 1 μM octreotide.

spleen tissue and thymus tissue, but not in isolated cells from these organs. Sst_2 mRNA was found in thymus tissue and isolated thymus cells (thymocytes), while PBMC, spleen tissue and isolated spleen cells (splenocytes) expressed sst_4 mRNA.

Table 3. Expression of sst subtype mRNA in mouse tissues and cells, with emphasis on the immune system.

Tissue	sst_1	sst_2	sst_3	sst_4	sst_5
Brain cortex	+	+	+	+	+
PBMC	-	-	-	+	-
Lymph node cells	-	-	-	-	-
Spleen cells	-	-	-	+	-
Thymus cells	-	+	-	-	-
Lymph nodes	+	-	-	-	-
Spleen	+	-	-	+	-
Thymus	+	+	-	-	-

Summary of RT-PCR results after amplification of 1/40 of the cDNA obtained from 10 mg tissue or $5x10^6$ cells.
(PBMC = peripheral blood mononuclear cells)

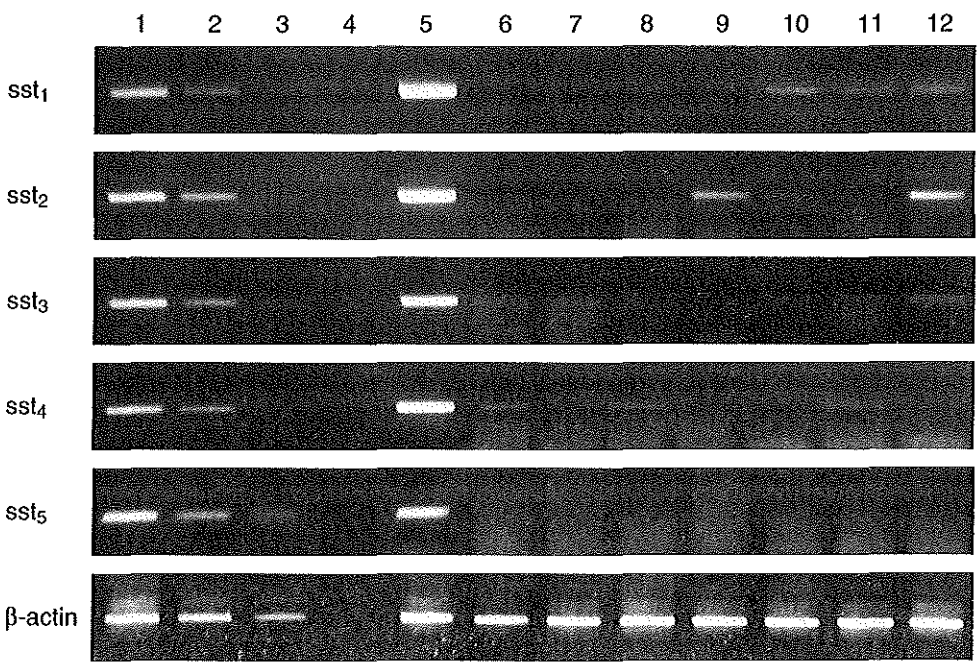

Figure 3. RT-PCR study of the expression of mRNA for somatostatin receptor subtypes 1-5 (sst_{1-5}) in mouse lymphoid organs and cells suspensions. Lane 1: genomic DNA 10^3 copies (β-actin box: 10^5 copies); lane 2: genomic DNA 10^2 copies (β-actin box; 10^4 copies); lane 3: genomic DNA 10 copies (β-actin box: 10^3 copies); lane 4: water; lane 5: brain cortex; lane 6: peripheral blood mononuclear cells (PBMC); lane 7: lymph node cell suspension; lane 8: spleen cell suspension; lane 9: thymus cell suspension; lane 10: whole lymph node; lane 11: whole spleen; lane 12: whole thymus.

Immunohistochemistry
Using a polyclonal rabbit serum directed against a peptide sequence in the intracellular C-terminal domain of sst_{2A} [15], we carried out an immunohistochemical study of the expression of this sst subtype in formaldehyde-fixed, paraffin-embedded mouse lymphoid organs. Paraffin-embedded mouse brain was used as a positive control tissue. High expression of sst_{2A} was found in the brain, in the expected pattern [17]. No specific staining could be observed in spleen and lymph nodes, nor in the thymus.

Discussion

We studied the presence and tissue distribution of sst subtypes in the normal mouse using four different methods. Specific uptake of radioisotope (^{111}In) labelled octreotide (a somatostatin analogue with high affinity for sst_2 and sst_5) could be seen in the pituitary, a classical sst-positive organ (1.3 % ID/g). However, by far the most interesting finding was the high specific uptake by the murine thymus (1.2 % ID/g). No specific uptake of ^{111}In-labelled octreotide was seen in the spleen. The latter is in agreement with the findings of Stolz *et al.* [18], but these authors did not study uptake in the thymus. These findings seem different from what is observed in humans, in whom the spleen shows high specific uptake of ^{111}In-labelled octreotide that can be blocked *in vivo* by unlabelled octreotide [19]. In contrast, the human thymus shows no uptake of ^{111}In-labelled octreotide (although the number of adolescents and children that have been studied is small).

By autoradiography we found high affinity binding sites for [^{125}I-Tyr^3]-octreotide at the border of the thymic cortex and medulla. This area contains a large number of blood vessels and is the site where precursor T lymphocytes from the bone marrow enter the thymus. The binding observed may be due to the presence of sst on these blood vessels. Somatostatin has been shown to decrease plasma extravasation. It is possible that somatostatin, through interaction with sst on thymic blood vessels, might influence migration of immature thymocytes into the murine thymus. Alternatively, the binding may be due to sst on precursor T lymphocytes. The fact that homogeneous binding of [^{125}I-Tyr^3]octreotide was observed in thymic sections from NOD/*scid* mice, supports the latter possibility. Mice with the severe combined immunodeficiency (SCID) phenotype do not have mature T lymphocytes due to a differentiation block at the early thymocyte stage ($CD44^-CD25^+$ double negative cells; W. van Ewijk, personal communication), and the thymus of these mice does not contain a structured medulla. In the human thymus, autoradiography using radioiodinated octreotide demonstrated the presence of sst in a different region, i.e. the thymic medulla [20].

No specific binding could be detected in murine spleen and lymph nodes. In humans however, these organs show a high expression of octreotide binding sites [20, 21].

By RT-PCR we evaluated the expression of mRNA for the 5 sst subtypes in murine tissues. Pituitary (not shown) and brain cortex expressed mRNA for all 5 subtypes. RT-PCR of rat brain and pituitary also showed that all five receptor subtypes were expressed simultaneously in these organs [22]. Sst_1 expression was found in homogenates of all lymphoid tissue studied, but not in isolated cells prepared from these tissues. This indicates that sst_1 mRNA is expressed by cells in the stromal compartment. Additionally, we found only sst_2 mRNA in murine whole thymus and isolated thymocytes. This is one of the sst subtypes that has a high affinity for octreotide, and therefore the expression of mRNA for this sst subtype correlates with the *in vivo* and *in vitro* binding of radioactively labelled octreotide

in the murine thymus. On the basis of the autoradiography of the thymus of normal and SCID mice we speculate that only a sub-population of thymocytes, i.e. the most immature thymocytes ($CD4^-CD8^-$ double negative), that have only recently entered the thymus, express sst_2. A thymic cell suspension prepared by pressing the organ through nylon gauze typically contains 95-99% thymocytes. Of these, in normal mice, 85% are $CD4^+CD8^+$ double positive cortical thymocytes, 10-12% are single positive mature thymocytes found in the medulla and only 1-3% are the most immature double negative thymocytes, whereas in SCID mice almost all thymocytes have this immature phenotype. In light of this hypothesis it would be interesting to evaluate by RT-PCR the expression of mRNA for sst subtypes in sorted populations of thymocytes. Sst_2 mRNA was also detected by RT-PCR in precursor cell populations in the murine bone marrow (S. Oomen, unpublished observations).

Using an antiserum directed against sst_{2A} we tried to resolve which cells in the murine thymus expressed this subtype. However, we could not detect any staining in the thymus or in the other lymphoid organs. Mouse brain, the control tissue, did show peptide-displaceable staining with the antiserum, indicating that the antiserum can detect sst_{2A} in murine tissues. The discrepancy between the results of *in vivo* receptor binding and *in vitro* autoradiography studies of the thymus on the one hand and immunohistochemistry on the other hand, needs to be further investigated. One possible explanation is that sst_{2B} is preferentially expressed in the murine thymus. However, Elliott *et al.* [23] did not find major differences in expression levels of mRNA for sst_{2A} and sst_{2B} in murine thymocytes by RT-PCR. Another possibility is that formaldehyde-fixed lymphoid tissues are unsuitable for immunohistochemical analysis of sst_{2A} expression. Using another antiserum, we have previously detected sst_{2A} in human immune cells in formaldehyde-fixed, paraffin-embedded tissues [24]. The staining was found to be mainly intracellular. We do not know whether the antiserum used in the present study is capable of binding to intracellular forms of sst_{2A}.

Lymph nodes and spleen did not show specific binding of radioactively labelled octreotide *in vivo* or *in vitro* in this study. In accordance with this observation, these organs and cell suspensions prepared from them, did not express mRNA for the sst subtypes associated with octreotide binding (sst_2, sst_3 or sst_5). Expression of mRNA for the non-octreotide binding subtype sst_4 was observed in PBMC, spleen tissue and in isolated splenocytes in this study. The fact that mRNA for this subtype was not detected in lymph nodes or thymus, leads us to speculate that sst_4 mRNA is expressed by cells present only in circulating blood (and as a consequence also in the spleen), i.e. granulocytes or monocytes. Schwabe *et al.* [12], however, did not detect expression of sst_4 mRNA in murine spleen by RT-PCR, but no comparisons are possible between the detection limits in our study and that of Schwabe *et al.* Preliminary RT-PCR data from mice in which the sst_2 gene has been inactivated, also showed expression of mRNA for sst_1 and sst_4 in the spleen (unpublished observations). We cannot rule out strain differences or differences in the activation state of the immune system as a cause for the discrepancies.

Elliott *et al.* [23] showed that sst_2 mRNA is expressed in the murine splenocytes and thymocytes and in purified T lymphocytes and T lymphocyte cell lines. In the present study we did not detect sst_2 expression in splenocytes from normal BALB/c mice. Elliott *et al.* studied female CBA mice, so the discrepancies may be due to strain differences. In the present study, we used a realistic detection limit of 10^2 copies of the sequence of interest. We hypothesise that in normal mice the sst_2 mRNA expression in the spleen is below this detection limit and may only become detectable after immunological stimulation. In support of this hypothesis, we were able to detect sst_2 mRNA by RT-PCR in splenocytes from

MRL/*lpr* mice, which suffer from lymphoproliferation and generalised autoimmune disease, thus representing a fully activated immune system (results not shown).

It appears that sst subtype expression in the immune system differs between closely related species. We recently showed by RT-PCR that in the rat immune system, sst_3 and sst_4 were the main subtypes to be expressed [25]. In the human immune system, sst_2 appears to be the main sst subtype [5]. We recently identified expression of sst_{2A} in macrophages and monocytes in inflamed synovium from patients with rheumatoid arthritis [24]. In the human thymus, sst_2 mRNA was found to be expressed by thymic epithelial cells, but not by cultured thymocytes [26]. However, a number of human T cell leukaemia cell lines with an immature ($CD3^-$) phenotype and a number of B cell plasmacytomas were also shown to express sst_2 mRNA [27]. Human Epstein Barr virus-transformed B cells also express sst_2 mRNA ([28] and E.G.R. Lichtenauer-Kaligis, unpublished observations).

The expression of sst_2 mRNA in human $CD3^-$ T cell leukaemia cell lines may parallel the expression of sst_2 mRNA by a sub-population of murine thymocytes as found in this study. However, no human parallel has yet been identified for the sst_4 expression by murine PBMC and splenocytes. These findings have important consequences for future animal studies. Further studies using sorted sub-populations of human and murine immune cells will be necessary to establish whether the human and murine immune systems are comparable with respect to sst subtype expression and modulation of function by somatostatin and somatostatin analogues.

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Chapter 4

MODULATION OF AUTOIMMUNE DISEASE AND CHRONIC INFLAMMATION IN MICE USING A LONG-ACTING SOMATOSTATIN ANALOGUE

Modulation of autoimmune disease and chronic inflammation in mice using a long-acting somatostatin analogue

4.1 Treatment of arthritis and systemic lupus erythematosus in MRL/*lpr* mice with a slow-release formulation of the somatostatin analogue octreotide.

4.2 Treatment of murine autoimmune encephalomyelitis with a slow-release formulation of the somatostatin analogue octreotide.

Chapter 4.1

TREATMENT OF ARTHRITIS AND SYSTEMIC LUPUS ERYTHEMATOSUS IN MRL/*lpr* MICE WITH A SLOW-RELEASE FORMULATION OF THE SOMATOSTATIN ANALOGUE OCTREOTIDE

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Abstract

Somatostatin is a neuropeptide that is widely distributed throughout the body. It acts as a neurohormone and a neurotransmitter and may also have an immunomodulatory role. The genes for five subtypes of somatostatin receptors have been cloned, suggesting that the diverse effects of the peptide might be mediated by different receptors. Octreotide is a metabolically stable cyclic octapeptide analogue of somatostatin, used in the clinic, with high affinity for sst subtypes sst_2, sst_3 and sst_5. A slow-release formulation (LAR) of octreotide has recently been developed. We studied the effects of octreotide treatment in a murine model of T cell-mediated autoimmune disease. The MRL/*lpr* mouse strain develops a rheumatoid arthritis-like polyarthritis and a syndrome reminiscent of human systemic lupus erythematosus (SLE). We studied the effects of treatment with two different doses of slow-release octreotide on the arthritis, the immune glomerulonephritis and the concentration of autoantibodies, in the MRL/*lpr* mouse. We found no significant differences in severity of arthritis or glomerulonephritis between the treatment groups and controls. However, the highest dose of treatment did give a slight decrease in the serum titre of anti-dsDNA autoantibodies in these mice. On the basis of these experiments, we cannot reliably conclude whether treatment with octreotide-LAR is able to down-modulate murine spontaneous arthritis and SLE. It is hypothesised that a short course of octreotide by injection may have a stronger effect than slow release of octreotide from a subcutaneous depot, as prolonged treatment with somatostatin might cause agonist-induced receptor desensitisation.

Introduction

Somatostatin is a hypothalamic neuropeptide with diverse functions. It was first identified as a growth hormone release-inhibiting factor synthesised in the hypothalamus. It can also function as a neurotransmitter and as a paracrine regulator of endocrine and exocrine secretion [1, 2].

Somatostatin and synthetic, metabolically stable analogues have also been shown to have a suppressive effect on a number of immune functions (for a review see [3]). Specific receptors for somatostatin have been identified in lymphoid tissues and on the surface of various types of white blood cells and cell lines by classic ligand binding studies [3]. In

addition it was found that sites of active inflammation in a number of granulomatous (e.g. sarcoidosis) and autoimmune diseases (e.g. Graves' ophthalmopathy and rheumatoid arthritis), could be visualised after the administration of an isotope-coupled somatostatin analogue, [^{111}In-DTPA^{0}]-octreotide (octreotide scintigraphy) [4, 5].

The genes for five G-protein-coupled somatostatin receptor subtypes (sst_{1-5}) have been cloned (for a review see [6]). Octreotide is a metabolically stable cyclic octapeptide analogue of somatostatin with high affinity for sst subtypes sst_2, sst_5, and to a lesser extent sst_3 [7]. Recently, a slow-release formulation of octreotide (octreotide-LAR) has been developed, which gives a continuous release of octreotide for a period of 6 weeks after subcutaneous (s.c.) or intramuscular (i.m.) injection [8].

We are interested in studying the effects of octreotide treatment on autoimmune and other immune-mediated diseases. Thereby we wish to focus on the diseases in which the inflammatory lesions may be visualised by octreotide scintigraphy, although it is not clear in all cases which cells in the lesions are the target for octreotide binding. Because of the up-regulation of somatostatin receptor expression in inflamed joints in human rheumatoid arthritis [5, 9], we initially chose rat adjuvant arthritis as an experimental model. However, we showed that cells of the rat immune system express a different spectrum of somatostatin receptors (sst_3 and sst_4) than those found in human granulomatous and autoimmune disease (mainly sst_2). Also, we found no effects of treatment with Octreotide-LAR on the incidence or the severity of arthritis in this model [10].

Because sst_2 has been shown to be expressed in the murine immune system ([11] and unpublished observations), we subsequently chose to study the effects of octreotide treatment in a murine arthritis model. The MRL/*lpr* mouse strain develops a rheumatoid arthritis-like polyarthritis and a syndrome reminiscent of human systemic lupus erythematosus (SLE), characterised by immune glomerulonephritis, a high frequency of lupus-associated autoantibodies and high levels of circulating immune complexes [12]. Lupus-associated arthritis in humans can be visualised by administration of radioactively labelled octreotide [13]. Furthermore, somatostatin has been shown to decrease secretion of antibodies from B cells and plasmacytoma cell lines *in vitro* [14-16]. Therefore, we decided to study the effects of treatment with slow-release octreotide on the arthritis, the immune glomerulonephritis and the concentration of autoantibodies, in the MRL/*lpr* mouse.

Materials and methods

Animals

Female MRL/*lpr* mice, 12 weeks of age, were used in the experiments. This is the age at which clinical signs of arthritis usually become manifest [12]. The mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA) at 3 weeks of age and maintained under SPF conditions (filtertop cages) in the animal facilities of the department of Immunology. The animals had free access to gamma-irradiated pelleted food (Hope Farms, Woerden, The Netherlands) and water acidified to pH 2.2.

Treatment with octreotide-LAR

At the start of the experiment, mice were injected s.c. in the dorsal neck region with a suspension of octreotide-LAR, a slow-release formulation of the somatostatin analogue octreotide (a gift from Novartis Pharma) in standard injection vehicle (buffered sodium carboxyl-methylcellulose) or with injection vehicle only. The dosages of Octreotide-LAR

in the two treatment groups corresponded to 10 mg/kg octreotide and 30 mg/kg octreotide, respectively.

Monitoring of arthritis

The mice were observed for 4 weeks after the start of the experiment. Every other day, the mice were weighed and the arthritis was scored using a clinical scoring scale developed for monitoring collagen-induced arthritis in rats ranging from 0 to 4 per paw, giving a maximum score of 16 [17]. Each paw was scored separately according to the following system: 0 = no disease, 0.5 = erythema of one toe, 1 = erythema or swelling of one toe or another joint, 2 = erythema and swelling of more than one toe, 3 = erythema and swelling of ankle joint and other parts of the foot, 4 = general erythema and swelling of toes and ankle joint and stiffening of the joints.

Measurement of autoantibodies directed against double-stranded DNA

Once weekly, a few drops of blood were collected from the mice by bleeding of the tail vein. The coagulated blood was sedimented by centrifugation and the serum was transferred to polypropylene tubes and stored at -20°C until use. The sera were then thawed, diluted 1/10 in phosphate buffered saline (PBS) and screened for the presence of antibodies directed against double-stranded DNA (dsDNA) using *Critidia luciliae* substrate slides (The Binding Site Ltd., Birmingham, England) according to the manufacturer's instructions. The secondary antibody used was goat-anti-mouse immunoglobulin conjugated to fluorescein isothiocyanate (GαM-FITC; Becton & Dickinson, San Jose, CA, USA). In the first screening, a known positive and a negative human serum were included as controls, with the appropriate secondary antibody conjugate. As a murine negative control, pooled normal mouse serum was used. In subsequent tests PBS, a selected positive mouse serum and pooled normal mouse serum were always included as controls on each slide, and results obtained for the other sera on the slide were always compared with these controls. After screening all the serum samples at a dilution of 1/10, the anti-dsDNA antibody titre was determined for the sera collected at the end of the experiment. Starting from the 1/10, serial 2-fold dilutions were tested (down to 1/640). The titre was defined as $-^2\log(10*c)$, whereby c is the farthest dilution that still gave a positive staining of the *C. luciliae* kinetoplast.

Scoring of glomerulonephritis

The animals were sacrificed by CO_2 asphyxiation at the end of the 4-week observation period on day 32 after injection with Octreotide-LAR. The kidneys were removed, fixed in 4% buffered formaldehyde solution (Sigma Chemical Co., St. Louis, MO, USA) and embedded in paraffin. One µm thick sections were stained with PAS reagent or with haematoxylin-eosin and scored for glomerular damage. A minimum of 50 glomeruli per section was examined. The percentage of abnormal glomeruli was determined. Furthermore, for each abnormal glomerulus the type of abnormality was noted (fibrinoid necrosis in the glomerulus without extracapillary proliferation (i.e. crescent formation); fibrin leakage into the subcapsular space without extracapillary proliferation; segmental or circumferential crescent formation with or without necrosis in the tuft or crescent). The presence of interstitial mononuclear cell infiltrates was scored separately.

Statistics

Maximum arthritis scores of the mice in the two treatment groups were compared with the

maximum scores of the mice which received injection vehicle only, using a Mann-Whitney non-parametric test. Mean cumulative arthritis scores over the entire observation period of the treatment groups were compared with the mean cumulative score of the group that received injection vehicle only, using a Student's t-test. The groups were compared for the incidence of anti-dsDNA antibodies and interstitial infiltrates in the kidneys using a Fisher exact (χ^2) test. The anti-dsDNA titres of the groups were compared using a Mann-Whitney test. A P-value < 0.05 was considered statistically significant.

Results

We studied the effects of octreotide treatment in a murine model of autoimmune disease. Because of the up-regulation of somatostatin receptor expression in inflamed joints in human rheumatoid arthritis [9], we chose to study the effects of 4 weeks of treatment with a slow-release formulation of octreotide (octreotide-LAR in two different dosages) on the spontaneous arthritis of the MRL/*lpr* mouse. Besides the arthritis, this mouse strain also develops a lupus-like syndrome characterised by nephritis and the presence of lupus-associated autoantibodies. We also studied the effects of octreotide-LAR on these lupus-associated phenomena.

MRL/*lpr* mice sometimes develop a severe wasting disease; others develop severe clinical symptoms such as ascites due to the massive lymphoproliferation that characterises these mice. Four mice died of severe weight loss (>15%) during the experiment, two died without severe clinical signs of disease and one animal was sacrificed for ethical reasons. In total 4 mice in the group treated with injection vehicle and 3 mice in the group treated with 10 mg/kg octreotide died or were sacrificed during the experiment.

Effects of treatment with Octreotide-LAR on arthritis

The arthritis was monitored by visual scoring of signs of paw inflammation on a scale of 0 to 4 per paw. The results are summarised in Table 1. No significant differences were found between the groups in the maximal or cumulative arthritis scores.

Table 1. The severity of arthritis in MRL/*lpr* mice treated with octreotide-LAR.

Treatment	Maximum score (mean ± SD)	Maximum score (median)	Cumulative score (mean ± SD)
Injection vehicle (n=10)	5.7 ± 3.2	7	35.1 ± 6.2
10 mg/kg octreotide (n=10)	6.8 ± 2.6	7	35.9 ± 6.5
30 mg/kg octreotide (n=9)	7.3 ± 3.1	8	34.6 ± 7.2

Effects of treatment with Octreotide-LAR on anti-dsDNA antibodies

The incidence and titre of autoantibodies against dsDNA was measured using a standard immunofluorescence test with the kinetoplast of the unicellular flagellate *Critidia luciliae* as the substrate and goat-anti-mouse immunoglobulins conjugated to FITC as the detecting antibody. The results are summarised in Table 2. Treatment with Octreotide-LAR did not significantly reduce the incidence of anti-dsDNA antibodies. There was, however, a slight trend towards reduced titres with increasing dose of Octreotide-LAR: a non-parametric test

for a difference between the titres of mice treated with 30 mg/kg octreotide and mice treated with injection vehicle only yielded a one-tailed P-value of 0.09.

Table 2. The incidence and titre of anti-dsDNA antibodies in MRL/*lpr* mice treated with octreotide-LAR.

Treatment	Incidence					Titre	Median
	day 4	day 10	day 17	day 24	day 32	day 32	titre
Injection vehicle	10/10	9/9	8/8	6/6	6/6	5,2,0,>6,4,4	4
10 mg/kg octreotide	4/10*	5/10*	6/9	4/7	4/7	3,2,3,>6	3
30 mg/kg octreotide	7/9	7/9	7/9	7/9	8/9	3,0,2,2,4,3,1,2	2

* P<0.5, significantly different from group treated with injection vehicle only

Effects of treatment with octreotide-LAR on lupus nephritis

The glomerulonephritis and the presence of interstitial mononuclear cell infiltrates were scored histologically. The results are summarised in Tables 3 and 4. No significant differences were found between the treatment groups and the group treated with injection vehicle only. This was mainly due to a large variability within the groups.

Table 3. The incidence of glomerulonephritis in MRL/*lpr* mice treated with octreotide-LAR.

Treatment (number of animals scored)	Percentage abnormal glomeruli (mean ± SD)	Incidence of interstitial infiltrates
Injection vehicle (n=6)	35 ± 51	2/6
10 mg/kg octreotide (n=7)	29 ± 43	1/7
30 mg/kg octreotide (n=9)	27 ± 33	1/9

Discussion

In this study we treated groups of MRL/*lpr* mice with octreotide, a metabolically stable octapeptide somatostatin analogue with high affinity for the somatostatin receptor subtypes sst_2 and sst_5 and to a lesser extent sst_3. These mice develop arthritis and a lupus-like syndrome characterised by glomerulonephritis and autoantibodies.

Somatostatin and somatostatin analogues have been shown to have a suppressive effect on the immune system [3]. Somatostatin receptors have been identified in the inflamed joints of patients with rheumatoid arthritis by *in vivo* receptor scintigraphy and *in vitro* receptor autoradiography [5, 9]. Recently, we showed by immunohistochemical staining that sst_{2A} is expressed by monocytes, macrophages and epithelioid cells in synovial tissue from rheumatoid arthritis (RA) patients [18] and in granulomas from sarcoidosis patients [19]. These cells are thought to be important effector cells in RA and may therefore be an interesting target for treatment with somatostatin or somatostatin analogues. Intra-articular administration of somatostatin was shown to decrease pain and synovial swelling in human RA [20, 21]. Furthermore, somatostatin was shown to down-modulate proinflammatory cytokine production by human synovial cells *in vitro* [22]. However, in the present study, systemic treatment with a metabolically stable somatostatin analogue did not reduce clinical signs of spontaneously occurring arthritis in MRL/*lpr* mice.

Table 4. Glomerular abnormalities in MRL/*lpr* mice treated with octreotide-LAR.

Treatment	Total number of glomeruli scored	Normal	Extracapillary proliferation:					Fibrinoid necrosis without extracapillary proliferation:	Fibrin leakage into Bowman's space
			Total:	circumferential crescent	segmental crescent	fibrinoid necrosis in the tuft	fibrinoid necrosis in the crescent		
Injection vehicle									
1	50	50							
2	50	46	2		2	2		1	1
3	50	0	36	28	8	12	2	12	2
4	52	0	26	15	11	19	7	11	3
5	50	50							
6	50	50							
10 mg/kg octreotide									
1	54	3	2		2	1		49	0
2	50	47						3	
3	50	46						3	1
4	50	50							
5	50	47			1	1		2	1
6	55	5	17	3	14	17	0	33	0
7	50	50							
30 mg/kg octreotide									
1	50	50							
2	51	5	42	33	9	36	1	3	1
3	49	47	1		1		1	1	0
4	54	31	15	1	14	3		6	2
5	50	50							
6	50	48						1	1
7	50	50							
8	50	20	24	12	12	14	5	3	3
9	50	28	5	4	2	2	1	17	

We have shown by RT-PCR that sst_2, the sst subtype with the highest affinity for octreotide, is expressed in MRL/*lpr* splenocytes (unpublished observations). Therefore we do not believe that the absence of a measurable effect of octreotide treatment is due to absence of the appropriate receptor. However, there are other possible explanations for the lack of effect of the current treatment protocol. It has been shown in other animal models that the timing of treatment is very important [23], i.e. that treatment before onset of the disease can ameliorate disease severity, whereas treatment after onset can actually cause an exacerbation. Furthermore, it is conceivable that a short course of intensive treatment by injections of octreotide might be more effective than the continuous slow infusion as used in this study, as prolonged treatment with somatostatin can cause agonist-induced receptor desensitisation [24]. It is also possible that the general immune activation in the MRL/*lpr* mouse is just too massive for octreotide to have an effect. The *lpr* mutation causes a defect in Fas-mediated apoptosis in these mice. If somatostatin works mainly through stimulation of apoptosis of immune cells, this could explain the absence of effect, as apoptosis-induction has only been linked to sst_3 [25], an sst subtype with only low affinity for octreotide.

We found no significant amelioration of immune glomerulonephritis in MRL/*lpr* mice by treatment with Octreotide-LAR, nor was there a reduction in the incidence of interstitial mononuclear cell infiltration. There was a large variation in the percentage of damaged glomeruli within the groups, probably due to the fact that the lupus-like syndrome in these mice occurs spontaneously and the disease kinetics may therefore differ between individual mice. The difference in disease kinetics was also observed in the large differences in weight loss between individual mice over the course of the experiment (not shown).

Somatostatin has been shown to decrease immunoglobulin secretion from B-lymphocytes and plasmacytoma cell lines *in vitro* [14-16]. Furthermore, somatostatin receptors were shown to be present in the germinal centres of B cell follicles [26, 27]. Autoantibodies are thought to be an important cause of clinical symptoms in diseases such as glomerulonephritis and vasculitis in human SLE [28]. MRL/*lpr* mice develop a lupus-like syndrome characterised by nephritis and high levels of circulating autoantibodies. In this study we showed that treatment with 30 mg/kg octreotide gave a slight reduction in the median titre of anti-dsDNA autoantibodies, which was, however, not statistically significant. It is also interesting to note that in this treatment group none of the mice died during the observation period. The production of lupus-associated autoantibodies is dependent upon the hyperactivation of the T cell compartment in this and other models [29]. It is not known whether the observed effect of octreotide treatment was mediated via the T cell compartment or directly via the B lymphocytes.

Experimental lupus erythematosus can be induced in genetically susceptible mice by injection of monoclonal lupus-associated autoantibodies [30]. It would be interesting to study the effects of octreotide on disease incidence and progression in this model, as individual variations in disease kinetics are expected to be smaller in an induced disease and time of onset of the disease can be controlled, making it possible to study the effects of different treatment protocols.

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Chapter 4.2

TREATMENT OF MURINE EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS WITH A SLOW-RELEASE FORMULATION OF THE SOMATOSTATIN ANALOGUE OCTREOTIDE

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Abstract

Somatostatin is a neuropeptide that is widely distributed throughout the body. It acts as a neurohormone and a neurotransmitter and may also have an immunomodulatory role. The genes for five subtypes of somatostatin receptors have been cloned, suggesting that the diverse effects of the peptide might be mediated by different receptors. Octreotide is a metabolically stable cyclic octapeptide analogue of somatostatin, used in the clinic, with high affinity for sst subtypes sst_2, sst_3 and sst_5. A slow-release formulation (LAR) of octreotide has recently been developed.

We studied the effects of octreotide treatment on murine experimental autoimmune encephalitis (EAE), a model for MS. In the first experiment, treatment with a subcutaneous (s.c.) depot of octreotide-LAR equivalent to 30 mg/kg octreotide gave a significant decrease in disease severity. In the second experiment, octreotide treatment did not reduce the severity of the disease in any of the treatment groups, not even in animals treated with 100 mg/kg octreotide.

Therefore, on the basis of these experiments, we cannot conclude whether treatment with octreotide in the form of Octreotide-LAR is able to down-modulate murine EAE. It is hypothesised that a short course of octreotide injections may have a stronger effect than slow release of octreotide from a s.c. depot, as prolonged treatment with somatostatin might cause agonist-induced receptor desensitisation. In conclusion, great care should be taken to establish the appropriate dosage and treatment regimens when using somatostatin for the treatment of autoimmune and other immune-mediated diseases.

Introduction

Somatostatin is a 14 amino acid neuropeptide that is widely distributed throughout the body. It was first identified as a growth hormone release-inhibiting factor synthesised in the hypothalamus. Outside the central nervous system (CNS), somatostatin is expressed in a variety of endocrine and non-endocrine tissues. In the CNS it can act as a neurotransmitter and neurohormone, while in peripheral tissues it regulates endocrine and exocrine secretion and acts as a modulator of motor activity in the gastrointestinal tract [1, 2].

Somatostatin and synthetic, metabolically stable analogues have been shown to have a suppressive effect on a number of immune functions (for a review see [3]). Specific receptors for somatostatin have been identified in lymphoid tissues and on the surface of various types of white blood cells and cell lines by classic ligand binding studies [3]. In addition it

was found that sites of active inflammation in a number of granulomatous (e.g. sarcoidosis) and autoimmune diseases (e.g. Graves' ophthalmopathy and rheumatoid arthritis), could be visualised by the administration of an isotope-coupled somatostatin analogue, [^{111}In-DTPA^0]-octreotide (octreotide scintigraphy) [4]. Recently it has been shown that inflammatory lesions in the brains of patients with multiple sclerosis (MS) could also be visualised by this technique [5], although the identity of the sst-expressing cells in the lesions is as of yet unknown.

The genes for five G-protein-coupled somatostatin receptors (sst_{1-5}) have been cloned (for a review see [6]). Octreotide is a metabolically stable cyclic octapeptide analogue of somatostatin with high affinity for sst subtypes sst_2, sst_5, and to a lesser extent sst_3 [7], which is used clinically for the treatment of hormone-secreting tumours and gastrointestinal problems. Recently, a slow-release formulation of octreotide has been developed, which continuously releases octreotide for a period of 6 weeks after subcutaneous (s.c.) or intramuscular (i.m.) injection [8].

We are interested in studying the effects of octreotide treatment on autoimmune and other immune-mediated diseases. The immune-mediated diseases that can be visualised by octreotide scintigraphy are T cell mediated autoimmune diseases and granulomatous diseases, although it is not clear in all cases which cells in the lesions are the target for octreotide binding. Recently, we demonstrated the expression of sst_{2A} by a subset of macrophages in synovial biopsies of patients with rheumatoid arthritis [9] and by macrophages, epithelioid cells and giant cells in the granulomas of patients with sarcoidosis [10]. We therefore hypothesise that macrophages are a target for treatment of these diseases using octreotide. We tested this hypothesis using an animal model of T cell mediated autoimmune disease. Because of the up-regulation of somatostatin receptors in the inflammatory lesions of patients with MS, we chose to study the effects of treatment with the slow-release formulation of octreotide on murine experimental autoimmune encephalomyelitis (EAE), a well-established animal model for MS. We have previously shown that rats do not express sst_2, the somatostatin receptor subtype with the highest affinity for octreotide within their immune system [11]. Therefore, EAE in the latter species is not a suitable model.

EAE is a T cell mediated autoimmune demyelinating disease, which can be induced in mice, rats and primates by sensitisation with spinal cord homogenate or myelin proteins [12]. It is also possible to induce EAE in these animal species with peptides representing epitopes of myelin basic protein (MBP) or proteolipid protein (PLP). In peptide-induced EAE, central nervous system (CNS) inflammation is seen before onset of clinical signs of the disease. The inflammatory infiltrate consists mainly of $CD4^+$ T lymphocytes and macrophages. The cytokines produced within the lesions are predominantly of the Th1 subset (e.g. IL-2, IFN-γ) [13]. CNS-infiltrating macrophages are thought to be important effector cells in EAE and may play an active role in demyelination [14, 15]. *In vitro*, somatostatin has been shown to inhibit lymphocyte proliferation and the release of certain cytokines (TNF-α, lymphotoxin and IFN-γ). Inhibition of the release of cytokines (TNF-α, IL-1β, IL-6 and IL-8) by monocytes has also been reported [16], although there are also conflicting reports [17]. Octreotide was shown to decrease the secretion of IFN-γ from murine T lymphocytes and to decrease the granuloma size in a mouse model [18]. Therefore we expect octreotide treatment to have anti-inflammatory effects in murine EAE, possibly by decreasing IFN-γ secretion from infiltrating T lymphocytes or by down-modulating macrophage effector functions. However, because somatostatin receptors are also expressed in the CNS, systemic effects of octreotide mediated via these receptors might also play a role.

mRNA for sst_2, the main target for octreotide, has been shown to be expressed in the murine brain and in murine splenocytes, thymocytes and isolated T lymphocytes [19]. We have not been able to confirm the presence of sst_2 mRNA in splenocytes from uninfected mice (manuscript in preparation); however, this receptor subtype may be up-regulated during inflammation.

Materials and methods

Animals

Female SJL/J mice were bred and maintained under SPF conditions with free access to gamma-irradiated pelleted food (Hope Farms, Woerden, The Netherlands) and water acidified to pH 2.2 in the animal facilities of the Department of Immunology of the Erasmus University. Animals which were 8-10 weeks of age, were used in the experiments. The experimental protocol was approved by the Animal Welfare Committee of the Erasmus University.

Induction and monitoring of EAE

EAE was induced by immunisation with a peptide derived from PLP, a major protein constituent of central nervous system myelin, and additional treatment with *Bordetella pertussis* to affect the integrity of the blood-brain barrier. This causes a monophasic ascending paralysis starting around day 11 after induction, from which most animals recover spontaneously around day 20 [12].

A solution of 0.5 mg/ml PLP139-151 peptide in phosphate-buffered saline (PBS) was emulsified 1:1 with complete Freund's adjuvant (CFA; Difco 311-60-5, with *Mycobacterium tuberculosis* strain H37RA; Difco Laboratories, Detroit, MI). On day 0, mice were injected s.c. with a total of 50 µg peptide, divided over four sites on the flanks, dispersed in 50 µl of emulsion per site. This dose of antigen had been previously shown to induce 100% disease incidence. On days 1 and 3, the mice received 10^{10} heat-killed *B. pertussis* intravenously (*Bordetella pertussis* vaccine; National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands).

Clinical disease was monitored daily from day 7 onward by weighing the mice, and by grading symptoms of paralysis using an internationally accepted ordinal clinical scoring scale, ranging from 0 to 5 (0 = no disease, 0.5 = tail paresis or partial paralysis, 1 = complete tail paralysis, 2 = paraparesis: limb weakness and tail paralysis, 2.5 = partial limb paralysis, 3 = complete hind- or front limb paralysis, 3.5 = paraplegia, 4 = quadriplegia, moribund, 5 = death due to EAE). The data were expressed as group mean ± standard deviation (SD). Animals that died due to EAE received score 5 on the day of death and no score during the remaining days of the experiment [20].

Treatment with octreotide-LAR

Three days before induction of EAE, mice were injected s.c. in the dorsal neck region with a suspension of octreotide-LAR, a slow-release formulation of the somatostatin analogue octreotide (Novartis Pharma AG, Basel, Switzerland) in standard injection vehicle (buffered sodium carboxyl-methylcellulose). Control mice were injected with vehicle only (designated 0 mg/kg octreotide). Two separate experiments were performed, in which the mice received different doses of octreotide. Experiment 1: 0 mg/kg octreotide, 3 mg/kg octreotide and 30 mg/kg (n=4); experiment 2: no treatment, 0 mg/kg octreotide, 10 mg/kg octreotide, 30 mg/kg

octreotide, 100 mg/kg octreotide.

Disease statistics
Incidence was compared using a Fisher exact test. Mean day of onset of disease, mean maximum weight loss and mean maximum disease score (as a measure of severity) in the different groups were compared using a Mann-Whitney non-parametric test. A P-value ≤ 0.05 was considered to be significant.

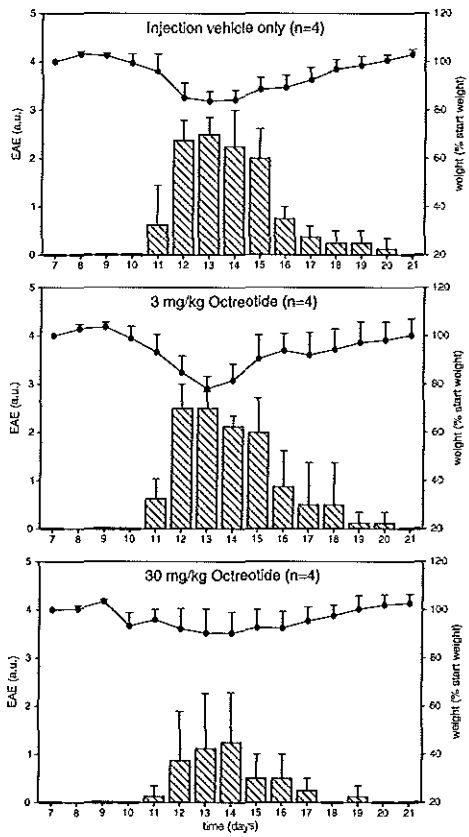

Figure 1. The effect of treatment with Octreotide-LAR on the disease severity in murine EAE; experiment 1. Groups of SJL mice were treated with octreotide as a subcutaneous depot of Octreotide-LAR or with injection vehicle only, followed by EAE induction for all groups. Graphs show group averages, with the line indicating the weight of the animals as a percentage of the start weight, and the bars represent clinical signs of disease, scored on a scale from 0 to 5.

Results

We wished to assess whether treatment with a slow-release formulation (Octreotide-LAR) of octreotide, a metabolically stable somatostatin analogue, had a limiting effect on the induction and disease progression of murine EAE. In the first experiment (summarised in Figure 1), groups of 4 mice were treated with injection vehicle only or with a s.c. depot of Octreotide-LAR equivalent to 3 mg/kg or 30 mg/kg octreotide, followed by EAE induction.

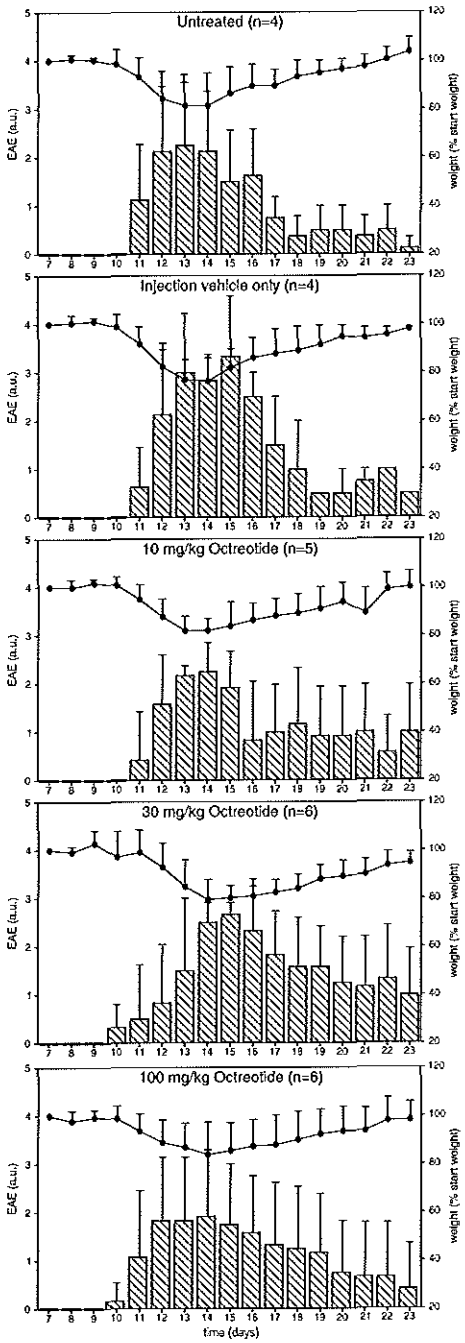

Figure 2. The effect of treatment with Octreotide-LAR on the disease severity in murine EAE; experiment 2. Groups of SJL mice were treated with an extended dose range of octreotide as compared with experiment 1. An untreated control group was also included. Graphs show group averages, and are organised as described in the legend to Figure 1.

In the control group and the 3 mg/kg group, all animals developed full-blown disease following normal disease kinetics. There was no significant difference in the severity of the disease between these two groups. In the 30 mg/kg group, however, one mouse was totally protected and a second one showed only a minimal disease score of 0.5 on day 14 after induction. The other two mice developed a similar disease course as the control animals.

In view of these promising results, a second experiment was carried out, using higher doses of Octreotide-LAR (summarised in Figure 2). Groups of 4-6 mice were either left untreated (n=4), treated with injection vehicle only (n=4), or with a s.c. depot of Octreotide-LAR equivalent to 10 mg/kg (n=5), 30 mg/kg (n=6) or 100 mg/kg octreotide (n=6), followed by EAE induction for all groups. In the untreated group, 3 out of 4 animals developed disease following normal disease kinetics. In the 10 mg/kg and the 30 mg/kg group, all animals developed a similar disease course as the control animals. In the 100 mg/kg group 2 animals were completely protected, whereas the other 4 developed a similar disease course as the control animals. In the group treated with injection vehicle only, all 4 mice developed disease, and 2 mice died from EAE.

Incidence, mean day of onset, mean maximum weight loss and disease severity were compared for the different groups in the two experiments. No differences were found in either experiment in the incidence or the day of onset of disease or in the maximum weight loss of the animals. Only in the first experiment a significant difference in severity (i.e. mean maximum score) was observed, with severity significantly lower in the group treated with 30 mg/kg than in the group which received injection vehicle only ($P = 0.03$). In the second experiment, octreotide treatment did not reduce the severity of the disease in any of the treatment groups. The results are summarised in Table 1.

Table 1. Treatment of murine experimental autoimmune encephalomyelitis with octreotide-LAR.

Treatment	Incidence	Deaths	Mean day of onset ± SD	Mean max. weight loss (%) ± SD	Mean max. score ± SD
Experiment 1					
Injection vehicle	4/4	0	11.5 ± 0.6	17.8 ± 3.9	2.8 ± 0.3
3 mg/kg octreotide	4/4	0	11.2 ± 0.5	21.9 ± 5.9	2.6 ± 0.5
30 mg/kg octreotide	2/4	0	12.3 ±1.5	13.6 ± 9.3	1.3 ± 1.2 *
Experiment 2					
None	3/4	0	11.3 ± 0.6	20.8 ± 14.8	2.4 ± 1.6
Injection vehicle	4/4	2	11.8 ± 1.0	26.9 ± 8.4	3.9 ±1.3
10 mg/kg octreotide	5/5	0	12.0 ± 0.7	21.1 ± 7.3	2.9 ± 0.4
30 mg/kg octreotide	6/6	0	12.2 ± 1.8	24.5 ± 7.2	2.8 ± 0.3
100 mg/kg octreotide	4/6	0	11.0 ± 0.8	18.6 ± 14.2	2.0 ± 1.6

* Significant difference ($P < 0.05$) compared to the group treated with injection vehicle only

Discussion

Because of the up-regulation of somatostatin receptors in the inflammatory lesions of patients with MS, and the putative immunoregulatory effects of somatostatin, we chose to study the effects of treatment with the slow-release formulation of the somatostatin analogue octreotide

on murine EAE. We have shown previously in rats that octreotide is released continuously during 6 weeks from a s.c. depot of Octreotide-LAR (plasma levels 1.5-2.5 ng/ml) [11]. Octreotide does not usually pass the blood-brain barrier, but in EAE the integrity of the blood-brain barrier is affected by the disease and the induction regimen using *B. pertussis*, so we expected octreotide to have local effects in the brain in addition to the systemic effects in this model.

In the first experiment, treatment with a s.c. depot of Octreotide-LAR equivalent to 30 mg/kg octreotide, gave a significant decrease in disease severity. In the second experiment, octreotide treatment did not significantly reduce the severity of the disease in any of the treatment groups. However, in this experiment, disease severity in the group treated with injection vehicle was uncommonly high: two animals even died of the disease. It is possible that the level of disease in the second experiment was too high for modulation to be possible [20].

Therefore, on the basis of these experiments, we cannot conclude whether treatment with octreotide in the form of Octreotide-LAR is able to down-modulate murine EAE. This could be due to the treatment regimen, i.e. to the fact that octreotide is released continuously throughout the course of the disease. Muhvic *et al.* [21] studied the effects of octreotide in a rat EAE model with a chronic-relapsing disease course. In that study, EAE was induced in DA rats by injection of calf brain white matter in CFA without the use of *B. pertussis*. These authors found large differences between groups treated with octreotide at various stages of disease (total octreotide dose was equal for all groups; 15 µg/kg). A short course of treatment on the day of induction of the disease delayed the onset of disease and shifted the peak of the disease from day 11 to day 14 after induction. Continuous treatment for 3 days after immunisation for disease induction also shifted the peak of the disease. Furthermore, this treatment abolished disease remissions and established a monophasic non-remitting disease course. This latter effect was seen even more clearly in animals treated with octreotide on the day of first clinical signs of the disease. Treatment with octreotide on the day of induction of EAE or continuously for 3 days rendered rats of the EAE-resistant OA strain susceptible to disease. The authors speculate that octreotide treatment might have a somatostatin receptor-mediated suppressive effect on suppressor cell populations, the differential activity of which is responsible for the prevention of disease in the resistant strain rats and for the remission of the disease in the susceptible strain rats. Alternatively, the effects observed might have been due to modulation of the functional activity of cells within the CNS that also possess receptors for somatostatin. As we have shown that mRNA for sst_2 is not expressed in the rat immune system, not even after systemic immune activation [11], we do not expect that octreotide treatment would modulate the function of (suppressor) immune cell populations via this receptor subtype. However, at high pharmacological doses octreotide might interact with sst_3, a subtype that is expressed in the rat immune system. We were unable to modulate the disease activity of rat adjuvant arthritis using octreotide-LAR [11]. It is difficult to compare the pharmacological dose and the bioavailability of octreotide between our experiments and the study of Muhvic *et al.* [21]. Unlike murine EAE, the use of pertussis toxin is not required for the induction of EAE in the rat, so the permeability of the blood-brain barrier may be different in the two models.

Prolonged treatment with somatostatin can cause agonist-induced receptor desensitisation [22]. Future studies should therefore include injection of octreotide, next to groups treated with a depot of octreotide-LAR. Thus, great care should be taken to establish the

appropriate dosage and treatment regimens when using somatostatin for the treatment of autoimmune and other immune-mediated diseases.

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Chapter 5

LOCALIZATION OF SOMATOSTATIN RECEPTORS IN HUMAN IMMUNE-MEDIATED DISEASE - AN IMMUNOHISTOCHEMICAL STUDY

Localization of somatostatin receptors in human immune-mediated disease - an immunohistochemical study

5.1 Immunohistochemical localization of somatostatin receptor sst_{2A} in human rheumatoid synovium.
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5.2 Immunohistochemical localization of somatostatin receptor sst_{2A} in sarcoid granulomas.
European Journal of Clinical Investigation 1999, in press.

5.3 Somatostatin receptors in inflammatory bowel disease: *in vitro* localization using a polyclonal anti-somatostatin receptor subtype 2A antiserum and *in vivo* visualization by [^{111}In-DTPA-D-Phe^1]-octreotide scintigraphy.

Chapter 5.1

IMMUNOHISTOCHEMICAL LOCALIZATION OF SOMATOSTATIN RECEPTOR SST_{2A} IN HUMAN RHEUMATOID SYNOVIUM

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Abstract

Objective. To identify the somatostatin receptor-expressing cells in rheumatoid synovium using a recently developed antiserum directed against the somatostatin receptor subtype 2A (sst_{2A}).
Methods. We carried out immunohistochemical studies of synovial biopsies from 7 patients with rheumatoid arthritis (RA) and one non-RA patient, using a rabbit polyclonal antiserum directed against sst_{2A} and monoclonal antibodies directed against phenotypic markers.
Results. Sst_{2A} was expressed by the endothelial cells of the synovial venules but also by a subset of synovial macrophages.
Conclusion. The identification of somatostatin receptors on macrophages, which are thought to be important effector cells in RA, may offer mechanistic insights into the potential therapeutic effect of somatostatin (analogs) in rheumatoid arthritis.

Introduction

Somatostatin is a neuropeptide that is widely distributed throughout the body. In the central nervous system somatostatin can act as a neurotransmitter; in peripheral tissues it regulates endocrine and exocrine secretion. Somatostatin and somatostatin analogues have also been shown to have a dampening effect on a number of immune functions [1].

Rheumatoid arthritis (RA) is an autoimmune disease of unknown etiology. T cells are assumed to be the main immune driven cell in RA. However, the role of macrophages is also under investigation: positive correlations were found between the number of synovial macrophages and radiological deterioration or joint pain [2, 3].

Previously, we described the presence of somatostatin receptors (sst) in the affected joints of patients with RA by *in vivo* somatostatin receptor scintigraphy [4]. *In vitro* autoradiography of inflamed synovium revealed sst_2 associated with the vasculature [5]. However, it was not possible to identify exactly which cell types in this tissue expressed the receptors.

The sst_2 receptor exists in two forms, sst_{2A} and sst_{2B}, generated by alternative splicing [6]. In this study we used a polyclonal antiserum directed against sst_{2A} [7] to more accurately identify the cells expressing sst_{2A} in RA synovium.

Materials and methods

Patients and biopsies
Synovium biopsies were obtained for diagnostic purposes or upon joint surgery from 7 patients with RA according to the criteria of the American Rheumatism Association [8], and from one patient with persistent edema. All patients provided informed consent. Three of the RA biopsies and the biopsy from the chronic edema patient were processed for cryosectioning; the other 4 biopsies were fixed in formalin and embedded in paraffin.

Autoradiography
The presence of sst in synovial tissue was investigated by autoradiography on cryosections using an iodinated octapeptide somatostatin analogue with high affinity for sst_2, [^{125}I-Tyr^3]-octreotide, as described [5].

Immunohistochemistry
Immunohistochemical analysis was performed according to standard methods using a polyclonal rabbit anti-sst_{2A} antiserum (R2-88) [7] with alkaline phosphatase (AP) conjugated goat anti-rabbit immunoglobulin (Dakopatts, Glostrup, Denmark) as the detecting antibody. For double staining, cryosections were additionally incubated with the appropriate monoclonal antibody with rabbit anti-mouse immunoglobulin conjugated to horseradish peroxidase (HRP) (Dakopatts) as the detecting antibody. AP activity was revealed first using naphtol AS-MX phosphate with Fast Blue as the chromogen. Subsequently, HRP activity was revealed using 3-amino-9-ethylcarbazole (red) as the chromogen (all chemicals from Sigma, St.Louis, MO, USA). The origins of the antibodies used in this study are summarized in Table 1. Controls for immunohistochemistry included: (1) omission of the primary antibody, (2) incubation with normal rabbit serum, (3) pre-absorption of the sst_{2A} antiserum with the immunizing peptide.

Table 1. Antibodies used in this study: specificity, source and working dilution.

Antibody	Specificity	Source	Dilution
R2-88	sst_{2A} (somatostatin receptor subtype 2A)	Dr. A. Schonbrunn	1:1000
Leu4	CD3 (T cells)	Becton & Dickinson, San Jose, CA, USA	1:50
My4	CD14 (monocytes, macrophages)	Coulter Clone, Hialeah, FL, USA	1:100
L133.1	CD31 (endothelium, myeloid cells)	Dakopatts, Glostrup, Denmark	1:100
EBM11	CD68 (macrophages)	Dakopatts	1:100
HLA-DR	HLA-DR (human MHC class II)	SeraLab, Crawley Down, UK	1:200

Results

Autoradiography
Autoradiographic analysis of rheumatoid arthritis synovial biopsies revealed a distinct binding pattern of [^{125}I-Tyr^3]-octreotide (Figure 1A). Non-specific binding was very low (Figure 1B), indicating that the binding seen in Figure 1A was specific for sst. No specific sst-positive cell type could be identified, but the sst-positive regions surround the blood vessels.

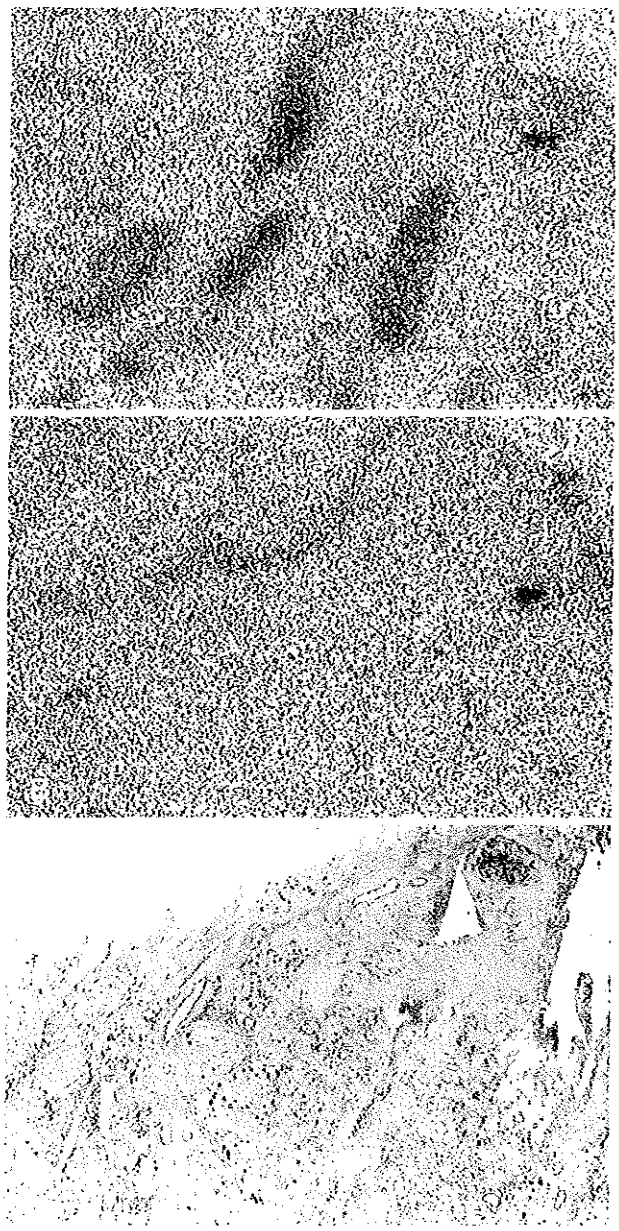

Figure 1. Visualization of somatostatin receptors (sst) in synovial tissue by autoradiography and immunohistochemistry. A. Autoradiograph showing total binding of [^{125}I-Tyr^3]octreotide in the absence of unlabeled octreotide. B. Autoradiograph showing residual nonspecific binding of [^{125}I-Tyr^3]octreotide in the presence of 1 μM unlabeled octreotide. C. Immunohistochemical staining using a polyclonal antiserum directed against sst_{2A} (all photos original magnification x63). Immunohistochemistry has a higher resolution than autoradiography and shows staining of the blood vessel endothelial cells as well as staining of cells in the surrounding infiltrate.

Immunohistochemistry

Immunohistochemical analysis of RA synovial biopsies revealed sst_{2A} expression in the endothelial cells of venules and capillaries, but also in other cells throughout the inflamed tissue (Figure 1C). Most of these cells have a macrophage-like or dendritic morphology, but cells with a fibroblast-like morphology were also observed. The biopsy from the chronic edema patient also showed sst_{2A} expression in the endothelial cells of venules and capillaries, without signs of inflammation.

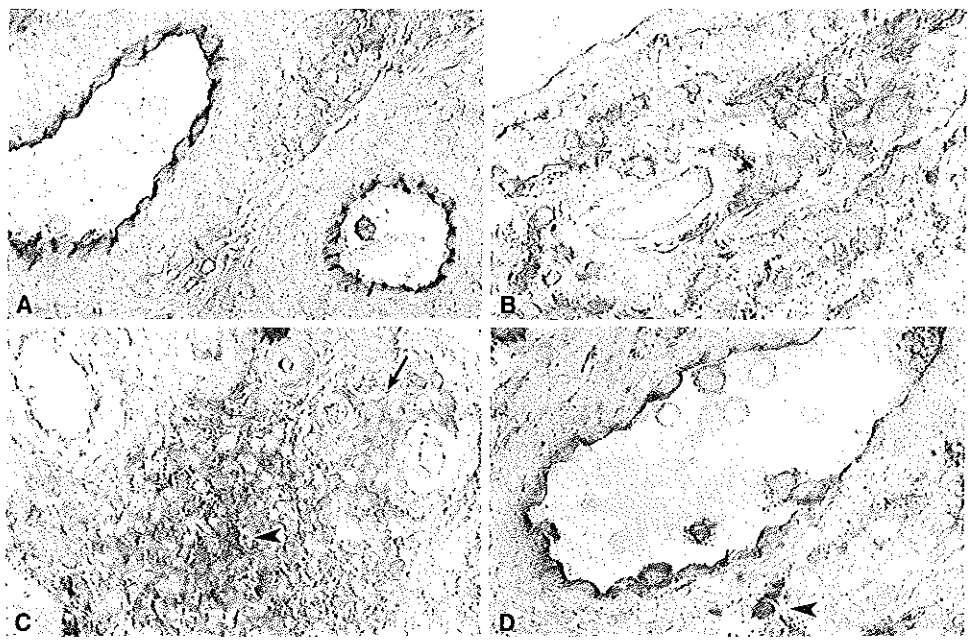

Figure 2. Identification of cell types in rheumatoid synovium expressing sst_{2A}. Immunohistochemical double staining of synovial tissue for somatostatin receptor subtype 2A (sst_{2A}; blue) and phenotypic markers (red). A. The endothelial cells, which are positive for sst_{2A}, also express CD31 (original magnification x400). B. CD3-positive T lymphocytes are sst_{2A} negative (original magnification x400). C. A number of CD14-positive monocytes also express sst_{2A} (arrowhead), whereas others are single positive for CD14 (arrow) (original magnification x400). D. Some sst_{2A}-positive endothelial cells also express HLA-DR, which points to their activated state. A few dispersed cells in the synovium also co-express sst_{2A} and HLA-DR (arrowhead); they are thought to be antigen presenting cells (original magnification x630).

Pre-absorption of the sst_{2A} antiserum with the immunizing peptide (0.3 µg/ml) completely abolished binding to endothelial cells in cryosections, and markedly reduced binding to the monocyte/macrophage-like cells. Pre-absorption with 3 µg/ml of the immunizing peptide gave complete inhibition of binding in paraffin-embedded sections, whereas pre-absorption with 1 µg/ml gave only partial inhibition (not shown).

Immunohistochemical double staining

Immunohistochemical double staining was performed to further characterize the sst_{2A}-positive cells. The results are shown in Figure 2. The endothelial cells, which are positive for

sst_{2A}, also express CD31 (Figure 2A), confirming that they are endothelial cells, in contrast to other cells associated with the vessel wall. The CD3-positive T lymphocytes are sst_{2A} negative (Figure 2B). In contrast, dispersed CD14-positive monocytes/macrophages (Figure 2C) and CD68-positive macrophages (not shown) show expression of the sst_{2A} receptor. The expression of HLA-DR by sst_{2A}-positive cells, especially the endothelial cells (Figure 2D), points to their activated state. A few dispersed cells in the synovium also co-express sst_{2A} and HLA-DR; they are thought to be antigen presenting cells.

Discussion

Using autoradiography we detected sst associated with the vasculature in rheumatoid synovium, but the resolution of this technique is too low to positively identify the specific cell types expressing these receptors [5].

The immunohistochemical technique employed in the present study has a higher resolution, so we were able to identify sst on endothelial cells of the blood vessels, and additionally on a subset of macrophages in inflamed synovium.

Somatostatin and somatostatin analogs have been shown to have a dampening effect on the immune system [1]. Recently, inhibition of the release of cytokines by monocytes was reported [9], although there are also conflicting reports [10].

Somatostatin might be produced by macrophages [11] or released from nerve termini and could conceivably act as a local autocrine regulator. Recently, somatostatin was shown to be produced by fibroblast-like synovial cells isolated from patients with RA. Both sst_1 and sst_2 mRNA (the RT-PCR primers used were specific for sst_{2A}) were expressed by these cells and sst_2 mRNA was up-regulated after stimulation of the cells with tumor necrosis factor alpha [12]. These results suggest a role for this receptor subtype in RA.

Sst on endothelial cells are hypothesized to have a role in decreasing extravasation of plasma and leukocytes, and may have an inhibitory effect on the proliferation of vascular endothelium [13]. They may also play a role in chronic edema, as observed in this study. It is interesting that the staining for sst_{2A} appeared to be confined to the luminal surface of the endothelial cells. This may mean that these receptors interact with circulatory somatostatin as opposed to somatostatin that is produced locally by synovial cells or released from nerve cells.

In this study we identified sst_{2A} on rheumatoid synovial venules and on a subset of macrophages. Because of their importance in inflammation, suppression of macrophage function and/or endothelium activation may result in decreased synovial inflammation. Intra-articular administration of somatostatin 14 was shown to decrease pain [14] and synovial swelling in patients with RA [15]. It is tempting to hypothesize that a systemically administered somatostatin analog might be used in the treatment of rheumatoid arthritis.

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Chapter 5.2

IMMUNOHISTOCHEMICAL LOCALIZATION OF SOMATOSTATIN RECEPTOR SST_{2A} IN SARCOID GRANULOMAS

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Abstract

Background. In a previous study we demonstrated the presence of receptors for somatostatin, a neuropeptide with immunoregulatory properties, in the inflammatory lesions of patients suffering from sarcoidosis and other granulomatous diseases by *in vivo* somatostatin receptor scintigraphy and *in vitro* autoradiography. However, it was not possible to identify exactly which cell types expressed the somatostatin receptors and which subtype was expressed. In this study we used a polyclonal antiserum directed against the sst_{2A} receptor to more accurately identify the sst_{2A}-expressing cells in sarcoidosis and other granulomatous diseases.
Patients and methods. Tissue biopsies from 12 patients with sarcoidosis, 1 patient with giant cell arteritis and 1 patient with Wegener's granulomatosis were studied by immunohistochemistry with the sst_{2A}-specific antiserum. Two of the sarcoidosis patients were treated with the somatostatin analogue octreotide (100 μg TID).
Results. Epithelioid cells, multinucleated giant cells and a subset of CD68+ macrophages stained positive for sst_{2A} in 9 out of 12 of the sarcoid biopsies and in both non-sarcoid granuloma biopsies. Treatment with octreotide resulted in clinical improvement in 1 of 2 treated patients.
Conclusion. The identification of somatostatin receptors on granuloma macrophages, epithelioid cells and giant cells, and the successful treatment of one patient with sarcoidosis with a somatostatin analogue, may offer new possibilities for treatment of granulomatous diseases.

Introduction

Sarcoidosis is a disease of unknown etiology, characterized by non-necrotizing granulomas, which can be found in various organs [1]. The major cellular constituent of mature granulomas, whether organized into epithelioid cells or not, is formed by non-lymphocytic mononuclear cells. In sarcoid granulomas they are represented by macrophages, epithelioid cells and giant cells, predominantly of the Langhans type [2].

Mononuclear phagocytes are present in granulomas in various stages of differentiation and activation. Some are phagocytic and capable of receptor-mediated endocytosis. Others may promote antigen sensitization by processing and presenting antigens to T lymphocytes. A secretory function has also been suggested [2]. Cytokines produced by macrophages, such as IL-1, IL-6 and TNF-α and the Th1 cytokines IL-2 and IFN-γ are thought to be

important in the local immune regulation of granulomas [3].

Other secretory products, such as neuropeptides, may also be engaged in the modulation of the granulomatous response [4]. Somatostatin is a 14 amino acid neuropeptide that is widely distributed throughout the body. Five G-protein-coupled somatostatin receptors (sst) have been cloned. Outside the central nervous system, somatostatin is present in a variety of endocrine and non-endocrine tissues. In the central nervous system the peptide can act as a neurotransmitter and neurohormone; in peripheral tissues it down-regulates endocrine and exocrine secretion and acts as a modulator of motor activity in the gastrointestinal tract [5]. Somatostatin and synthetic, metabolically stable analogues have been shown to have a suppressive effect on a number of immune functions (reviewed in [6]). In a previous study we demonstrated the presence of somatostatin receptors in the inflammatory lesions of patients suffering from sarcoidosis and other granulomatous diseases by *in vivo* somatostatin receptor scintigraphy and *in vitro* autoradiography [7]. The receptors were shown to be of the sst_2 or sst_5 subtype on the basis of their high affinity for the somatostatin analogue octreotide [7]. However, it was not possible to identify exactly which cell types in this tissue expressed the somatostatin receptors.

The sst_2 receptor exists in two forms, sst_{2A} and sst_{2B}, generated by alternative splicing of the sst_2 mRNA [8]. The two forms differ in their intracellular C-terminal domain and may differ in G-protein coupling and second messenger cascade activation. In the present study we used a polyclonal antiserum directed against the sst_{2A} receptor [9] to more accurately identify the sst_{2A}-expressing cells in sarcoidosis. We concentrated on the sst_{2A} receptor subtype, as the sst_2 receptor subtype has the highest affinity for synthetic, metabolically stable somatostatin analogues and would therefore be the most likely target for treatment with these analogues. Also, there is evidence that sst_2 is the major somatostatin receptor subtype expressed in the human immune system (reviewed in [6]).

Patients and methods

Patients and biopsies

Tissue biopsies, which were obtained for diagnostic purposes (after informed consent), were used for immunohistochemical investigation of sst_{2A} expression. Twelve patients with sarcoidosis, 1 patient suffering from giant cell arteritis and 1 patient suffering from Wegener's granulomatosis were enrolled in this study. Two lymph node biopsies (from patients 1 and 3) were processed for cryosectioning; the other biopsies were fixed in 4% (v/v) buffered formalin and embedded in paraffin.

The patient characteristics, the tissue studied and the *in vivo* somatostatin receptor status are shown in Table 1. The *in vivo* somatostatin receptor status was investigated by somatostatin receptor scintigraphy, as described previously [7].

Autoradiography

The presence of sst in sarcoidosis was investigated by autoradiography on 10 μm frozen sections using an iodinated octapeptide somatostatin analogue, [^{125}I-Tyr^3]-octreotide (Novartis Pharma, Basle, Switzerland), which was prepared as described previously [7, 10]. Tissue sections were mounted on gelatin-coated glass slides and stored at -20 °C for at least 3 days to improve adhesion of the tissue to the slide. Sections were air-dried, preincubated in 170 mmol L^{-1} Tris buffer adjusted to pH 7.6 with HCl (Tris-HCl buffer) for 10 min at room

Table 1. Characteristics of the patients and tissues studied

Patient	Sex	Age	Tissue	Diagnosis	Treatment*	Octreoscan	Anti-sst_{2A} staining	Peptide inhibition**
1†			Lymph node	Sarcoidosis	Unknown	N.D.	Positive	N.D.
2	F	77	Orbita tumor or inflammatory process	Sarcoidosis	None	Positive	Positive	N.D.
3	M	38	Lymph node	Sarcoidosis	None	Positive	Positive	0.3 μg/mL : ± 1 μg/mL : ±
4	M	56	Lymph node	Sarcoidosis	None	Positive	Negative	3 μg/mL : ±
5	F	38	Lymph node	Sarcoidosis	NSAID	N.D.	Negative	N.D.
6	F	38	Lymph node	Sarcoidosis	None	N.D.	Positive	1 μg/mL : + 3 μg/mL : +
7‡	M	34	Lymph node	Sarcoidosis	None‡	N.D.	Negative	N.D.
8	F	47	Skin	Sarcoidosis	None	Positive	Positive	1 μg/mL : ± 3 μg/mL : +
9	F	38	Lymph node	Sarcoidosis	None	Positive	Positive	3 μg/mL : +
10	F	35	Lymph node	Sarcoidosis	None	Positive	Positive	1 μg/mL : ± 3 μg/mL : +
11	M	36	Nasopharynx	Sarcoidosis	None	Positive	Positive	1 μg/mL : ± 3 μg/mL : +
12	F	65	Lung	Sarcoidosis	None	Positive	Positive	1 μg/mL : ± 3 μg/mL : +
13	M	29	Sinus frontalis, nasal mucosa, orbita	Wegener's granulomatosis	None	Positive	Positive	3 μg/mL : +
14	F	78	Arteria temporalis	Giant cell arteritis	None	N.D.	Positive	1 μg/mL : ± 3 μg/mL : +

N.D.= not determined

* Treatment during or within 3 months prior to biopsy

** ± partial inhibition of immunostaining, + complete inhibition of immunostaining;

† This biopsy was a kind gift from Dr. J. Lindeman, pathologist, Department of Pathology, Slotervaartziekenhuis Amsterdam

‡ This patient had received intensive cancer chemotherapy 5 years previously

temperature (RT) and then incubated for 60 min at room temperature (RT) with the iodinated ligand (3.0 MBq/mL, about 250 pmol L^{-1}). The incubation solution was Tris-HCl buffer containing 1% (w/v) bovine serum albumin (BSA), 40 µg/mL bacitracin (as a peptidase inhibitor; Merck, Darmstadt, Germany) and 5 mmol L^{-1} $MgCl_2$. Non-specific binding was determined by incubating a sequential section in the presence of 1 µmol L^{-1} of unlabeled octreotide (Novartis Pharma). After incubation, the sections were washed twice for 5 min in cold incubation buffer containing 0.25 % BSA, then in buffer alone and dried quickly. The sections were exposed to ^{3}H-Ultrafilms (Amersham, Little Chalfont, UK) for 3 weeks in X-ray cassettes.

Immunohistochemistry

Immunohistochemical analysis was performed according to standard methods using a polyclonal rabbit anti-somatostatin receptor 2A antiserum (anti-sst_{2A}; R2-88, a kind gift from co-author Dr. A. Schonnbrunn, see [9]) and monoclonal antibodies directed against phenotypic markers. Mouse monoclonal antibodies used in this study were Leu4 (detects the CD3 antigen on T cells; Becton & Dickinson, San Jose, CA, USA) and EBM11 (detects the CD68 antigen in macrophages; Dakopatts, Glostrup, Denmark).

Paraffin-embedded tissue sections (4 µm) mounted on glass slides coated with 3-amino propyl tri-ethoxy silane (APES; Sigma, St. Louis, MO, USA) were deparaffinized, rehydrated and boiled for 3 min in citric acid buffer (0.05 mol L^{-1}) using a microwave oven. They were rinsed in Tris-buffered saline (TBS) and processed further as described below for frozen sections. Frozen tissue sections (5 µm) mounted on uncoated glass slides were dried, fixed in 10% (v/v) buffered formalin, rinsed in TBS and preincubated for 15 min at RT with 10% (v/v) normal goat serum. Incubation with anti-sst_{2A} (dilution 1:500) was carried out overnight at 4°C; incubation with monoclonal antibodies was carried out for 1 h at RT. Antibody binding was detected using an alkaline phosphatase (AP)-conjugated secondary antibody. AP activity was revealed by naphtol AS-MX phosphate with New Fuchsin, which gives a red colour, as the chromogen, in the presence of levamisole to block endogenous alkaline phosphatase activity, followed by a nuclear stain of contrasting colour (Mayer's haematoxylin (blue); Merck).

For double staining, frozen sections which were processed as described above up to and including the incubation with AP-conjugated goat-ant-rabbit immunoglobulin (GαR-Ig-AP; Dakopatts), were incubated with the appropriate mouse monoclonal antibody, followed by incubation with rabbit-anti-mouse immunoglobulin conjugated to horseradish peroxidase (RαMIg-PO; Dakopatts). AP activity was revealed first as described above, but now using Fast Blue as the chromogen. Subsequently, peroxidase activity was revealed using 3-amino-9-ethylcarbazole, which gives a red colour, as the chromogen (all chemicals from Sigma).

Controls for immunohistochemistry included: 1) omission of the primary antibody, 2) pre-absorption of the sst_{2A} antiserum with the immunizing peptide (1-3 µg/mL).

Results

Autoradiography

Autoradiographic analysis of frozen sections of a sarcoid lymph node revealed a distinct binding pattern of [^{125}I-Tyr^3]-octreotide (Figure 1A). The labeled regions correspond to the pale-staining granuloma regions containing epithelioid cells but not to the surrounding darker staining regions containing mainly lymphocytes, as shown by haematoxylin-eosin staining

(Figure 1C, D). Residual non-specific binding of [^{125}I-Tyr^3]-octreotide in the presence of excess unlabelled octreotide was very low (Figure 1B), indicating that the binding seen in Figure 1A was specific for somatostatin receptors (sst). No specific sst-positive cell type could be identified with this technique, due to the limited resolution of the high-energy gamma radiation.

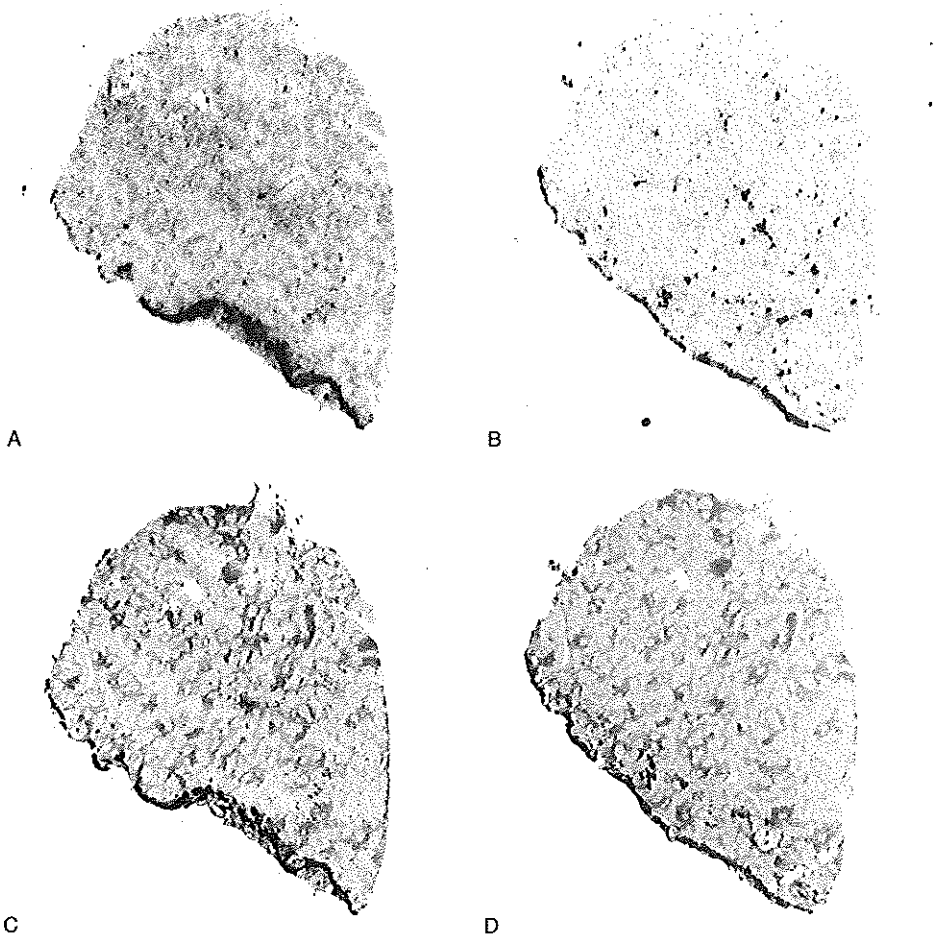

Figure 1. Visualization of somatostatin receptors (sst) in a sarcoid lymph node (patient 3) by autoradiography. A. Autoradiogram showing total binding of [^{125}I-Tyr^3]octreotide in the absence of unlabeled octreotide. B. Autoradiogram showing residual non-specific binding of [^{125}I-Tyr^3]octreotide in the presence of 1 μM unlabeled octreotide. C and D. Histology of the biopsy: haematoxylin-eosin staining of sections A and B respectively (all original magnification x5). The pale-stained granulomatous areas show specific binding of [^{125}I-Tyr^3]octreotide, whereas the surrounding darker-stained lymphocytic areas do not.

Immunohistochemistry

Immunohistochemical analysis of tissue biopsies containing sarcoid granulomas, using the specific antiserum, revealed sst_{2A} expression in epithelioid cells (Figure 2A) and giant cells (Figure 2B) within the granuloma and dispersed cells in the surrounding mononuclear infil-

trate in 9 out of 12 biopsies. Almost all of these cells had a macrophage-like or dendritic morphology, but cells with a fibroblast-like morphology were also observed. The staining appeared to be mainly intracellular. Occasionally, staining of the endothelium of high endothelial venules and small capillaries was observed. Also in the two other granulomatous diseases (giant cell arteritis and Wegener's granulomatosis) the epithelioid cells and giant cells expressed sst_{2A} receptors.

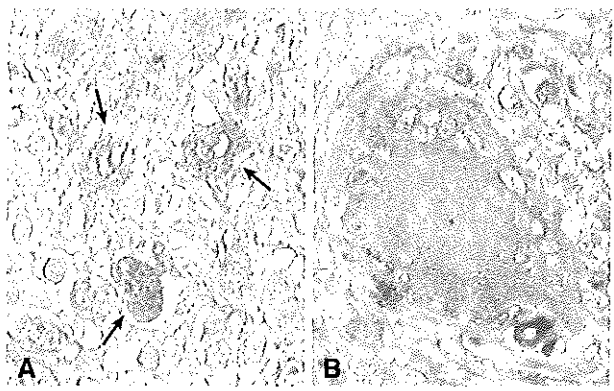

Figure 2. Immunohistochemical staining of sst_{2A} receptors in biopsies from patients with chronic granulomatous disease. A. Sst_{2A} expression (red) in epithelioid cells (arrows) in a lymph node biopsy of a sarcoidosis patient (patient 9). B. Sst_{2A} expression (red) in a multinucleated giant cell in an arteria temporalis biopsy of a patient with giant cell arteritis (patient 14).

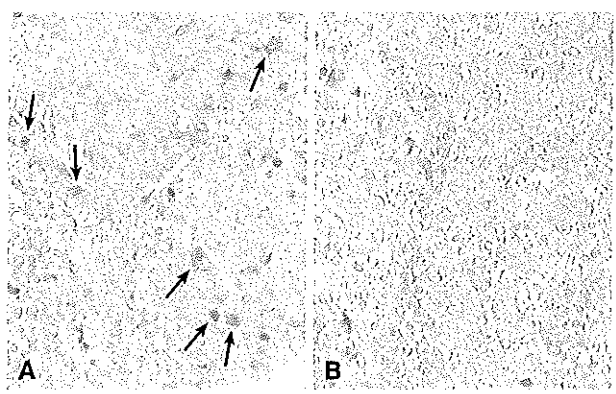

Figure 3. A characteristic example (patient 9) of the inhibition of immunostaining by the antiserum directed against sst_{2A} after pre-absorption with the immunizing peptide in sections of a sarcoid lymph node. A. Total staining in the absence of peptide. B. Staining of the adjoining section after pre-absorption with 3 μg/mL peptide (both original magnification x250).

Pre-absorption of the antiserum with the immunizing peptide gave a dose-dependent inhibition of binding: 1 μg/mL gave partial inhibition of immunostaining (not shown), whereas 3 μg/mL gave virtually complete inhibition of staining (see Figure 3). Staining of endothelium cells was inhibited at lower peptide concentrations than staining of epithelioid cells and giant cells (not shown).

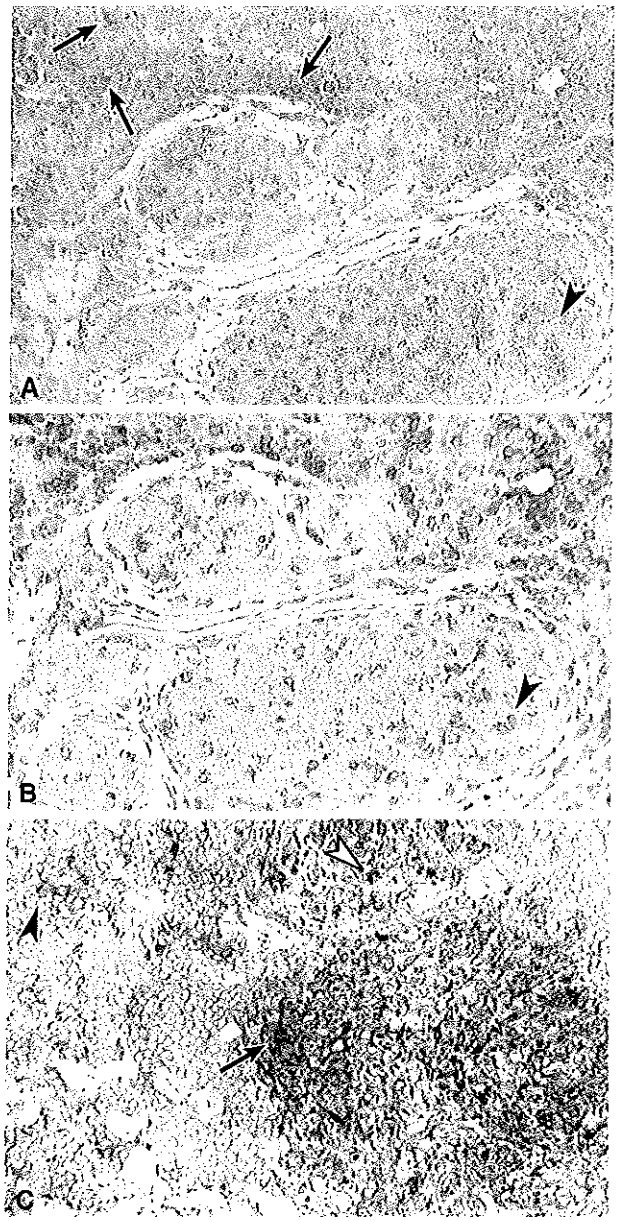

Figure 4. Identification of cell types in a sarcoid lymph node (patient 3) which express sst_{2A} by immunohistochemical single and double staining. A. Sst_{2A} (red) is expressed by cells within the granuloma, but also by some cells in the surrounding mononuclear infiltrate (arrows). B. CD3-positive T lymphocytes (red) in the adjoining section do not correspond to the sst_{2A}-expressing cells (black arrowheads in A and B). C. The majority of cells within the granuloma that express sst_{2A} (blue), also express CD68 (red), resulting in a violet double staining (black arrows). A number of CD68-positive but sst_{2A}-negative cells can be observed within the infiltrate surrounding the granulomas (red cells; black arrowhead). Cells that express sst_{2A} but not CD68 are rare; they may represent plasma cells (blue cells; white arrowhead) (all original magnification x250).

The biopsies from patients 4, 5 and 7 (see Table 1) were not stained by the antiserum directed against sst_{2A}. All three of these biopsies were characterized by partial fibrosis and the presence of only a limited mononuclear infiltrate consisting of small lymphocytes. Moreover, patient 5 was treated with non-steroid anti-inflammatory therapy prior to biopsy and patient 7 had undergone intensive chemotherapy for an embryonal cell carcinoma 5 years previously. It is not known whether these treatments influence the sst expression.

We carried out immunohistochemical staining with monoclonal antibodies directed towards phenotypic markers, in order to further characterize the sst_{2A}-positive cells. The results are shown in Figure 4. The sst_{2A}-positive cells within the granuloma and in the mononuclear infiltrate surrounding the granuloma (Figure 4A) did not correspond to the CD3-positive T lymphocytes in the adjacent section (Figure 4B). The majority of cells within the granuloma and in the surrounding infiltrate that express sst_{2A} also expressed CD68 in immunohistochemical double staining, demonstrating that they are of macrophage origin (Figure 4C).

Two patients with sarcoidosis and positive immunostaining for sst_{2A} receptors (patients 3 and 10; see Table 1) were treated with octreotide, a somatostatin analogue with high affinity for sst_2 and sst_5, in a dose of 100 µg subcutaneously, three times daily for 4 months. Biopsies from both patients stained positive for sst_{2A} using the specific antiserum (see Table 1). Patient 10, who suffered from pulmonary sarcoidosis with mediastinal lymphadenopathy, showed no clinical response to this therapy: the size of the lymph nodes and the serum levels of angiotensin-converting enzyme (ACE) did not change. The initial 1,25-dihydroxy-vitamin D level decreased from 187.5 pmol L^{-1} to 117.2 pmol L^{-1} after one month of treatment but increased again during therapy the next two months to 170.0 pmol L^{-1} (reference value 39.4-101.9 pmol L^{-1}). In contrast, patient 3 responded very well: the skin granulomas (refractory for 4 years; treatment had been discontinued) had disappeared after 3 months of octreotide therapy and the lymphadenopathy had improved markedly. Serum levels of 1,25-dihydroxy-vitamin D and ACE were within the normal range in this patient. *In vivo* somatostatin receptor scintigraphy performed 2 weeks after cessation of octreotide treatment showed a marked decrease in uptake of radioactivity as compared to the scintigraphy performed before the start of therapy (Figure 5). It remains a possibility that this decrease in uptake of radioactivity was due to receptor desensitization caused by the octreotide treatment even though the treatment had been discontinued. Not much is known yet about the kinetics of receptor desensitization. However, this issue is being addressed in a larger study currently being carried out in our hospital.

Discussion

In a previous study [7], we detected somatostatin receptors in a lymph node biopsy containing sarcoid granulomas by autoradiography. Autoradiography using radionuclide-labeled octreotide detects sst_5 and also both sst_{2A} and sst_{2B}, as there is no difference in the ligand-binding properties of these two splice variants [8].

The immunohistochemical technique employed in this study has a higher resolution and thus we were able to identify sst_{2A} expression by epithelioid cells and giant cells within the granuloma, and additionally on a subset of macrophages in the surrounding infiltrate.

We found positive staining with this antiserum in 9 out of 12 sarcoid granuloma biopsies. This percentage corresponds to the high percentage of sarcoidosis patients reported positive for *in vivo* somatostatin receptor scintigraphy [11]. Three biopsies were negative.

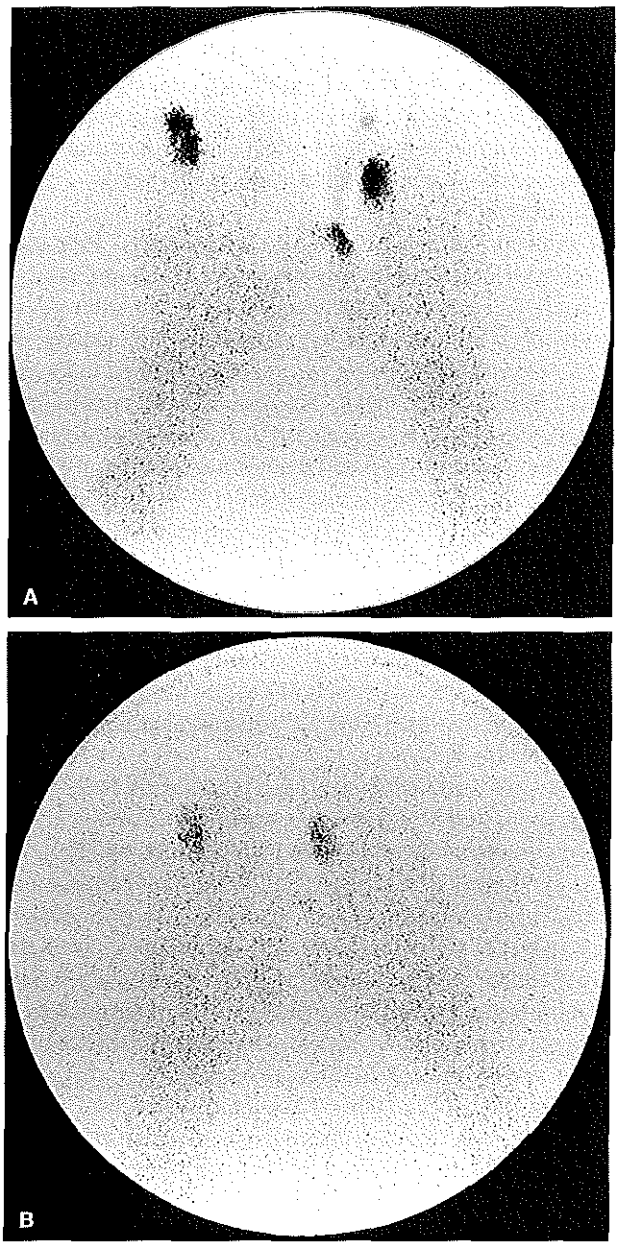

Figure 5. *In vivo* somatostatin receptor scintigraphy (Octreoscan) of skin nodules of a sarcoidosis patient (patient 3). A. Uptake of radiolabel before the start of octreotide treatment. B. Uptake of radiolabel is greatly reduced after 3 months of treatment with octreotide. Scintigraphy was performed 2 weeks after cessation of octreotide treatment.

All three were characterized by fibrosis and the absence of an active mononuclear infiltrate. Previously we have shown by autoradiography that a sclerotic sarcoid lymph node did not express somatostatin receptors [7]. One of these patients was treated with anti-inflam-

matory medication, while another had previously undergone intensive cancer chemotherapy. It is not clear whether these treatments might have influenced the sst expression.

The specific antiserum was directed only to the sst_{2A} splice variant. Therefore it is not yet clear whether sst_{2B} is also expressed in granulomatous diseases such as sarcoidosis. As of yet, no antisera are available to study the other human receptor subtypes possibly of interest for binding of somatostatin analogues such as octreotide, i.e. the subtypes 2B, 5 and to a lesser extent subtype 3.

The antiserum showed staining of intracellular granules. This may point either to newly synthesized receptor molecules in vesicles of the Golgi and trans-Golgi network or alternatively to internalized receptor molecules. We favor the latter possibility. An immunohistochemical study of rat brain showed two distinct staining patterns using this particular antiserum. In some regions the staining was mainly confined to the plasma membrane, in other regions it was mainly intracellular. The regions with mainly intracellular staining contained high amounts of endogenous somatostatin, and it was suggested that the intracellular staining might be due to ligand-induced receptor internalization [12]. Granuloma macrophages may be capable of producing somatostatin [13, 14], suggesting that the receptors detected in this study may be involved in autocrine signaling. Alternatively, sensory nerves may be a source of somatostatin within the tissue.

In the present study we report for the first time the treatment with octreotide of 2 patients with sarcoidosis, one of whom showed an excellent clinical response. These observations suggest that octreotide treatment may be beneficial in sarcoidosis and possibly in other granulomatous diseases. The expression of sst_{2A} by macrophages and epithelioid cells in sarcoidosis, giant cell arteritis and Wegener's granulomatosis suggests that these cells may be a target for octreotide therapy.

Somatostatin and somatostatin analogues have been shown to have a suppressive effect on the immune system (reviewed in [6]). *In vitro*, somatostatin inhibits lymphocyte proliferation and the release of certain cytokines (TNF-α, lymphotoxin and IFN-γ). Recently, inhibition of the release of cytokines (TNF-α, IL-1β, IL-6 and IL-8) by monocytes was reported [15], although there are also conflicting reports [16]. We have previously demonstrated expression of sst_{2A} by a subset of macrophages in synovium biopsies from rheumatoid arthritis patients [17] and by medullary epithelial cells [18] and macrophages (L.J. Hofland and D. Ferone, personal communication) in the human thymus. Macrophages and other cells of the mononuclear phagocyte lineage may therefore be a target for somatostatin.

Somatostatin and somatostatin receptors are expressed in *Schistosoma* egg-induced hepatic granulomas in mice [13, 19]. CD4+ T lymphocytes isolated from these granulomas expressed mRNA for sst_2 [19], whereas macrophages isolated from the granulomas were shown to express mRNA for preprosomatostatin [14]. Administration of octreotide decreased the secretion of IFN-γ by 70% and diminished the size of the granulomas in the mouse schistosomiasis model [20]. In the present study, the results of octreotide treatment were promising for 1 out of 2 sarcoidosis patients; however, further investigations are necessary to establish the indications and efficacy and the appropriate dosage of octreotide in the treatment of granulomatous diseases.

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Chapter 5.3

SOMATOSTATIN RECEPTORS IN INFLAMMATORY BOWEL DISEASE: *IN VITRO* LOCALIZATION USING A POLYCLONAL ANTI-SOMATOSTATIN RECEPTOR SUBTYPE 2A ANTISERUM AND *IN VIVO* VISUALIZATION BY [^{111}IN-DTPA-D-PHE^1]-OCTREOTIDE SCINTIGRAPHY

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Abstract

Somatostatin is a small neuropeptide, which acts on multiple non-neural targets, including the gastrointestinal tract and the immune system. In inflammatory bowel disease (IBD) mucosal somatostatin is diminished and its receptor density is increased.

In a combined study of endoscopy, pathology, immunohistochemistry and [^{111}In-DTPA-D-Phe^1]-octreotide scintigraphy, we describe the somatostatin receptor (sst) expression in 14 patients with clinically active IBD (11 Crohn's disease (CD), 3 ulcerative colitis (UC)), localized in colon and/or terminal ileum. Patients underwent colonoscopy and octreotide scintigraphy on the same day. Mucosal biopsy samples and in 3 cases also bowel resection specimens were studied by immunohistochemistry, using a polyclonal rabbit anti-sst_{2A} antiserum.

Mild to moderately active chronic inflammation was seen in colon and/or ileum biopsy specimens. Scintigraphy showed bowel uptake of [^{111}In-DTPA-D-Phe^1]-octreotide in IBD. Such uptake was also seen in non-IBD control subjects. No correlation was found between classical disease activity, endoscopy and histology on the one hand, and scintigraphy positivity on the other.

Immunohistochemistry showed sst_{2A}-positive staining of blood vessel endothelium in the submucosa and mucosa in both CD and UC, with a slight preference for actively inflamed parts of the mucosa. In several CD cases, superficial epithelium was positive, with a striking paranuclear staining. In UC patients, sst_{2A}-positive fibroblast-like cells and macrophages were seen.

We conclude that sst_{2A}-positive cells are found in intestinal biopsies in active IBD. This receptor expression can be visualized by immunohistochemical techniques. Although bowel uptake of [^{111}In-DTPA-D-Phe^1]-octreotide was found, its specificity was poor, as it was also found in control subjects and had no relation with clinical and endoscopical disease activity.

Introduction

Somatostatin is a small neuropeptide, which has two principal bioactive configurations consisting of 14 or 28 amino acids. Somatostatin acts on specific G-protein coupled transmembrane receptors in multiple targets in the central nervous system, endocrine glands and exocrine pancreas, the gastrointestinal tract and the immune system [1]. Somatostatin receptors (sst) can be demonstrated by *in vitro* receptor autoradiography and by *in vivo* somatostatin receptor scintigraphy with [^{111}In-DTPA-D-Phe^1]-octreotide (octreotide scintigraphy) [2, 3]. [^{111}In-DTPA-D-Phe^1]-octreotide binds with high affinity to sst subtypes sst_2 and sst_5 and has a low biliary excretion, making abdominal imaging possible. Octreotide scintigraphy has been tested extensively in recent years as a tool in the diagnosis of hematological malignancies, and granulomatous and autoimmune diseases [4]. Receptor density in general correlates with disease activity and octreotide scintigraphy has been proposed as a method for evaluation of these disorders [5-8].

Previously, sst expression has been found by autoradiography in gut-associated lymphoid tissue, leukocytes, and at several intestinal mucosal and submucosal sites in the gut [9, 10]. Increased density of vascular sst was observed in blood vessels surrounding inflammatory lesions in Crohn's disease (CD) and ulcerative colitis (UC), and in peri-inflammatory venules in rheumatoid arthritis, suggesting a role for somatostatin in the regulation of inflammatory processes [11, 12]. Somatostatin has suppressive effects on the immune system *in vivo* and *in vitro* [10, 13-15]. mRNA for all five sst subtypes was found in rat intestinal tissue [16], however in humans and mice sst_2 mRNA predominates [17].

In the present study we investigated sst_2 expression in the intestinal wall by immunohistochemistry with an anti-sst_{2A} antiserum and compared the findings with the results of octreotide scintigraphy in 14 patients with active inflammatory bowel disease (IBD), because in patients with neuroendocrine tumors and immunological diseases positivity on octreotide scintigraphy correlates well with the presence of sst_2 [18].

Patients and methods

Patients and clinical evaluation

Fourteen consecutive patients with clinically active IBD, 3 with UC and 11 with CD of colon and/or terminal ileum, who were scheduled for colonoscopy to monitor disease activity, were enrolled in this study after obtaining informed consent. Clinical disease activity was assessed using a Powell-Tuck scoring system (PTS) for UC or a modified Crohn's Disease Activity Index (mCDAI) [19, 20]. Each patient was his own control with respect to actively inflamed and non-inflamed mucosal areas.

All patients were prepared for endoscopy with oral laxative solution (Klean-prep®). Colonoscopy was performed 18 hrs later. Endoscopic evaluation of CD and UC was performed according to operative criteria [21-23]. A digital video endoscopy report was obtained from each colonoscopy and reviewed without knowledge of scintigraphy findings.

Octreotide scintigraphy

One day before colonoscopy, 200 to 250 MBq (10 μg) of [^{111}In-DTPA-D-Phe^1]-octreotide was administered intravenously. Planar abdominal images were obtained and single positron emission computed tomography (SPECT) was performed 24 hrs after injection of the ra-

diopharmaceutical, as described previously [2, 24]. Scintigraphic images were examined in a blinded fashion.

Histological evaluation
Biopsies were taken from inflamed and non-inflamed mucosa for microscopic evaluation. At least 5 biopsies were taken from each patient from right, middle and left sided colon, rectosigmoid, and if possible from terminal ileum. After haematoxylin-eosin staining, inflammation was classified as 0 (absent), 1 (mild chronic), 2 (moderate chronic and/or mild active), 3 (severe chronic, moderate active), or 4 (ulcerative).

Immunohistochemistry
For immunohistochemistry, biopsies were obtained from the most inflamed parts of colon or ileum. Moreover, bowel resection specimens were obtained from 3 patients (2 CD, 1 UC). Biopsy or bowel resection specimens were fixed in buffered formalin 10% (v/v) and embedded in paraffin wax. Sections (5 μm) were mounted on 3-amino propyl silane-coated glass slides. Immunohistochemical analysis was performed according to standard methods using a polyclonal rabbit anti-sst_{2A} antiserum [25], as described previously [26].

Results

Patient characteristics
Patient characteristics are presented in Table 1. One patient (patient 9) had been treated with radiotherapy for prostate carcinoma and had developed a radiation proctitis. Six patients had undergone surgery for CD at least two years before this study. Thirteen patients used 5-ASA, steroids, azatioprine, or a combination of these, whereas one did not receive drug treatment at the time of this study. The patients from whom bowel resection specimens were obtained, had all been treated with one of these medications in the month before surgery.

Clinical and histological evaluation
The results of endoscopy, histology and scintigraphy are shown in Table 2. Laxation was sufficient in 12 patients. Two UC patients still had some coecal fecal stasis. Histology could be obtained from most colon segments and terminal ileum. In five patients (8, 10, 11, 12, and 14) the terminal ileum could not be reached. Histology only showed granulomata in ileum biopsies in 1 patient (patient 3). Patchy inflammation was seen in all CD patients. Inflammation was also found in biopsies of intestinal segments, which had a normal appearance at endoscopy. In UC a marked chronic ulcerative inflammation was seen in the most severely inflamed parts.

Octreotide scintigraphy
Octreotide scintigraphy in non-IBD controls (n=5) showed positivity in the colon region in all patients and small intestine positivity in 4. Apart from uptake in liver, spleen and kidneys, no additional abdominal uptake was observed. In CD, all patients showed intestinal uptake of the radiopharmaceutical 24 hrs after injection. In UC one patient did not show any intestinal uptake. One other patient showed small as well as large intestine positivity.

In 6 patients with CD, increased uptake of radioactivity was found at sites of endoscopic disease. In 5 of these patients increased uptake was also seen at endoscopically

normal sites. In 3 patients, endoscopic disease was not accompanied by increased uptake. One patient (patient 1) showed no endoscopic changes, but scintigraphy showed uptake in the right-sided colon and small intestine. This patient suffered only from periods of intractable diarrhea and 'silent' perianal disease. Histology and scintigraphy did not correlate well. In 4 patients, biopsies from segments which were found positive at scintigraphy showed no abnormalities (patients 2,4,7,9). In 6 patients inflamed intestinal sections did not show up at scintigraphy (patients 1,3,4,6,7,8).

Table 1. Clinical characteristics of the patients in this study

Patient number	Age	Sex	Disease activity score*	Therapy
1	70	f	10	5ASA
2	48	f	12	none
3	34	f	5	5ASA
4	46	m	10	CS, azath
5	45	f	12	5ASA, CS, azath
6	46	f	8	CS[1]
7	39	f	5	azath, CS[1]
8	42	f	7	5ASA
9	60	m	9	5ASA
10	43	f	2	5ASA
11	35	f	10	SASP
12	32	m	11	5ASA (7 yrs)+ CS (1 yr)
13	70	m	12	CS (10 yrs)
14	59	m	9	SASP

* Patients 1-11: diagnosis Crohn's disease; disease activity scored using modified Crohn's disease activity index (mCDAI; ref. 20)
Patients 13-15: diagnosis ulcerative colitis; disease activity scored using the Powell-Tuck disease activity score (PTS; ref. 19)

[1] Budesonide ileal release orally

5ASA = 5-aminosalicylic acid; SASP = salicylazosulfapyrimidine; CS = corticosteroids; azath = azathioprine

Immunohistochemistry

Results of immunohistochemistry with an anti-sst_{2A} antiserum are shown in Table 3. Immunohistochemistry showed positivity in all but one specimen (patient 9). Endothelium of mucosal or submucosal capillaries and venules was positive in 10 out of 11 of the positive patients, most pronounced in or in the proximity of leukocyte infiltrates (see Figures 1A and B). Superficial epithelium showed slight sst_{2A} positivity in 6 CD patients (not shown). A remarkable paranuclear staining was seen in these cases. Crypt epithelium positivity was observed in 2 CD patients and all 3 UC patients (see Figure 1C). Focally, sst_{2A}-positive plasma cells, ganglion cells and macrophages were seen in CD patients. All UC biopsies showed positive macrophages. Sst_{2A} expression by fibroblast-like cells was also occasionally observed (see Figure 1D).

Discussion

Somatostatin is a small neuropeptide, which acts in various tissues and has mainly inhibitory effects [1]. Its specific receptors are found in the CNS, the digestive tract and the immune system. Five distinct sst (sst_{1-5}) have been identified, each with different binding characteristics for somatostatin and different somatostatin analogues including octreotide [27, 28]. *In vivo* sst expression can be revealed by scintigraphy with [^{111}In-DTPA-D-Phe^1]-octreotide, which binds mainly to sst_2 and sst_5 [18]. The application of octreotide scintigraphy has been well established in the diagnosis and staging of neuroendocrine tumors [29]. Receptor positivity is also found in breast cancer, small cell lung cancer, malignant lymphomas and granulomatous disease [29, 30]. Normally, uptake of [^{111}In-DTPA-D-Phe^1]-octreotide is seen in the pituitary, thyroid, liver, spleen and kidneys [30]. It has a largely renal clearance and only small amounts show up in the feces [24]. Octreotide is used for the staging and monitoring of abdominal lesions like gastrinomas and other neuroendocrine tumors, because these diseases show high sst density [31]. However, a small amount of intestinal background activity due to fecal elimination of [^{111}In-DTPA-D-Phe^1]-octreotide is seen in most cases [18, 32-35].

In this study we show for the first time intestinal octreotide scintigraphy in IBD. In 10 CD patients and in one UC patient octreotide scintigraphy was found positive. In non-IBD control subjects, large intestine positivity was also seen. This could be due to ineffective bowel cleansing. As scintigraphy and colonoscopy were performed on the same day in our study of IBD patients, the effectiveness to the laxative protocol could be visually monitored. Laxation had been sufficient in all patients who underwent colonoscopy. However, even small quantities of radioactivity released into the bowel may be visible on scintigraphy despite a laxation that is judged sufficient for colonoscopy. In IBD patients no unequivocal relation of receptor localization and either clinical or endoscopic disease activity was observed; normal endoscopic findings often coincided with increased uptake of radioactivity. There was also no unequivocal relation between increased uptake of radioactivity and inflammatory changes at histological evaluation of mucosal biopsies. We therefore conclude that octreotide scintigraphy is unsuitable for identifying the localization of intestinal inflammatory lesion and monitoring disease activity in IBD.

We used a newly developed polyclonal antiserum directed against the intracellular C-terminal domain of sst_{2A} [25] to study the expression of this sst subtype in mucosal biopsies and bowel resection specimens from IBD patients. Sst_{2A} positivity was observed in all but one of the specimens. In patients taking steroids, sst_{2A} was expressed in the same density as in those on 5-ASA monotherapy. This indicates that steroids do not per se down-regulate sst expression.

In the biopsies the endothelium of blood vessels in the mucosa and often also in the submucosa expressed sst_{2A}. This positivity was observed mainly near leukocyte infiltrates. By autoradiography sst expression can be detected in IBD associated with mucosal venules, particularly those near actively inflamed sites, but not in arteries [12]. The authors speculated that somatostatin might counterregulate the proinflammatory, vasodilatative effects of other neuropeptides such as vasoactive intestinal peptide and substance P [12]. Increased sst density in IBD might also be the result of a feedback effect of the diminished mucosal somatostatin content that has been described in connection with IBD [36-38]. However, a marked density of sst has also been described in vessels surrounding human colon adenocarcinoma, without signs of inflammation [39].

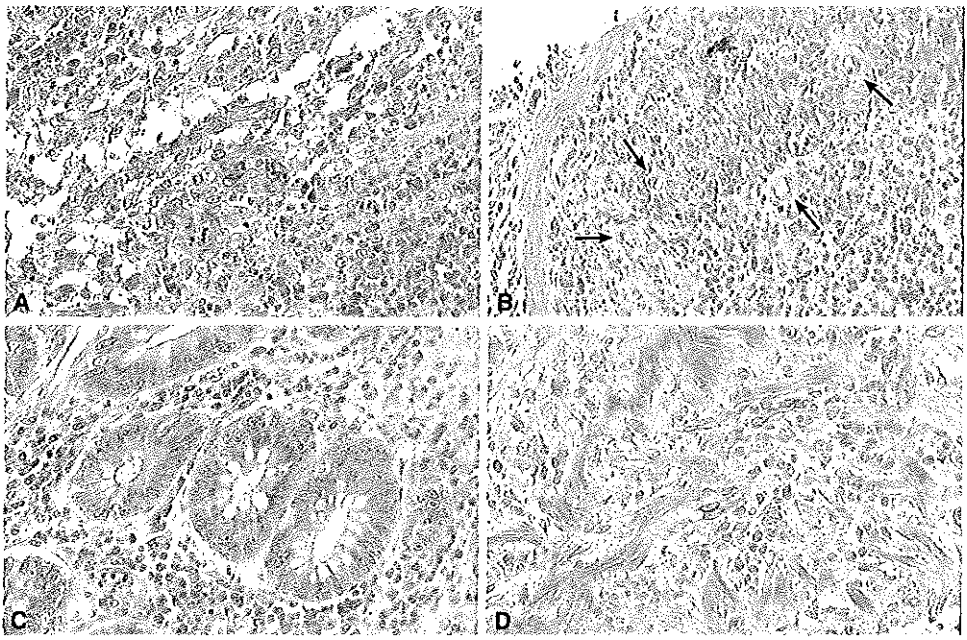

Figure 1. Immunohistochemical staining of mucosal biopsies of patients with inflammatory bowel disease, using a polyclonal antiserum directed against somatostatin receptor subtype 2A. A. Positively stained endothelium cells of a small venule in the mucosa of patient 11. B. Positively stained capillary endothelium within a mononuclear cell infiltrate in the lamina propria of patient 3. C. Paranuclear staining of crypt epithelium cells in the mucosa of patient 11. D. Positively stained fibroblasts in close apposition to collagen fibrils within the lamina propria of patient 14. (All: original magnification x400).

Most IBD specimens also showed sst_{2A} positivity at superficial and crypt epithelium. Epithelium staining was not only seen at the cell membranes, but also paranuclear. This could be due to non-specific binding, especially in goblet cells. However, earlier autoradiography studies also describe an increased density of sst in intestinal epithelium [40, 41]. The effect of somatostatin on intestinal epithelium is associated with inhibition of regeneration, but it might also have an effect on mucosal permeability or secretion [42, 43].

An interesting observation is the dispersed fibroblast-like cells in UC that stain positive for sst_{2A}. Sst_2 expression has been described in lung and intestinal fibroblasts [44]. Somatostatin may have an inhibitory effect on fibroblasts [45], although in another study such an effect was not observed [46]. In all UC biopsies, sst_{2A}-positive macrophages were observed. We have previously demonstrated expression of sst_{2A} by a subset of macrophages in synovium biopsies from rheumatoid arthritis patients [26] and in granuloma macrophages in sarcoidosis and other granulomatous diseases [47]. Recently, inhibition of the release of cytokines (TNF-α, IL-1β, IL-6 and IL-8) by monocytes was reported [48], although there are also conflicting reports [49]. We speculate that fibroblasts and macrophages may be a target for IBD therapy with somatostatin or somatostatin analogues.

Table 2. Results of endoscopy, histology and scintigraphy

Patient number	Location	Endoscopy	Histology*	Scintigraphy
1	Ileum	NI	1	pos
	R. colon	N	1	pos
	M. colon	N	1	neg
	L. colon	N	1	neg
	Rectosigmoid	N	1	neg
2	Ileum	N	0	neg
	Ileoascendostomy	Ulcers	2	pos
	R. colon	N	0	pos
	M. colon	N	0	neg
	L. colon	N	0	pos
	Rectosigmoid	N	0	pos
3	Ileum	N	2	neg
	Ileoascendostomy	Ulcers	2	pos
	R. colon	N	2	pos
	M. colon	N	2	neg
	L. colon	N	2	pos
	Rectosigmoid	N	2	neg
4	Ileum	N	0	pos
	R. colon	N	1	neg
	M. colon	N	1	neg
	L. colon	Erythema	1	neg
	Rectosigmoid	Ulcerative stenosis	2	neg
5	Ileum	Aphthae	0	neg
	R. colon	N	1	pos
	M. colon	N	1	pos
	L. colon	Aphthae	1	pos
	Rectosigmoid	Aphthae	1	pos
6	Ileum	NI	1	neg
	Ileoascendostomy	Ulcerative stenosis	1	pos
	R. colon	N	1	pos
	M. colon	N	1	pos
	L. colon	Aphthae	1	pos
	Rectosigmoid	Aphthae	1	pos
7	Ileum	Ulcerative stenosis	1	neg
	R. colon	N	0	pos
	M. colon	N	0	pos
	L. colon	N	1	pos
	Rectosigmoid	N	1	
8	Ileum	NI		neg
	Ileoascendostomy	Ulcers	2	pos
	R. colon	N	0	pos
	M. colon	N	0	neg
	L. colon	N	0	neg
	Rectosigmoid	N	0	neg

9	Ileum	N	0	pos
	Ileoascendostomy	Aphthae	0	pos
	R. colon	N	0	pos
	M. colon	N	0	neg
	L. colon	N	0	neg
	Rectosigmoid	Radiation proctitis	0	neg
10	Ileum	NI		neg
	R. colon	Pseudopolyps	1	pos
	M. colon	N	0	neg
	L. colon	N	0	neg
	Rectosigmoid	N	0	neg
11	Ileum	NI		ND
	R. colon	Ulcers	2	
	M. colon	Ulcers	2	
	L. colon	Ulcers	2	
	Rectosigmoid	Ulcers/ Pseudopolyps	2	
12	Ileum	NI		ND
	R. colon	NI		
	M. colon	N		
	L. colon	Erythema	1	
	Rectosigmoid	Ulcers	2	
13	Ileum	N	1	neg
	R. colon	Erythema	2	neg
	M. colon	Erythema	2	neg
	L. colon	Atrophy	2	neg
	Rectosigmoid	Sporadic ulcers	2	neg
14	Ileum	NI		pos
	R. colon	Superficial ulcers	1	pos
	M. colon	Ulcers	2	pos
	L. colon	Ulcers	2	neg
	Rectosigmoid		2	neg

* 0 = absent; 1 = mild chronic; 2 = moderate chronic and/or mild active;
3 = severe chronic, moderate active; 4 = ulcerative
R. = right; M = middle; L. = left
NI = not inspected; N = normal
pos = positive; neg = negative

Table 3. Results of sst_{2A} immunohistochemistry on biopsy and resection specimens

Patient number	Biopsy	Positively stained cells/ structures
1	Colon	Endothelium (++)
		Endothelium in submucosa
		Plasma cells (focal)
		Superficial epithelium (paranuclear)
		Crypt epithelium (focal)
2	Ileo-asc. anastomosis	Superficial epithelium (paranuclear)
3	Colon	Endothelium
		Plasma cells (focal)
		Superficial epithelium (paranuclear)
4	Colon	Endothelium
7	Ileo-asc. anastomosis	Superficial epithelium (paranuclear) especially near infiltrate
		(endothelium not biopsied)
7	Ileum resection	Superficial epithelium (paranuclear)
		Endothelium near infiltrate or ulcers
8	Ileo-asc. anastomosis	Endothelium
		Superficial epithelium (paranuclear)
		Ganglion cells (focal)
		Fibroblast-like cells (focal)
9	Ileo-asc. anastomosis	None
10	Colon	Endothelium
		Superficial epithelium (paranuclear)
11	Colon	Crypt epithelium
		macrophages (focal)
		capillary endothelium (++)
11	Colon resection	Crypt epithelium
		Endothelium (mucosa)
		Endothelium (musc. ext.) and surrounding infiltrate
12	Colon	Endothelium
		Submucosal endothelium
		Crypt epithelium
		Fibroblasts / macrophages
13	Colon	Endothelium (focal)
		Crypt epithelium (focal)
		Fibroblast-like cells (focal)
14	Colon	Endothelium
14	Colon resection	Endothelium (++) (mucosa: focal)
		Fibroblast / macrophages (near musc. mucosae)
		Crypt epithelium (focal)

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Chapter 6

GENERAL DISCUSSION

Chapter 6

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Somatostatin is a neuropeptide with generally inhibitory actions in the nervous and endocrine systems. Additionally, somatostatin has been shown to have immunomodulatory effects, which are also predominantly suppressive in nature. Somatostatin receptors have been shown to be present in the inflammatory lesions of a number of human immune-mediated diseases by *in vivo* somatostatin receptor scintigraphy [1]. The synthetic, metabolically stable octapeptide somatostatin analogue octreotide was used in these human studies, indicating that the detected somatostatin receptors were of the subtypes sst_2, sst_5 or possibly sst_3. Although it is not known which cells within the inflammatory lesions express these sst, they represent possible targets for treatment of these diseases by local or systemic administration of somatostatin analogues.

Somatostatin receptor subtype expression in the immune system of experimental animals
For the purpose of preclinical studies into the effects of somatostatin analogues in the treatment of autoimmune diseases and chronic inflammatory diseases, animal models have to be established which mirror the human diseases as closely as possible. Because synthetic, metabolically stable somatostatin analogues such as octreotide interact only with the sst subtypes sst_2 and sst_5, it is important to study the sst subtype expression in the immune system of the animal model of choice. In chapter 2 we showed by RT-PCR and receptor binding studies that sst_3 and sst_4 are the main sst subtypes expressed in the rat immune system, and that the sst subtypes with high affinity for octreotide, i.e. sst_2 and sst_5, are not expressed. We concluded that the rat is not a suitable model for studying the immunomodulatory effects of somatostatin analogues such as octreotide.

We subsequently investigated the sst subtype expression in the immune system of normal mice, using *in vivo* receptor scintigraphy and *in vitro* receptor autoradiography with radioisotope-labelled octreotide, and RT-PCR of whole lymphoid organs and isolated cells (chapter 3). We found a high intensity of binding sites for octreotide in the murine thymus, but not in lymph nodes or spleen. In agreement with these results, we found expression of mRNA for sst_2 in the thymus and isolated thymocytes, but not in the other lymphoid organs. We hypothesised that the observed sst_2 expression is confined to the most immature thymocytes. Sst expression has also been detected in $CD34^+$ progenitor cells in murine bone marrow using fluorescently labelled somatostatin-28. No sst expression was found in the bone marrow of sst_2-deficient mice, indicating that sst_2 is the main sst subtype expressed by haematopoietic progenitor cells in the mouse (S. Oomen, unpublished observations). Studies by the group of Weinstock have shown that sst_2 is expressed by T-lymphocytes in murine schistosome granulomas [2], indicating that up-regulation of this sst subtype may be induced in the mouse by inflammatory stimuli. The presence of this sst subtype makes mice promising animal models for studying the possible beneficial effects of octreotide treatment in immune-mediated disease.

Effects of systemic octreotide treatment in animal models of autoimmune and chronic inflammatory diseases

Somatostatin, and to a lesser extent somatostatin analogues, have been shown to be beneficial in several *in vivo* models of immune-mediated disease (see chapter 1), e.g. experimental arthritis in different animal species. We therefore investigated whether systemic octreotide, in the form of the slow-release formulation Octreotide-LAR [3], was capable of modulating the disease course in animal models of chronic inflammatory diseases such as arthritis, and autoimmune diseases such as systemic lupus erythematosus and experimental autoimmune encephalomyelitis (chapters 2 and 4). No effects of systemic octreotide treatment were found on either the incidence or the severity of adjuvant arthritis in Lewis rats. This could be explained by the absence of expression within the rat immune system of sst subtypes with high affinity for octreotide (chapter 2).

Sst_2, a sst subtype with high affinity for octreotide, is expressed within the murine immune system (chapter 3 and [2]). Therefore we studied the effects of systemic octreotide treatment in murine models of immune-mediated disease (chapter 4). Systemic octreotide did not influence the severity of spontaneously occurring arthritis or immune-mediated kidney damage in MRL/*lpr* mice. A slight (marginally significant) decrease in the titres of autoantibodies directed against dsDNA was observed. This correlates with the suppressive effects of somatostatin on the humoral immune response seen in other murine models, but also in rats and in *in vitro* studies using human B lymphocyte cell lines [4-8]. In a murine model of autoimmune encephalomyelitis, the effects of systemic octreotide treatment were ambiguous: in one experiment a significant reduction in disease severity was observed in mice which had received 30 mg/kg octreotide (as Octreotide-LAR), whereas in another experiment with an extended dose range, no effects were observed. Only one treatment regimen, i.e. a subcutaneous depot of Octreotide-LAR, was used in these studies. However, a short course of octreotide by injection in the early stages of the disease might give different results [9]. Clearly, these studies warrant further investigation, possibly using simpler disease models in which it is easier to distinguish local (immunomodulatory) and systemic (via endocrine or nervous system) effects of octreotide treatment. Potential models include air pouch inflammation [10], skin granulomas and localised DTH reactions. Other animal species in which somatostatin treatment has been shown to be beneficial, e.g. rabbits, [11], might also be included in these investigations.

Cellular localisation of sst_2 in human immune-mediated diseases

In a number of immune-mediated diseases in which the inflammatory lesions can be visualised *in vivo* by octreotide scintigraphy, the localisation of the octreotide binding sites was investigated in tissue biopsies by *in vitro* receptor autoradiography [12-14]. In chronic inflammatory diseases such as RA and IBD, octreotide-binding sites were found to be associated with venules in the vicinity of the inflammatory infiltrates; in granulomatous diseases the binding sites were associated with the epithelioid cell areas. However, the resolution of the autoradiography technique is not high enough to identify single sst-expressing cells. Despite the positive correlation of octreotide binding with the expression of mRNA for sst_2 [15, 16], a role for sst_5 or sst_3 in the observed binding cannot be ruled out. Nevertheless, as sst_2 appears to be the major sst subtype expressed in the human immune system [17, 18], it is assumed that the observed octreotide binding in immunological diseases is mediated mainly by sst_2. In chapter 5 we investigated by immunohistochemistry the cellular localisation of sst_2 protein in tissue biopsies from a number of such diseases, using a polyclonal

antiserum directed against a conserved epitope in the intracellular C-terminal domain of the sst_2 receptor. In biopsies of rheumatoid synovium and inflamed gastrointestinal mucosa of patients with IBD, we detected sst_{2A} expression in endothelial cells of venules (especially HEV) and capillaries in the vicinity of mononuclear cell infiltrates. This correlated with earlier autoradiography studies [12, 13]. In addition, we detected sst_{2A} expression in fibroblast-like cells and in a subset of cells of the monocyte/macrophage lineage [19]. In biopsies from patients suffering from granulomatous diseases such as sarcoidosis and Wegener's granulomatosis, sst_{2A} expression was also associated with cells of the mononuclear phagocyte lineage, including epithelioid cells and multinucleated giant cells within the granulomas [20]. In none of the biopsies did we observe sst_{2A} expression by T lymphocytes, while in the IBD specimens sst_{2A}-expressing plasma cells were occasionally observed. On the basis of classical ligand-binding studies and RT-PCR studies it had previously been assumed that lymphocytes were the major cell types expressing sst_2 in inflammation [17, 18]. Based on our present studies this does not appear to be the case. Preliminary results of RT-PCR studies on isolated human peripheral blood cells support these findings: resting and activated peripheral blood T- and B-lymphocytes express mRNA for sst_3, whereas only activated peripheral blood monocytes express mRNA for sst_2 (E.G.R. Lichtenauer-Kaligis, unpublished observations). As the antiserum we used in these studies was directed against the sst_{2A} variant of the sst_2 receptor, it is not yet clear whether sst_{2B} is also expressed in these diseases.

The presence of sst_2 on blood vessel endothelial cells and on macrophages makes these cells potential targets for octreotide treatment. Somatostatin receptors found on endothelial cells are hypothesised to have a role in decreasing extravasation of plasma and leukocytes [10], and may also have an inhibitory effect on the proliferation of vascular endothelium [21, 22]. It is interesting to note that in our studies the staining for sst_{2A} appeared to be confined to the luminal surface of the endothelial cells. This may suggest that these receptors interact with circulatory somatostatin in addition to somatostatin that is produced locally by synovial cells or macrophages, or released by nerve cells. Locally released somatostatin has been shown to suppress neurogenic inflammation at remote sites via the circulation [23, 24]. Somatostatin might be able to modulate macrophage functions such as chemotaxis or the release of proinflammatory cytokines and reactive oxygen species (see chapter 1).

Potential for octreotide treatment in human immune-mediated diseases

Because of their importance in inflammation, suppression of macrophage functions and/or endothelium activation may result in decreased inflammation. Intra-articular administration of somatostatin-14 was shown to decrease pain and synovial swelling in patients with RA [25, 26]. It is tempting to hypothesise that a systemically administered somatostatin analogue might be of use for the treatment of rheumatoid arthritis. Systemic octreotide treatment was beneficial in one patient with sarcoidosis; however, in another patient octreotide had no effect [20]. There may also be a role for new subtype-specific somatostatin analogues in the treatment of these diseases [27-29].

Our studies point to a species difference in the expression of sst subtypes in cells of the immune system: in humans, sst_2 expression is associated with cells of the monocyte/macrophage lineage, whereas in mice this subtype appears to be expressed in T lymphocytes. Animal models may be useful for studying the functional role of sst expression in the immune system, but care must be taken in extrapolating findings from an animal model to disease in humans.

Suggestions for future research

A number of questions remain regarding the studies described above. First of all, it will be important to establish which cell populations in the murine thymus are responsible for the observed sst_2 expression ([2] and chapter 3). This could be studied using sorted cell populations, cell lines, or more sensitive methods of *in situ* detection of sst expression, both at the mRNA and the protein level. Furthermore, with a view to what has been found in humans, it should be investigated whether sst_2 is expressed or can be induced in cells of the murine monocyte/macrophage system. Sst_2-deficient mice [30, 31] also provide an excellent model to explore the role of sst_2 in the murine immune system and in immune-mediated disease. In view of the fact that mRNA for sst_2 is expressed in the thymus of normal mice, and somatostatin is expressed by dendritic cells in the thymic medulla [32], especially the T lymphocyte compartment of these mice needs to be investigated. Mice deficient for the other sst subtypes are expected to be under development also, enabling studies into the distinct roles of the different sst subtypes, e.g. sst_4 which has been found to be expressed in murine PBMC and spleen (chapter 3).

The functional significance of sst_2 expression by cells of the monocyte/macrophage system in human inflammatory disease (chapter 5) is not yet clear. The effects of somatostatin and somatostatin analogues such as octreotide on these cells need to be further investigated *in vitro* and *in vivo* using appropriate model systems such as isolated human cells and well-characterised cell lines. As cells may express different sst subtypes simultaneously [33], new subtype-specific somatostatin analogues may be valuable in such functional studies [27-29].

The presence and distribution of other sst subtypes in human immune-mediated diseases also needs to be further investigated, preferentially by immunohistochemistry, as this technique allows localisation of the receptor protein at the single cell level in mixed cell populations. New antisera are currently being developed that should make such studies possible [34, 35].

The somatostatin-substance P immunoregulatory circuit: a model

Systemic or local treatment with somatostatin or somatostatin analogues has been shown to be beneficial in a number of models for autoimmune disease and chronic inflammation (see chapter 1). The precise mode of action is not known in most of these cases, but in many of the models, somatostatin appears to antagonise the effects of another neuropeptide, substance P. Antagonism between somatostatin and substance P has been shown to be a regulatory mechanism in neurogenic inflammation [23, 24], in gastrointestinal inflammation [36] and in murine schistosomiasis mansoni [6, 37, 38]. A somatostatin-substance P immunoregulatory circuit has been proposed to be operational within murine schistosome granulomas [39]. In this chapter I would like to extend the model of the somatostatin-substance P immunoregulatory circuit to include data derived from model systems other than murine schistosomiasis, including humans. The extended model is shown schematically in Figure 1 and the different components of the model are described below. The regulatory system described in this model is part of a larger regulatory network in which other neuropeptides and cytokines may also play a role.

Antidromic release of substance P from peripheral terminals of afferent sensory nerves contributes to tissue inflammation by stimulating plasma extravasation and possibly inflammatory cell recruitment [40-43]. Somatostatin, which can also be released from sensory nerves, antagonises this neurogenically induced inflammation by decreasing plasma

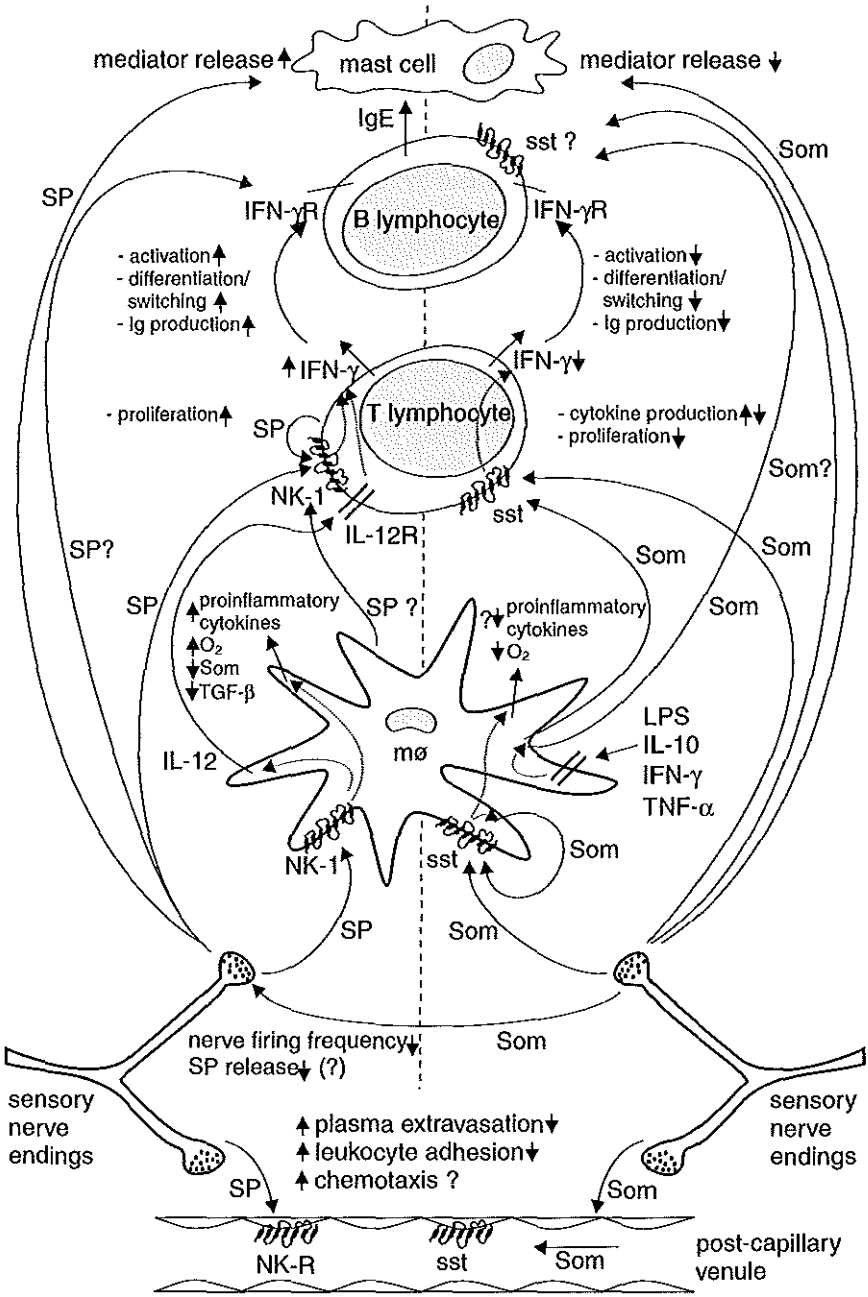

Figure 1. The somatostatin-substance P immunoregulatory circuit. The cartoon shows different components of a putative somatostatin-substance P immunoregulatory circuit operating within inflamed tissues. It includes data derived from studies in murine, rat and human model systems. The somatostatin-substance P immunoregulatory circuit is part of a larger regulatory network in which other neuropeptides may also play a role. Som = somatostatin; SP = substance P; sst = somatostatin receptor; R = receptor; NK-R = neurokinin receptor; NK-1 = neurokinin receptor subtype 1 (with high affinity for SP); O_2 = reactive oxygen species; Mϕ = macrophage.

and leukocyte extravasation [10, 44-46]. Sensory nerves become hyperreactive during inflammation, resulting in increased firing frequency and increased release of neuropeptides [47]. Somatostatin can diminish this central sensitisation by decreasing the firing frequency of sensory afferents [48, 49] and might also reduce neuropeptide release [50].

Nerve cells are not the only sources of neuropeptides in inflammatory lesions: somatostatin has been shown to be produced by macrophages within murine schistosome granulomas, by dendritic cells in the murine thymus and by activated human synovial cells [51-54]; substance P may be produced by eosinophils in murine granulomas, by rat alveolar macrophages and neutrophils and by human peripheral blood monocytes, macrophages and granulocytes [52, 55-57]. These peptides might have an autocrine regulatory role, as murine macrophage cell lines, human peripheral blood monocytes and cells of the mononuclear phagocyte lineage within inflammatory lesions express receptors for somatostatin of the sst_2 subtype ([19, 20, 39] and unpublished observations), while mouse and human monocytes and macrophages express substance P receptors of the NK-1 subtype [55, 58, 59]. Activation of somatostatin receptors may induce somatostatin production, although this mechanism has not yet been demonstrated in immune cells [60]. Somatostatin antagonises many of the effects of substance P, although the mechanism of action is not always clear. The mutual antagonism could be mediated at the level of intracellular second messenger systems: somatostatin receptor activation leads to inhibition of adenylyl cyclase, whereas activation of the NK-1 substance P receptor increases the activity of this enzyme.

In general, the effects of substance P are pro-inflammatory and the effects of somatostatin are anti-inflammatory. Many of these effects are mediated by cells of the mononuclear phagocyte system, which may be evolutionarily geared toward responding to neuropeptides [61]. Substance P enhances the secretion of IL-1, IL-6, IL-10, TNF-α and reactive oxygen intermediates from cells of the mononuclear phagocyte system [58, 62-65]. In addition, substance P decreases the release of the anti-inflammatory substances TGF-β and somatostatin [64, 66] from macrophages. It increases the secretion of IFN-γ from T lymphocytes, possibly by increasing the secretion of IL-12 from macrophages [37, 67]. The released IFN-γ in turn stimulates the activation, differentiation and Ig-production of B-lymphocytes [6]. In addition to the effects on cytokine secretion, substance P enhances the proliferation of T lymphocytes, probably via an authentic substance P receptor [4, 38, 59, 68-70]. T lymphocytes may also produce substance P [57, 70]. B lymphocytes may express substance P receptors of the NK-1 subtype [71], and direct effects of substance P on B cell differentiation and immunoglobulin secretion by isolated B lymphocytes and B cell lines *in vitro* have also been reported [72-75]. Substance P can also mediate the degranulation of mast cells [76], thus possibly playing a role in hypersensitivity and allergy.

Proinflammatory stimuli such as LPS, IFN-γ or TNF-α can induce the expression of preprosomatostatin mRNA [54]. As somatostatin has mainly anti-inflammatory effects, this may be a regulatory mechanism in the control of the inflammatory response. The main target for somatostatin may again be the mononuclear phagocyte system. Somatostatin reduces the secretion of reactive oxygen species from macrophages [77], but the effects on the secretion of cytokines are ambiguous: both stimulation and inhibition of the secretion of IL-1β, IL-6 and TNF-α by cells of the mononuclear phagocyte lineage have been observed by different researchers [78-82]. Somatostatin also reduces the proliferation [4, 83, 84] and the secretion of IFN-γ by T lymphocytes [85], and increases the secretion of IL-2 [2, 85-87]. Furthermore, somatostatin may suppress the IgE-induced degranulation of mast cells [88], thereby possibly limiting hypersensitivity and allergic responses. All these studies

thus yield compelling evidence that the balance between the concentrations of somatostatin and substance P may profoundly influence the outcome of a local inflammatory response.

Regulation of the default class of immune response within a tissue: a hypothesis.

In different tissues of the body, immune responses of a particular T-helper (Th) cell class are preferentially induced. In the gastrointestinal tract, the default class of immune response is preferentially suppressive (Th3), with IL-10 and TGF-β as the most important cytokines [89, 90]. In the lung, the default class is Th2, with mainly IL-4 production [91], whereas in the skin the default class is Th1, geared towards DTH reactions and granuloma formation, with IFN-γ as the hallmark cytokine [92]. The induction of a particular class of effector lymphocytes is determined by the cytokine environment which is present during the initial priming phase in the local lymphoid tissue [93], and which may be regulated primarily by the antigen presenting cells [91, 94]. After priming, the effector cells migrate to the appropriate target tissues. The migration of effector lymphocytes of a certain class is strongly influenced by the pattern of vascular addressins and chemokines expressed in the target tissues [95-98]. Lymphocytes originating in a distinct compartment have been shown to be capable of homing to sites of inflammation in other tissues under the appropriate circumstances [99, 100]. Therefore, there is probably an important role for the tissue microenvironment in determining the preferential effector class of a local inflammatory response [101, 102].

The tissue microenvironment consists of a varying collection of resident and visiting lymphoid and non-lymphoid cells, tissue-specific cells, neurons, extracellular matrix components, hormones, cytokines, chemokines, neurotransmitters and other humoral substances, membrane-bound, intracellular and secreted receptors and ions. I propose that an important determinant of the final effector class of a local immune response in different tissues is the balance of neuropeptides secreted by local sensory nerves or produced by local cells, in other words, that the default effector class is anatomically "hard-wired" into the tissues. This ensures a system that is relatively insensitive to perturbations, but that is nevertheless plastic enough to be able to induce the deviations from the default class of immune response that have been observed to occur under extreme circumstances, such as in chronic inflammation and autoimmune disease.

Nerves are probably the major source of neuropeptides in the tissues: sensory nerves, mainly mechanosensitive and chemosensitive nociceptors, are abundant in the skin, the joints and in almost all visceral organs [42, 103, 104]. Neuropeptides released antidromically from these nerves after stimulation may contribute to local inflammation; neurogenic influences on inflammation have been described in the skin, the lung, the joints and the gastrointestinal tract [36, 47, 50, 105-109]. Moreover, changes in the concentrations of major sensory neuropeptides have been found to be associated with a number of chronic inflammatory conditions such as RA, psoriasis and IBD [40, 47, 109-112], showing that the correct balance of neuropeptides is important for maintaining immune homeostasis in the tissues.

The main basis for the hypothesis that the balance of neuropeptides can direct the effector class of an immune response, is that sensory neuropeptides such as substance P and somatostatin can induce the secretion of "forbidden" cytokines from distinct Th1 and Th2 clones, thereby breaking their commitment to a distinct class of response [113]. Substance P most likely skews towards a Th1 response, possibly by inducing secretion of IL-12 [67],

and somatostatin may be more involved in a Th2 response [64, 113]. By this mechanism an immune response may be skewed towards the default type appropriate to the particular tissue in which the response occurs, despite possible inappropriate skewing during the priming phase in the lymphoid organs.

Factors such as drug treatment, stress or chronic stimulation could possibly affect this regulatory system in such a way as to allow an immune response of an inappropriate class to occur, thus contributing to the development of chronic inflammation or autoimmune disease. Local factors, such as the microflora in the gut [114] or drug treatment [115], may enhance or suppress neuropeptide release. Stress can induce increased release of substance P [116], and this could in turn reduce the production of somatostatin [64]. Inflammation can sensitise nerves [49, 117] and may cause recruitment of previously "silent" nerve fibres, e.g. high threshold chemosensitive and mechanosensitive nociceptors [42]. In addition, the neuropeptide content of the nerve fibres may be altered and increased innervation may be induced by the release of trophic factors such as nerve growth factor from activated or damaged resident tissue cells [41, 101, 112, 118, 119]. Neuropeptide receptors on target cells can be up- or down-regulated by chronic exposure to the ligand (see chapter 1), or changes in level or activity of local peptidases could alter the concentration of bioactive ligand [52, 120]. Such changes could severely affect the balance of neuropeptides locally present within the tissue, potentially leading to induction of an inappropriate effector class of immune response, and consequently disease.

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SUMMARY

Somatostatin is a neuropeptide with diverse functions in different organ systems. It was originally detected as a factor produced in the hypothalamus that inhibited the release of growth hormone from the pituitary. Somatostatin is present in two biologically active forms: a 14-amino acid form (somatostatin-14) and an N-terminally extended 28-amino acid form (somatostatin-28). Somatostatin is found throughout the body, but the highest concentrations are found in the central nervous system, endocrine glands and the gastrointestinal tract. In the central nervous system somatostatin functions as a neurotransmitter, while in peripheral tissues it suppresses endocrine and exocrine secretion and modulates the motility of the gastrointestinal tract. There is evidence that somatostatin also has a role in the regulation of the immune response. For instance, the inflammatory lesions in patients suffering from autoimmune diseases or chronic inflammation can be visualised by injection of a radioactively labelled synthetic somatostatin analogue, called octreotide. This indicates that cells present in the inflammatory lesions express receptors for somatostatin. At present five subtypes of somatostatin receptors (sst_{1-5}) have been identified; however, synthetic somatostatin analogues bind only to the receptor subtypes sst_2 and sst_5, and to a lesser extent sst_3.

The presence of somatostatin receptors within the inflammatory lesions of patients suffering from chronic inflammation and autoimmune diseases suggests that these diseases might react to treatment with synthetic somatostatin analogues such as octreotide. **Chapters 2 and 4** describe experiments in which we investigated whether systemic octreotide treatment could influence the course of the disease in animal models of chronic inflammatory diseases such as rheumatoid arthritis (RA), and autoimmune diseases such as multiple sclerosis (MS) and systemic lupus erythematosus (SLE). Systemic treatment with octreotide in the form of a single application of a subcutaneous depot had no effect on the course of the disease in experimentally induced arthritis in Lewis rats (**chapter 2**) and spontaneously occurring arthritis in MRL/*lpr* mice (**chapter 4.1**). There was also no significant effect on the course of the disease in animal models for autoimmune diseases such as experimentally induced autoimmune encephalomyelitis (a model for MS) in SJL mice (**chapter 4.2**) and spontaneously occurring autoimmune nephritis (a model for SLE) in MRL/*lpr* mice. The concentration of autoantibodies directed against double stranded DNA in MRL/*lpr* mice was decreased slightly but not significantly by the octreotide treatment. In rats, the lack of effect of the octreotide treatment was found to be due to the fact that in the immune system of rats only somatostatin receptor subtypes sst_3 and sst_4 are expressed (**chapter 2**). The binding of octreotide to these receptor subtypes is negligible.

In the mouse however, sst_2, the receptor subtype with the highest affinity for octreotide, is expressed in cells of the immune system, especially in thymocytes (**chapter 3**). In the literature it has been shown that sst_2 is also expressed in mouse splenocytes and isolated T lymphocytes; this expression probably only reaches detectable levels after activation of the cells. The lack of effect of octreotide treatment in the two murine disease models (**chapter 4**) therefore cannot be attributed to the absence of the appropriate receptor. It is possible that a different treatment protocol would give better results; this needs to be investigated.

In a number of chronic inflammatory diseases and autoimmune diseases, somatostatin receptors can be detected using radioactively labelled octreotide. However, the resolution of this technique is too low to positively identify which cells within the inflammatory lesions express the somatostatin receptors. By immunohistochemical staining using a specific an-

tiserum it was possible to detect the receptors on individual cells. **Chapter 5** describes the localisation, using immunohistochemical staining, of somatostatin receptors of subtype sst_{2A} on blood vessel endothelial cells, but also on cells of the mononuclear phagocyte system (monocytes and macrophages) in inflamed tissues of patients with RA (**chapter 5.1**), sarcoidosis and other granulomatous diseases (**chapter 5.2**) and inflammatory bowel disease (**chapter 5.3**). Cells of the mononuclear phagocyte system are thought to play an important role in the induction and maintenance of the inflammatory process that characterises these diseases. Because these cells express somatostatin receptors of the sst_2 subtype, they are possible targets in the treatment of these diseases using synthetic somatostatin analogues such as octreotide. One patient who suffered from sarcoidosis was successfully treated with octreotide; however, in another sarcoidosis patient the same treatment had no effect.

From the studies described in this dissertation it can be concluded that cells of the mononuclear phagocyte system in inflamed tissues of patients with immune-mediated diseases, express somatostatin receptors. Therefore these cells are likely targets in the treatment of these diseases using somatostatin analogues. Studies in animals models will be necessary to further investigate and validate this hypothesis. The rat is not a suitable model animal for such studies due to the lack of expression of the appropriate somatostatin receptor subtypes in cells of the rat immune system. It is not yet clear whether the mouse would be a suitable model animal. Although expression of sst_2 was detected in cells of the murine immune system, it is not known whether the cells expressing the receptors belong to the mononuclear phagocyte system, as is the case in humans.

SAMENVATTING

Somatostatine is een neuropeptide dat diverse functies heeft in verschillende orgaansystemen. Het is aanvankelijk geïdentificeerd als een factor afkomstig uit de hypothalamus die in de hypofyse de afgifte van groeihormoon remde. Somatostatine bestaat in twee biologisch actieve vormen: één van 14 aminozuren (somatostatine-14) en een N-terminaal verlengde vorm van 28 aminozuren (somatostatine-28). Somatostatine komt voor in het gehele lichaam, maar de hoogste concentraties worden gevonden in het centraal zenuwstelsel, endocriene klieren en het maag-darm stelsel. In het centraal zenuwstelsel is somatostatine werkzaam als neurotransmitter, terwijl het in perifere weefsels endocriene en exocriene secretie remt en de motiliteit van het maag-darm stelsel beïnvloedt. Er zijn aanwijzingen dat somatostatine ook een rol speelt in de regulatie van de afweerreaktie. Zo kunnen bijvoorbeeld de ontstekingshaarden in patiënten met autoimmuunziekten of chronische ontstekingen zichtbaar worden gemaakt door inspuiting van een radioactief synthetisch somatostatine analoog, het octreotide. Dit betekent dat cellen die in die ontstekingshaarden aanwezig zijn, receptoren dragen voor somatostatine. Er zijn tot nu toe 5 soorten somatostatinereceptoren bekend ($sst_{1\text{-}5}$); synthetische somatostatine analogen kunnen echter alleen binden aan de receptor subtypen sst_2 en sst_5, en in mindere mate aan sst_3.

De aanwezigheid van somatostatinereceptoren in de ontstekingshaarden in patiënten met chronische ontstekingen en autoimmuunziekten geeft aan dat deze ziekten mogelijk zouden reageren op behandeling met synthetische somatostatine analogen, zoals het octreotide. In de **hoofdstukken 2 en 4** zijn experimenten beschreven waarin is onderzocht of systemische behandeling met octreotide invloed had op het ziekteverloop in diermodellen van chronische ontstekingen zoals reumatoïde artritis (RA), en autoimmuunziekten zoals multiple sclerose (MS) en systemische lupus erythematose (SLE). Systemische behandeling met octreotide, in de vorm van een eenmalig toegediend onderhuids depot, had geen effect op het ziekteverloop in geïnduceerde artritis in Lewis ratten (**hoofdstuk 2**) en spontane artritis in MRL/*lpr* muizen (**hoofdstuk 4.1**). Ook was er geen significant effect op het ziekteverloop in diermodellen voor autoimmuunziekten zoals geïnduceerde autoimmuun encephalomyelitis (een model voor MS) in SJL muizen (**hoofdstuk 4.2**) en spontane autoimmuun nefritis (een model voor SLE) in MRL/*lpr* muizen. De concentratie van autoantistoffen gericht tegen dubbelstrengs DNA in MRL/*lpr* muizen werd licht maar niet significant verlaagd door de behandeling met octreotide. In de ratten kon het gebrek aan effect geweten worden aan het feit dat in het afweersysteem van ratten alleen de somatostatinereceptor subtypen sst_3 en sst_4 tot expressie komen (**hoofdstuk 2**). Octreotide bindt nauwelijks of niet aan deze receptor subtypen.

In de muis komt sst_2, het receptor subtype met de hoogste bindingsaffiniteit voor octreotide, wel tot expressie in cellen van het afweersysteem, met name in thymocyten (**hoofdstuk 3**). In de literatuur is beschreven dat sst_2 ook tot expressie komt in miltcellen en in geïsoleerde T lymfocyten van de muis; deze expressie is waarschijnlijk pas aantoonbaar na activatie van de cellen. Het gebrek aan effect van de behandeling met octreotide in de twee gebruikte muizenmodellen (**hoofdstuk 4**) kan dus niet geweten worden aan de afwezigheid van de geschikte receptor. Het is mogelijk dat een ander behandelingsprotocol meer effect zou opleveren; dit moet verder onderzocht worden.

In een aantal chronische ontstekingsziekten en autoimmuunziekten kunnen somatostatinereceptoren worden gedetecteerd met behulp van radioactief gemarkeerd octreotide. De resolutie van deze techniek is echter te laag om met zekerheid te kunnen vaststellen

welke cellen in de ontstekingshaarden de somatostatinereceptoren tot expressie brengen. Met behulp van een immunohistochemische kleuring, waarbij gebruik wordt gemaakt van specifieke antistoffen, kunnen de receptoren wel op afzonderlijke cellen worden gedetecteerd. In **hoofdstuk 5** is beschreven dat met behulp van een immunohistochemische kleuring, somatostatinereceptoren van het subtype sst_{2A} gedetecteerd zijn op endotheelcellen van bloedvaten, maar met name ook op cellen van het mononucleaire fagocytensysteem (monocyten en macrofagen) in ontstoken weefsels van patiënten met RA (**hoofdstuk 5.1**), sarcoïdose en andere granulomateuze ziekten (**hoofdstuk 5.2**), en in ontstekingsziekten van de darm (**hoofdstuk 5.3**). Cellen van het mononucleaire fagocytensysteem spelen waarschijnlijk een belangrijke rol in het ontstaan en het onderhouden van de ontstekingen waardoor deze ziekten gekenmerkt worden. Omdat zij somatostatine receptoren van het subtype sst_2 tot expressie brengen, zijn zij mogelijk ook een doelwit voor behandeling met synthetische somatostatine analogen zoals octreotide. Eén patiënt met sarcoïdose werd ook met succes behandeld met octreotide; bij een andere sarcoïdosepatiënt had dezelfde behandeling echter geen effect.

Uit de in dit proefschrift beschreven studies kan geconcludeerd worden dat cellen van het mononucleaire fagocytensysteem in ontstoken weefsel van patiënten met immuungemedieerde ziekten, somatostatinereceptoren tot expressie brengen. Deze cellen zijn daardoor waarschijnlijk het doelwit bij de behandeling van deze ziekten met somatostatine analogen. Studies in diermodellen zijn nodig om deze hypothese verder te onderzoeken en functioneel te onderbouwen. De rat is voor zulke studies geen geschikt proefdier vanwege het ontbreken van de geschikte receptor subtypes op de cellen van het afweersysteem. Het is nog niet voldoende duidelijk of de muis wel een geschikt proefdier is. Hoewel de expressie van sst_2 is aangetoond op cellen van het afweersysteem van de muis, is het nog niet bekend of het hierbij, net als in de mens, cellen van het mononucleaire fagocytensysteem betreft.

ABBREVIATIONS

ACTH	:	adrenocorticotrophic hormone
AP	:	alkaline phosphatase
BSA	:	bovine serum albumin
CD	:	Crohn's disease
cDNA	:	complementary DNA
CFA	:	complete Freund's adjuvant
CNS	:	central nervous system
Con A	:	concanavalin A
CRF	:	corticotrophin-releasing factor
DTH	:	delayed-type hypersensitivity
EAE	:	experimental autoimmune encephalomyelitis
FCS	:	foetal calf serum
FITC	:	fluorescein isothiocyanate
GH	:	growth hormone
HEV	:	high endothelial venules
HRP	:	horseradish peroxidase
i.d.	:	intradermal
i.m.	:	intramuscular
IBD	:	inflammatory bowel disease
ID	:	injected dose
IFA	:	incomplete Freund's adjuvant
IFN	:	interferon
Ig	:	immunoglobulin
IL	:	interleukin
LAR	:	long-acting release
LPS	:	lipopolysaccharide
MBP	:	myelin basic protein
mRNA	:	messenger RNA
MS	:	multiple sclerosis
PBMC	:	peripheral blood mononuclear cells
PBS	:	phosphate-buffered saline
PHA	:	phytohaemagglutinin
PLP	:	proteolipid protein
PNS	:	peripheral nervous system
PRL	:	prolactin
RA	:	rheumatoid arthritis
RT	:	room temperature
s.c.	:	subcutaneous
SCID	:	severe combined immunodeficiency
SD	:	standard deviation
SEM	:	standard error of the mean
SLE	:	systemic lupus erythematosus
SPECT	:	single positron emission computed tomography
sst	:	somatostatin receptor
TBS	:	Tris-buffered saline

TID	:	thrice daily
TMD	:	transmembrane domain
TNF	:	tumour necrosis factor
TSH	:	thyroid-stimulating hormone
UC	:	ulcerative colitis
VIP	:	vasoactive intestinal peptide

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