# REVIEW ARTICLE

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# Potential of targeting natural killer T cells for the treatment of autoimmune diseases

**Abstract** Natural killer (NK) T cells emerge as unique lymphocytes subsets implicated in the regulation of autoimmunity. Abnormalities in the numbers and functions of NKT cells have been observed in patients with diverse autoimmune diseases as well as in a variety of mouse strains that are genetically predisposed for the development of autoimmune diseases. Unlike conventional T cells that recognize peptides in association with major histocompatibility complex (MHC), NKT cells recognize glycolipid antigens presented by the nonpolymorphic MHC class I-like protein, CD1d. Recently, vigorous activation of NKT cells by synthetic glycolipids such as α-galactosylceramide (α-GC) or its sphingosine truncated derivative OCH have been shown to suppress autoimmune diseases such as experimental autoimmune encephalomyelitis (EAE), diabetes in nonobese diabetic (NOD) mice, and collagen-induced arthritis (CIA) by inducing T helper (Th) 2 bias of autoimmune T cells. In this review, we examine the potential roles of NKT cells in the pathogenesis of autoimmune disease regulation, and the recent advances in glycolipid therapy for autoimmune disease models. In addition, we summarize studies suggesting a role for NKT cells in human autoimmune disease, and discuss the potential of targeting NKT cells for the treatment of autoimmunity.

**Key words** α-Galactosylceramide (α-GC) · Autoimmune disease · Natural killer (NK) T cells · OCH · Th1/Th2

## Introduction

Natural killer (NK) T cells are usually defined as cells coexpressing the natural killer receptors such as NK1.1 or

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NKR-P1A (CD161) and an αβT cell receptor (TCR). Although NK1.1$^+$ TCR$^+$ lymphocytes are heterogeneous, the majority of NKT cells have a restricted TCR diversity, with an invariant TCRα chain, composed of Vα14-Jα281 segments in mice and Vα24-JαQ segments in humans, which is associated with TCR β chains using a restricted set of Vβ genes. These Vα14 invariant NKT cells recognize glycolipid antigens such as α-GC presented by a nonpolymorphic MHC class I-like molecule, CD1d[1–5] (Fig. 1). As little is known about CD1d nonrestricted NKT cells or α-GC-independent CD1d restricted NKT cells, in this review we focus on the α-GC responsive NKT cells, and the term "NKT cells" will be used for α-GC responsive NKT cells.

Subsets of mouse and human NKT cells have a similar memory phenotype, and recognize α-GC in association with CD1d molecules highly conserved through mammalian evolution. Whereas human and mouse NKT cells share many characteristics, the frequency is much lower in humans.[2–4] Consistent with their preactivation status, NKT cells release large amounts of cytokines, including IL-4 and IFN-γ promptly upon antigen stimulation, and these affect the functions of neighboring cell populations such as T cells, B cells, NK cells, and dendritic cells.[2,5] NKT cells are composed of two subsets: CD4$^+$ or CD4$^-$CD8$^-$ (double negative DN). CD4$^+$ and DN NKT cells appear to be different in terms of cytokine production in humans but not in mice.[2,6] The CD4$^+$ subset of human NKT cells produces both Th1 and Th2 cytokines upon antigen stimulation, whereas the DN subset produces Th1 cytokines and upregulates the production of perforin after exposure to cytokines.[6]

Natural antigens for NKT cells have not yet been identified. However, it is speculated that self glycolipid antigens probably function as activating ligands for NKT cells owing to the self-reactivity of NKT cells and the activated memory phenotype of NKT cells isolated from human umbilical-cord blood[7,8] and germ-fee mice.[9] α-GC is a synthetic glycolipid originally isolated from marine sponges *Agelas mauritanius*, and later, a synthetic analogue of this compound was developed for experimental studies and clinical trials (Fig. 1).[10] α-GC has been shown to be a potent stimulator of both murine and human NKT cells.[10–12] NKT cells
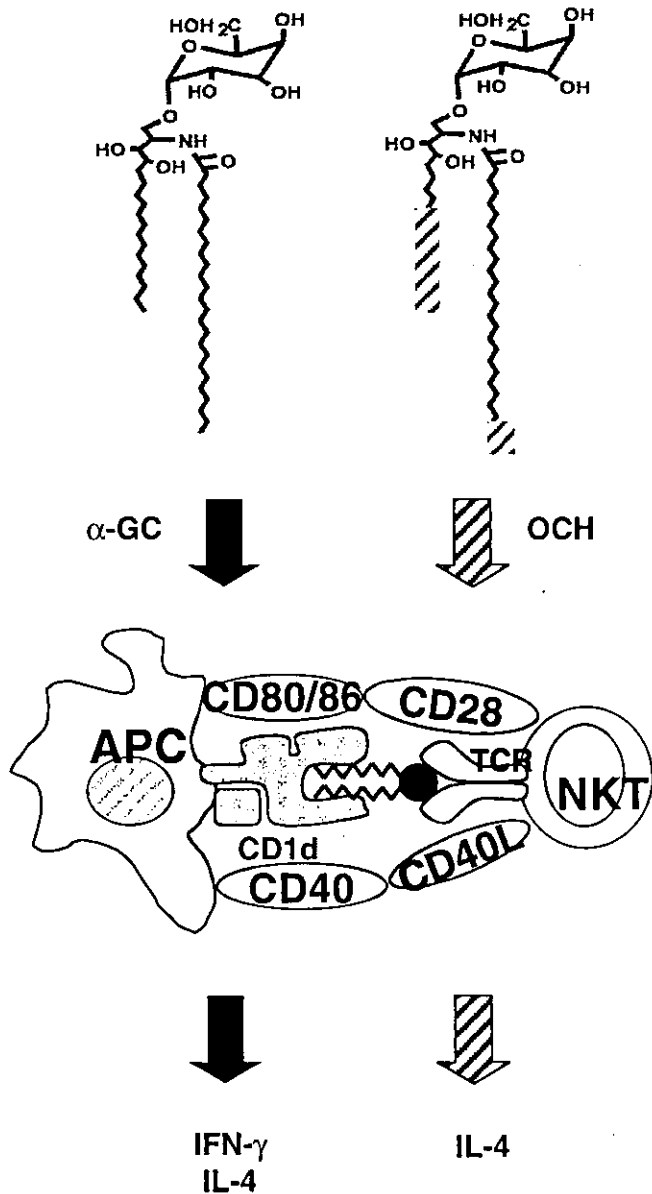
Fig. 1. Structure and function of α-galactosylceramide (α-GC) and an altered ligand OCH. NKT cells recognize glycolipid ligand presented by CD1d molecules. The α-anomeric conformation of sugar moiety, the configuration of the 2-hydroxyl group on the sugar moiety, and 3,4-hydroxyl groups of the phytosphingosine are important for NKT cell recognition of α-GC. The OCH analogue has a shorter sphingosine chain. Upon stimulation, NKT cells produce a variety of cytokines and exert effector functions. α-GC stimulates NKT cells to produce both anti-inflammatory (e.g., IL-4 and IL-10) and pro-inflammatory (e.g., IFN-γ) factors. This response can be modified by stimulation with an altered ligand such as OCH, or stimulation in the absence of CD28/B7.2 co-stimulation. These modifications are a potentially important therapeutic approach to suppressing Th1-mediated autoimmune diseases. APC, antigen presenting cell; TCR, T cell receptor

respond to sphingolipids substituted with an α-linked galactose or glucose, but not α-linked mannose and sphingolipids containing β-linked galactose or glucose.[10] Sphingolipids containing β-linked sugars resemble common mammalian lipids, whereas α-glycosyl sphingolipids have not been found in normal mammalian tissues. Recently, we have demonstrated that a sphingosine truncated analogue of α-GC, OCH, is a unique ligand for NKT cells.[13]

OCH stimulates NKT cells to preferentially produce IL-4, in contrast to α-GC which induces a variety of cytokines including IFN-γ, IL-2, tumor necrotic factor-α, IL-4, and IL-13 from NKT cells (Fig. 1).

## NKT cells in rheumatoid arthritis and collagen-induced arthritis (CIA)

Abnormalities of NKT cells in autoimmune diseases were first reported in scleroderma patients. In such patients, αβ+ DN T cells were increased and there was an oligoclonal expansion of Vα24⁺TCR⁺ cells among them.[14] However, the invariant Vα24JαQ T cells were reduced in scleroderma patients, although the invariant Vα24JαQ T cells were dominant among these cells from healthy donors. Van der Vliet et al.,[15] Kojo et al.,[16] and other groups investigated the number of NKT cells by using Vα24 and Vβ11 mAb to detect NKT cells in patients with several different autoimmune diseases, including rheumatoid arthritis (RA). They found lower numbers of Vα24⁺Vβ11⁺ NKT cells in the peripheral blood of patients than in that of controls. In this study, Kojo et al.[16] showed that half of the patients with autoimmune disease responded to α-GC in culture. In addition, Maeda et al.[17] reported the expansion of noninvariant Vα24 TCR⁺ cells but not Vα24JαQ T cells in the synovium of RA patients.

CIA is a murine experimental model of RA induced by immunization with type II collagen. The activation of NKT cells by glycolipid ligands was examined by Chiba et al.[18] The effect of α-GC on CIA was marginal. In contrast, Th2 skewing ligand OCH inhibited the clinical course of CIA. Histological analysis revealed that OCH treatment protected against the infiltration of inflammatory cells and the destruction of cartilage and bone. The suppressive effect of OCH was not observed for CIA induced either in CD1d knockout mice or in Jα281 knockout mice deficient in NKT cells, suggesting that OCH-mediated suppression requires NKT cells. Moreover, the injection of OCH strongly suppressed CIA in SJL mice even though these mice have defects in the numbers and functions of NKT cells, and even after the arthritis had already developed (Fig. 2). In contrast, the administration of α-GC did not suppress arthritis in SJL mice. The suppression of arthritis was associated with an elevation of the IgG1:IgG2a ratio, indicating the Th2 bias of type II collagen-reactive T cells. The injection of neutralizing antibody to either IL-10 or IL-4 reversed the beneficial effect of OCH treatment. These results imply that IL-10 and IL-4 are critical in the OCH-mediated suppression of CIA, and are consistent with the idea that OCH modulated CIA by stimulating the production of Th2 cytokines from NKT cells, although the source of IL-10 remains to be elucidated.

The role of NKT cells in CIA is still not clear. The clinical score of CIA induced in NKT cell-deficient mice
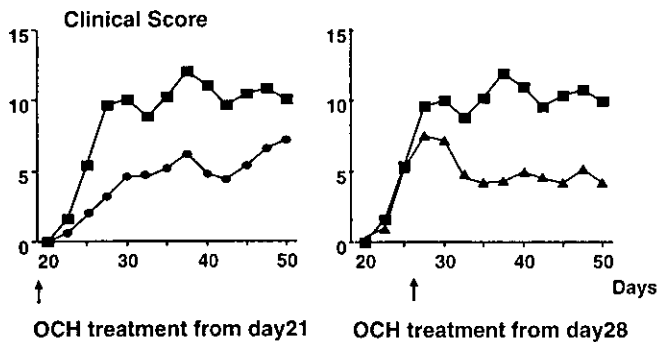
**Clinical Score**

OCH treatment from day21    OCH treatment from day28

**Fig. 2.** The suppressive effect of *OCH* on collagen-induced arthritis (CIA) in SJL mice. Clinical score of CIA in SJL mice treated with 500 μg/kg of vehicle (*squares*) or OCH (*circles*) twice per week starting from day 21 or day 28

appeared lower than that in wild-type mice. The finding of a natural antigen for NKT cells would give us further insight into the precise role of NKT cells in the pathogenesis of autoimmune diseases such as arthritis.

## NKT cells in systemic lupus erythematosus and lupus murine models

Sumida and co-workers[19,20] observed the expansion of noninvariant Vα24 TCR[+] clones in patients with active systemic lupus erythematosus (SLE). These authors and other groups found lower numbers of Vα24[+]Vβ11^{+} NKT cells in the peripheral blood of patients with SLE than in that of controls.[15,16]

In lupus murine models such as MLR lpr/lpr mice, it has been reported that a selective reduction in NK1.1^{+} T cells precedes the development of disease. Mieza et al.[21] also found a decrease in the expression of invariant Vα14 TCR mRNA of NKT cells before the onset of lymphocyte accumulation and autoimmune disease in MRL lpr/lpr mice, C3H gld/gld mice, and NZB/W F1 mice when compared with control mice. Morshed et al.[22] reported that the number of NKT cells increased after the onset of disease, and then the transfer of NK1.1+ T cells from diseased mice to young F1 mice (before the onset of renal failure) induced proteinuria and swelling of the glomeruli. Moreover, Zeng et al.[23] demonstrated that treatment of NZB/W F1 mice with anti-CD1d monoclonal antibody augmented Th2-type responses, increased serum levels of IgE, decreased levels of IgG2a and IgG2a antidouble-stranded DNA (dsDNA) antibodies, and ameliorated lupus. They also showed that multiple injections of α-GC treatment induced an enhanced Th1-type response and exacerbated lupus associated with decreased serum levels of IgE and increased levels of IgG2a and IgG2a anti-ds DNA antibodies. This exacerbation of disease was associated with reduced IL-4 and tumor necrotic factor-α production and an expansion of marginal zone B cells. These results suggested that the activation of NKT cells by α-GC augmented Th1-type responses and

autoantibody production that contribute to lupus development in NZB/W F1 mice. In contrast, CD1d deficiency did not lead to disease acceleration in MRL lpr/lpr mice,[24] and pristine-induced lupus nephritis was accelerated when induced in CD1d-deficient mice.[25] They also demonstrated that repeated injections of α-GC resulted in the expansion of NKT cells and ameliorated dermatitis in MRL lpr/lpr mice.[26] Therefore, they postulated that NKT cells may play a protective role in lupus models. Since lupus models are not simply explained by only Th1-mediated or Th2-mediated pathology, the complexity of these models may explain the differences in results in these studies.

## NKT cells in multiple sclerosis and experimental autoimmune encephalomyelitis (EAE)

Multiple sclerosis (MS) is an autoimmune demyelinating disease of the central nervous system (CNS). A reduction in Vα24JαQ cells among Vα24^{+} cells from the peripheral blood of patients with MS compared with healthy subjects has been found by using a single-strand conformation polymorphism method to detect TCR gene rearrangements.[27] In support of this finding, Van der Vliet et al.[15] showed a decrease in the number of NKT cells by screening Vα24[+]Vβ11^{+} cells in the blood using monoclonal antibodies specific for Vα24^{+} and Vβ11^{+}TCR. Araki et al.[28] demonstrated that DN NKT cells in the periphery were greatly reduced in remission, whereas the reduction of CD4^{+} NKT cells was marginal. Furthermore, CD4^{+} NKT line cells expanded from MS in remission produced a larger amount of IL-4 than those from healthy subjects or from MS in relapse, suggesting that the Th2 bias of CD4^{+} NKT cells may play a role in the regulation of Th1-type autoantigen-reactive T cells. Conversely, Gausling et al.[29] did not find a significant difference in the number of DN Vα24^{+} NKT cells in peripheral blood lymphocytes between MS patients and healthy controls. Considering that the proportion of Vα24JαQ T cells in normal individuals varies among studies, it may not be easy to compare these studies, and the basis for the discrepancy between the numbers of NKT cells is not clear.

EAE is a Th1-mediated autoimmune inflammatory disease affecting the CNS that serves as a model for MS. EAE can be induced in susceptible mouse strains by immunization with CNS proteins or peptides in adjuvant, or by the passive transfer of T cells reactive against such CNS antigens. The presence of a Vα14 transgene reduced myelin oligodendrocyte glycoprotein (MOG)-induced EAE in nonobese diabetic (NOD) mice.[30] The disease severity in CD1d deficient mice has been reported as being reduced, unaltered, or enhanced.[30-34] Even though the basis for these inconsistencies is not clear, breeding genetic alterations onto C57BL/6(B6) background, the variability in the B6 strains used, and the marked impact of colony health on the disease severity of EAE could explain some differences. Although the role of NKT cells in the course of EAE is not yet clear, the stimulation of NKT cells to produce Th2

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cytokines would be a powerful strategy to deliver protective cytokines to autoimmune-mediated inflammatory lesions, since NKT cells are known to invade rapidly and to accumulate in inflammatory lesions in a manner similar to inflammatory cells and produce cytokines.

The results obtained from α-GC treatment of EAE generated conflicting results. The administration of α-GC was found either to prevent disease, to have no effect, or to accelerate disease.[13,31-35] These differences could be due to the differences in the protocols of the α-GC treatment and the differences in strains and antigens used for the induction of EAE. The timing and the route of administration appear to be critical to modulation of the disease. Since NKT cells produce both IFN-γ and IL-4 upon stimulation with α-GC, α-GC may have different effects on EAE depending on the stage of the disease and the strains used. NKT cell-derived IFN-γ would mask the protective effect of the IL-4 simultaneously produced by the NKT cells, and sometimes even worsen the disease. We have shown several lines of evidence supporting this idea.[35] First, α-GC treatment inhibited EAE induced in IFN-γ-deficient mice. Second, α-GC treatment augmented the clinical signs of EAE induced in IL-4-deficient mice. Third, blockade of CD86 polarized NKT cells toward a Th2-like phenotype with a concomitant suppression of EAE, and the activation of APCs by treatment with CD40 biased them toward a Th1-like phenotype and exacerbated the EAE.

Thus, EAE could be prevented if ligand stimulation led to the selective production of Th2 cytokines by NKT cells in vivo. Manipulating NKT cells through adoptive transfer may not be practical in humans, and therefore a more attractive strategy would be the direct activation of these cells in vivo. Therefore we synthesized several analogues of α-GC and found that a sphingosine-truncated analogue, OCH, induced selective IL-4 production by NKT cells (see Fig. 1). As expected, the administration of OCH prevented the development of EAE in both clinical and pathological parameters. The inhibitory effect of OCH was not observed for EAE induced either in NKT cell-deficient or IL-4-deficient mice, confirming that IL-4 produced by NKT cells is critical for OCH-mediated suppression of EAE.[13] In addition to B6 mice, SJL mice are highly susceptible to EAE, and EAE induced by immunization with proteolipid protein (PLP)-derived peptides PLP$_{139-151}$ is used as a remitting–relapsing MS model. SJL mice have been reported to have markedly fewer NKT cells and markedly lower cytokine production upon activation.[36] Singh et al.[32] reported that SJL mice responded poorly to treatment with α-GC. When SJL mice were treated with α-GC, their morbidity and mortality were exacerbated although the onset of disease was delayed. In contrast, multiple injections of OCH protected SJL mice against EAE (S. Miyake and T. Yamamura, unpublished observation). Furthermore, OCH protected SJL mice against a relapse in EAE, suggesting that OCH holds possibilities as a therapeutic agent to prevent relapses in MS. On the whole, treatment with OCH might be preferable to α-GC, as OCH preferentially induces IL-4 production and inhibits disease in several different strains of mice.

## NKT cells in type I diabetes and NOD mice

Studies of the frequency of human NKT cells in peripheral blood in patients with type I diabetes have shown conflicting results. In initial studies analyzing identical twin/triplet sets discordant for disease, there was a lower frequency of invariant Vα24Jα18 sequences among DN Vα24$^+$ T cells in diabetic siblings than in nondiabetic siblings.[37] In support of these data, Kukreja et al.[38] showed a reduction in the number of NKT cells in newly diagnosed patients using an antibody specific for the conserved-determining region (CDR) 3 of the Vα24-Jα18 rearrangement. However, more recent papers reported unaltered or increased NKT cells in recent-onset patients with type I diabetes.[39,40] Wilson et al.[37] also showed that DN Vα24JαQ T cell clones isolated from diabetics had an impaired ability to produce IL-4. In contrast, Lee et al.[39] reported that IL-4 production by NKT cells was similar among these groups as assessed by intracytoplasmic staining following short-term PMA and ionomycin stimulation. At this stage, it is hard to interpret the discrepancies between these results, since the methods for detecting NKT cells and the functional assays used differ between studies. In addition, the patients analyzed in the different reports were of different ethnic groups and different age groups.

NOD mice develop a spontaneous autoimmune diabetes which is similar to the human disease insulin-dependent type 1 diabetes mellitus. Many studies have indicated that Th1-type CD4$^+$ cells and CD8$^+$ T cells have been implicated in the development of diabetes in NOD mice. In parallel with these effector cells, it has been suggested that the regulatory cells, including NKT cells, inhibit the development of diabetes. Deficiencies in the number and function of NKT cells have been found in NOD mice.[41] Although the correlation between a defect in NKT cells and a susceptibility to diabetes in NOD mice is still being debated,[3-5,42,43] the putative involvement of NKT cells in the control of islet β-cell reactive T cells in NOD mice was suggested by examples of diabetes being prevented following the infusion of NKT cell-enriched thymocyte preparations,[44] and also by the increase in NKT cells in Vα14Jα281 transgenic NOD mice.[45]

Several recent studies have investigated the effect of treating NOD mice with α-GC.[43,46-48] When started at around 3 or 4 weeks of age, repeated injections at least once a week delayed the onset and reduced the incidence of diabetes. After treatment, splenocytes from NOD mice produced a greater amount of IL-4 in response to islet antigens, and the IgG1/IgG2a (Th2/Th1) ratio of anti-GAD antibody increased. It therefore appears that the mechanism of protection is similar to that observed by increasing the numbers of NKT cells in NOD mice and by α-GC treatment in other autoimmune disease models such as EAE and CIA. We also observed the protective effect of OCH treatment in NOD mice as well as that of α-GC treatment. The protective effect of OCH against insulitis was more profound than that of α-GC (M. Mizuno and S. Miyake, unpublished observation).

## Prospects for glycolipid therapy for autoimmune diseases

There is still controversy about whether defects in NKT cells cause autoimmune disease or occur as a secondary consequence of the autoimmune process. However, given the efficacy of glycolipid ligands such as OCH and α-GC in mouse models, the stimulation of NKT cells with glycolipid seems to be an attractive strategy for the treatment of autoimmune diseases. Although several studies have shown that the administration of α-GC caused liver damage, the hepatotoxicity was minimal in phase I trials of α-GC for patients with cancer. Considering the low toxicity in humans, it seems reasonable to use glycolipids for the prevention or therapy of selected human autoimmune disorders. α-GC has been shown to exacerbate EAE, depending on the strain of mouse and stage of disease tested, and to have only a marginal effect on CIA. In this situation, treatment with OCH might be preferable to α-GC for Th1-mediated diseases such as MS, type I diabetes, and RA, as OCH elicits a predominantly IL-4 response rather than IFN-γ. Both rodent and human NKT cells have been reported to recognize α-GC in the context of CD1d. OCH also stimulates human NKT cells, particularly CD4$^+$ NKT cells, and induces greater Th2 cytokine production from NKT cells compared with α-GC stimulation (M. Araki and T. Yamamura, unpublished observation). The evolutionary conservation and the homogeneous ligand specificity of NKT cells allow us to apply a glycolipid ligand such as OCH for the treatment of human disease without considering species barriers or the genetic heterogeneity of humans.

## Conclusion

Ligand stimulation of NKT cells is an attractive strategy for the prevention or treatment of autoimmune diseases. The mechanisms by which NKT cells exert their immunoregulatory functions are still largely unknown and a number of questions require further investigation, including the mechanism to recruit NKT cells and control their functions at inflammatory sites, and the interactions of other subsets of cells. The identification of the nature of natural ligands for NKT cells is a major question, and the answer would provide us with more information about NKT cells and autoimmunity. It could also provide us with an interesting natural source of useful stimulators for CD1-restricted regulatory cells.

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# More sympathy for autoimmunity with neuropeptide Y?

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Substantial evidence indicates a dysfunctional communication between the sympathetic nervous system and the immune system in Th1-mediated autoimmune diseases, such as rheumatoid arthritis and multiple sclerosis. In this Opinion, we propose that the sympathetic regulation of immunity is not only mediated by catecholamines but also involves neuropeptide Y (NPY), an additional postganglionic SNS transmitter that is shown to modulate various immunological functions *in vitro* and *in vivo*. Based on recent experimental findings, we believe that a more precise understanding of the role of NPY in the regulation of autoimmune Th1 cells will provide novel insights into the neuroimmunological basis of autoimmunity.

The precise pathophysiological mechanisms underlying organ-specific autoimmune disorders, such as multiple sclerosis (MS) and rheumatoid arthritis (RA), are largely unknown. Therefore, it is not surprising that treatment options for these diseases still remain unsatisfactory. Despite significant variations in the pathophysiology of autoimmune diseases, it is generally accepted that CD4^{+} Th1 lymphocytes have a key role in mediating target tissue destruction [1]. By producing typical Th1 cytokines, such as interferon-γ (IFN-γ), autoimmune T lymphocytes maintain and drive complex immune responses ultimately resulting in the destruction of the body's own structures, such as myelin sheaths in the case of MS or cartilage tissues in patients with RA.

It is now well established that the sympathetic nervous system (SNS) and the immune system communicate functionally with each other [2–5]. Interestingly, recent studies have indicated that the SNS is mechanically and/ or functionally affected in RA and MS [6,7], suggesting that the crosstalk between the SNS and the immune system is substantially disturbed in Th1-mediated autoimmune diseases. So far, experimental and clinical studies seeking to correlate the sympathetic defect and associated autoimmune disorders have predominantly focused on catecholamines, as they are widely considered to be the major sympathetic transmitters [8]. In fact, there is substantial evidence to suggest that catecholamines are able to suppress typical Th1-mediated autoimmune

responses [9]. This has led to the idea that catecholamines or catecholaminergic agents can be therapeutic options for the treatment of autoimmune disease. However, considerable evidence suggests that the 'adrenergic intervention' might be of limited value in providing protection against autoimmune diseases. Autoreactive T cells from patients with RA, for example, elicit significantly reduced functional responses to catecholamines due to altered expression of β-adrenergic receptors [10]. A further limitation is seen in the demonstration that, even though catecholamine levels in joint cavities of RA are elevated, active inflammation does not appear to be controlled [11].

Notably, catecholamines are not the only sympathetic transmitters that are of relevance for the communication between the SNS and the immune system. Postganglionic sympathetic nerves innervating primary and secondary lymphoid organs also release neuropeptide Y (NPY). Resembling the ability of other neuropeptides, such as vasointestinal peptide [12], to modulate crucial immunological functions in health and disease, NPY also has a crucial role in the communication between the SNS and the immune system [13]. In this Opinion, we will discuss the role of NPY in the regulation of autoimmunity and suggest that NPY, in addition to NPY receptor agonists and antagonists, represents an interesting innovative tool to shed new light on the pathology of Th1-mediated autoimmune diseases.

## Disturbances in the interactions between the SNS and the immune system in autoimmune disease

Early indications for a role of the SNS in modulating autoimmune responses came from studies showing that the chemical depletion of sympathetic transmitters modulates prototypical models of Th1-mediated autoimmunity, such as experimental autoimmune encephalomyelitis (EAE). Back in 1988, Chelmicka-Schorr et al. demonstrated that chemical sympathectomy augments disease severity of EAE induced by both active sensitization with myelin components and passive transfer of encephalitogenic T lymphocytes [14,15]. Similar results were obtained in experimental models of RA, in which chemical sympathectomy exacerbated the inflammation and osteopathic destruction of arthritic joints [16]. Although these direct observations support an anti-inflammatory role for the SNS, there are data available demonstrating that the

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Available online 20 August 2004

SNS can also be proinflammatory. This is of particular importance in experimental arthritis, where it was demonstrated that the SNS can indirectly promote the process of neurogenic inflammation by which joint damage is augmented [17].

Thus, the SNS modulates autoimmune responses in a bimodal manner: on the one hand there is a strong anti-inflammatory branch, whereas on the other hand the SNS also exerts proinflammatory action. It remains to be determined which factors are involved in the decision as to whether the pro- or the anti-inflammatory branch of the SNS predominates. One such factor could be the stage of the disease because converse effects of chemical sympath-ectomy have been observed in collagen-induced arthritis that was treated at different stages of the disease.

Functional defects of the SNS are not only present under experimental conditions. Clinical studies have also revealed considerable SNS dysfunction in patients with MS, such as impaired sympathetic skin responses and abnormal cardiovascular reflexes [7,18], suggesting that the defective crosstalk between the SNS and the immune system might further precipitate the manifestations of MS. In the same line of evidence, a selective reduction in sympathetic fibers has been demonstrated in the syno-vium of patients with RA [11]. Although it is unclear precisely how the SNS is affected in inflamed joints, the detectable changes in the SNS have been interpreted as evidence for the disruption of the local neuroimmunologi-cal control of inflammation in RA [19].

### Catecholamines in autoimmunity: not the whole truth?

Based on the fact that immune cells express functional $\beta_2$-adrenergic receptors [2–4], several investigators have studied the significance of the $\beta$-adrenergic pathway in autoimmune processes. *In vivo* experiments have shown that repetitive injections of $\beta_2$-adrenergic agonists effectively suppress clinical manifestations of both EAE and experimental arthritis [9,15]. Furthermore, it has been reported that pharmacological stimulation of the $\beta_2$-adrenergic pathway promotes the induction of oral tolerance by selectively inhibiting proinflammatory cyto-kines, such as IFN-$\gamma$ and interleukin-12 (IL-12), as well as promoting anti-inflammatory cytokines, such as IL-4, transforming growth factor-$\beta$ and IL-1R antagonist [20].

Even though these findings raise hopes of using catecholamines and adrenergic stimulants as new thera-peutic approaches, there are also concerns about introdu-cing 'adrenergic intervention' into the clinical arena. In fact, Miller *et al.* [11] showed that despite the reduction in sympathetic fibers in the joints of RA patients, the levels of norepinephrine are remarkably normal as a result of upregulated secretion of norepinephrine from synovia-borne TH$^+$ cells, suggesting an endogenous mechanism compensating for the loss of sympathetic innervation. Although the meaning of this observation remains speculative, it does not support the optimistic idea that further elevation of catecholamine levels leads to the suppression of inflammation.

An additional problem for an 'adrenergic intervention' comes from the well-known phenomenon of up- and downregulation of adrenergic receptors when the local levels of catecholamines are changed [4,21]. Accordingly, altered numbers and affinities of $\beta_2$-adrenergic receptors have been reported for T lymphocytes from RA [22] and MS patients [23], thus changing their responsiveness to the suppressive action of catecholamines.

### The complexity of the humoral crosstalk between the SNS and the immune system

A more precise look at sympathetic neurotransmission reveals that catecholamines are not the only transmitters released upon stimulation or depleted upon chemical sympathectomy. Instead, the release of catecholamines is usually accompanied by the secretion of other sympathetic mediators, such as NPY or ATP. Sympathetic transmission in general is a rather complex process, involving differen-tial corelease sequences, direct interactions between the transmitters in the synaptic cleft and alterations in presynaptic and postsynaptic receptor sensitivity [8,24]. This complexity also accounts for the immunological actions of the SNS. Both catecholamines and NPY, for example, evoke various immunological functions indepen-dently, yet they also interact functionally. More specifi-cally, NPY differentially regulates the functional efficacy of catecholaminergic effects during leukocyte mobiliz-ation. Weak catecholaminergic stimuli are facilitated in the presence of NPY, whereas exaggerated catecholamine effects can be inhibited [25]. Furthermore, the influence of NPY on catecholaminergic effects in the immune system also differs depending on which adrenergic receptor subtype is engaged to activate the downstream pathway [26]. Thus, NPY must be included in the picture, to improve our understanding of the precise mechanisms underlying the defective interaction between the SNS and the immune system in autoimmunity.

### A role for NPY in suppressing Th1-mediated autoimmunity

NPY, a 36 amino acid peptide, was discovered in 1982 by Tatemoto and has since been found to be present in various brain regions, where it is implicated in various central nervous system functions. In the periphery, NPY is released from sympathetic nerves alone and in combi-nation with catecholamines [24]. Interestingly, NPY has been implicated in a multitude of immunological functions and mechanisms [13]. For example, NPY increases the phagocytosis of *Candida albicans* [27], differentially regulates IL-1$\beta$ and IL-6 secretion by monocytes [28] and modulates T-lymphocyte adhesion [29]. Of particular interest are two independent reports showing that NPY inhibits the secretion of IFN-$\gamma$ and enhances IL-4 secretion of murine lymphocytes *in vitro*, indicating that NPY shifts the Th1/Th2 balance towards the Th2 phenotype [30,31]. Together with the finding that NPY levels are decreased in the cerebrospinal fluid of MS patients [32], a role for NPY in Th1-mediated central nervous system autoimmunity seemed likely. Therefore, we tested to see whether repetitive administrations of NPY exerted any impact on EAE, and found that it ameliorated symptoms and disease severity in a dose-dependent fashion [33]. This study provided the first direct indication that a sympathetic transmitter other

than a catecholamine contributes to the 'immunoregula-immunoregulatory' role of the SNS in Th1-mediated autoimmunity.

Furthermore, there have been several other observations suggesting a regulatory role for NPY in human Th1-mediated autoimmunity. The potential relevance of NPY for MS was very recently emphasized in a genetic study revealing a vulnerability locus on human chromosome 7p15 [34], a locus that actually encodes NPY. Additionally, a specific loss of NPY-containing fibers was found in the synovium of RA patients [6,11], and NPY concentrations in the joints of RA patients were found to be directly correlated with symptom severity, with higher levels of NPY associated with a shorter duration of pain [35]. These observations suggest that decreased local concentrations of NPY are involved in the progression of joint inflammation in RA. It remains to be investigated whether NPY also has the ability to modulate animal models of arthritis.

## Mechanisms by which NPY might influence Th1-mediated autoimmunity

Looking for underlying mechanisms for the suppressive action of NPY on EAE (Figure 1), we discovered that treatment with NPY changes the cytokine profile of autoreactive T lymphocytes towards the Th2 phenotype *ex vivo*. When stimulated with the specific autoantigen, autoreactive T lymphocytes from EAE mice that had been treated with NPY secreted significantly lower amounts of IFN-$\gamma$. Another *in vivo* indicator for a deviation towards the Th2 phenotype in treated mice is the finding that the majority of autoantigen-specific antibodies are of the IgG1 isotype [33]. It was also interesting to establish whether NPY exerted its effects directly on T lymphocytes or whether it acted via an indirect action mediated by antigen-presenting cells. Using differential coincubation protocols, we were able to show that NPY acts directly on T lymphocytes via the NPY $Y_1$ receptor subtype. Thus, it is our opinion that NPY induces a shift in autoreactive T lymphocytes towards the Th2 phenotype. As the induction of a Th2 shift is also known to be beneficial in arthritis, we speculate that a possible suppressive effect of NPY on experimental or clinical arthritis could also engage this mechanism. However, such investigations must also consider other cellular targets for NPY, such as natural killer cells, macrophages, mast cells or osteoclasts, because these cells also have an important role in the pathogenesis of arthritis.

As NPY is usually secreted in combination with catecholamines, it is most likely that the cooperativity between both SNS transmitters also has a significant role in influencing autoimmune responses. NPY is known to differentially modulate the immunological effects of catecholamines. This complex phenomenon depends on such parameters as the adrenergic receptor subtype predominantly activated ($\alpha$- versus $\beta$-adrenoreceptors), and the concentrations of catecholamines actually involved. Of particular interest is the finding that low, ineffective concentrations of catecholamines can be facilitated upon coadministration of NPY. For example, when applied in combination with NPY, catecholamine dosages

that are *per se* without an effect can evoke a biological response attributable to the action of catecholamines [25]. As autoimmunity represents a situation in which the catecholaminergic action is somehow ineffective, administration of exogenous NPY might represent a mechanism for facilitating and potentiating the subthreshold action of endogenous catecholamines.

## Ineffective activation of Y$_1$ receptors in autoimmunity?

It has been demonstrated that T lymphocytes from individuals with MS express higher amounts of the surface protein CD26 [36]. Notably, this protein is not only an activation marker but also exerts enzymatic activities, which are of major consequence for NPY [24]. The result of N-terminal truncation of NPY by CD26 is a peptide fragment with significantly altered receptor affinities, as the degradation product $NPY_{3-36}$ loses its ability to activate the $Y_1$ receptor and becomes a selective agonist at the $Y_2$ and $Y_5$ receptors [37]. Increased degradation of NPY by CD26 under autoimmune conditions might thus account for insufficient activation of $Y_1$ receptors, which in turn favors the development of Th1 responses. Interestingly, this possibility is consistent with the recent report that the pharmacological inhibition of CD26 suppresses the clinical course of EAE via the induction of a Th2 shift [38]. It remains to be investigated whether the suppressive action of CD26 inhibition results from increased $Y_1$ receptor activation and the corresponding Th2 shift.

Apart from the possibility of increased functional degradation of NPY and the resulting consequences for the Th1/Th2 balance of autoimmune responses, an additional mechanism might be at work to decrease the efficiency of $Y_1$ receptor activation in autoimmunity. Silva *et al.* [39] have recently reported that IFN-$\gamma$ preferentially upregulates the expression of $Y_5$ receptors without exerting any effect on the expression of $Y_1$ receptors in human umbilical cord endothelial cells. It might well be the case that the increased levels of IFN-$\gamma$ present in Th1-mediated autoimmune responses also selectively increase the expression of $Y_5$ receptors, which in turn shifts the balance between $Y_1$ and $Y_5$ receptor activation.

These indirect indications give us clues for further investigations into the role of NPY in the regulation of autoimmune processes and help us to develop pharmacological interventions for autoimmune diseases based on stimulating NPY receptors or inhibiting the degradation of NPY.

## Conclusion and future perspective

There is convincing experimental and clinical evidence demonstrating a dysfunctional communication between the SNS and the immune system in autoimmunity. Despite the early notion that catecholamines are the main players in the sympathetic control of Th1 immunity, we show here that other sympathetic transmitters, such as NPY, also have a significant role in autoimmunity. It is our view that the catecholaminergic defect only partially reflects the pathophysiological basis of the defective crosstalk between the SNS and the immune system. A
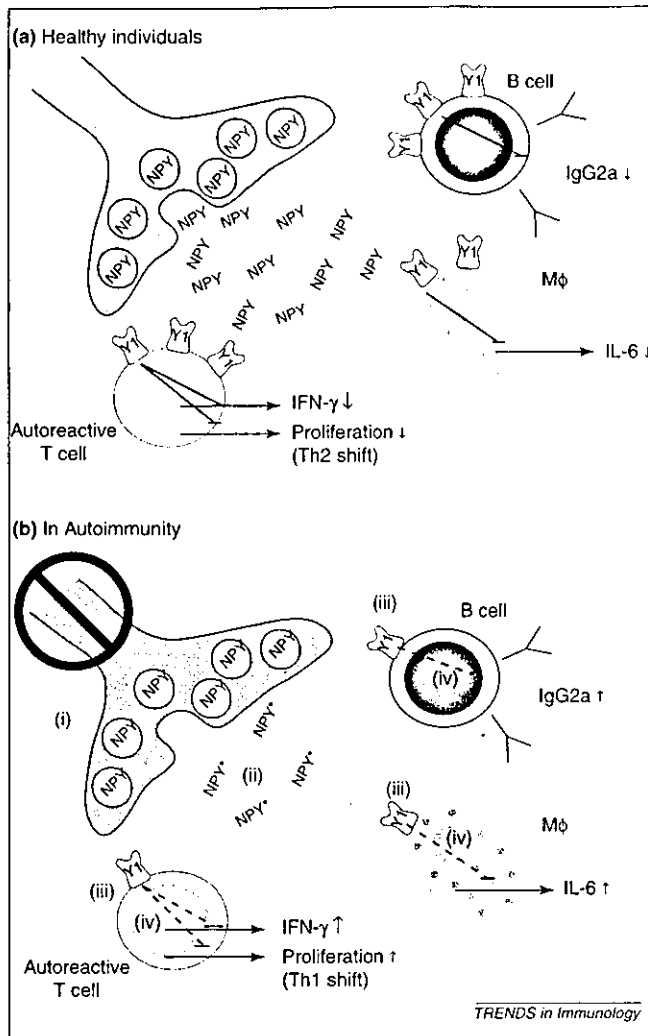
**Figure 1.** The effects of neuropeptide Y (NPY) on certain immune parameters in both physiological and autoimmune conditions. (a) Under physiological conditions, NPY is released from sympathetic nerve fibers innervating immune competent organs, such as lymph nodes and the spleen. Upon its release, NPY binds to $Y_1$ receptors, which are expressed on a variety of immune cells, where it induces functional effects, such as the promotion of Th2 responses, by inhibiting the release of interferon (IFN)-γ from T cells and promoting the production of IgG1 antibodies. NPY also inhibits the production of proinflammatory cytokines from monocytes. (b) Proposed model of the involvement of NPY in autoimmunity. There is a loss of NPY-containing sympathetic nerve fibers (i) innervating disease-relevant compartments, such as the joint cavity in rheumatoid arthritis (RA), and a significant decrease in NPY levels (ii) in the cerebrospinal fluid from patients with multiple sclerosis (MS). In analogy to the alterations in $β_2$-adrenoreceptor expression in RA and MS, we hypothesize that the number of NPY $Y_1$ receptors (iii) and their responsiveness (iv) might also be altered. This proposed multitude of mechanisms for reducing responsiveness to NPY induces a Th1 shift, as demonstrated by increased production of IFN-γ and IgG2a antibodies. We also speculate that in the absence of NPY, the production of interleukin-6 (IL-6) from monocytes (Mφ) is increased.

comprehensive understanding of SNS and immune system interactions during autoimmunity can only be achieved by shifting the focus towards alternative sympathetic transmitters, such as NPY or ATP, and their role in autoimmune processes.

Many issues of the complex network between the SNS and the immune system and their significance for autoimmunity remain to be clarified. What are the precise mechanisms and factors that determine whether the anti-inflammatory or the proinflammatory branch of the SNS predominates? What is the role of other SNS transmitters,

such as ATP? How do non-immunological properties, such as the angiogenetic properties of the SNS [40], contribute to this network?

Solving these issues might prove to be a stimulus for reigniting the possibility of a 'sympathetic intervention' for autoimmune disorders.

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# CASE REPORT

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Tsuneyo Mimori

# Systemic lupus erythematosus associated with recurrent lupus enteritis and peritonitis

**Abstract** We describe the case of a 41-year-old woman with systemic lupus erythematosus (SLE) who suffered from repeated reversible lupus enteritis characterized by marked edematous thickening of the small intestine. Ultrasonography (US) and computed tomography (CT) manifested as an 'accordion-like appearance' and a 'target-like appearance', respectively. Resolution of gastrointestinal tract wall thickening was observed on follow-up US performed a week after the increase in predinosolone (PSL). We conclude that careful evaluation of sonographic and radiographic findings helps to establish the diagnosis of lupus enteritis.

**Abbreviations** CT: Computed tomography · FANA: Fluorescent antinuclear antibody · GI: Gastrointestinal · SLE: Systemic lupus erythematosus · US: Ultrasound

## Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by the involvement of various organs [1]. Although gastrointestinal (GI) symptoms are

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frequently observed in this disorder, their incidence varies widely [2, 3, 4]. Among these symptoms, abdominal pain is the most common GI involvement [2], and a variety of causes has been implicated. Here, we describe a lupus patient who presented with recurrent enteritis characterized by marked edematous thickening of the wall of the small intestine assessed by ultrasonography (US) and computed tomography (CT).

## Case report

A 41-year-old woman was diagnosed as having SLE on the basis of facial erythema, photosensitivity, oral ulcer, pleuritis, and positive fluorescent antinuclear antibody (FANA). She was treated with prednisolone (PSL) and azathioprine in 1979. In 1981, she developed fever and proteinuria and was admitted to Keio University Hospital. A renal biopsy specimen showed membranous lupus nephritis (WHOV), which was improved by PSL and cyclophosphamide therapy. In 1984, she was readmitted to our hospital because of abdominal pain and vomiting, and was found to have ascites. The dose of PSL was then increased from 5 mg to 17.5 mg daily with 50 mg of cyclophosphamide daily, resulting in a dramatic improvement of her GI symptoms. Since that time she had experienced similar episodes, including abdominal pain and vomiting, and an increased dose of PSL was required three more times. In July 1998 she was again admitted to our hospital because of fever, abdominal colic pain and vomiting (Fig. 1).
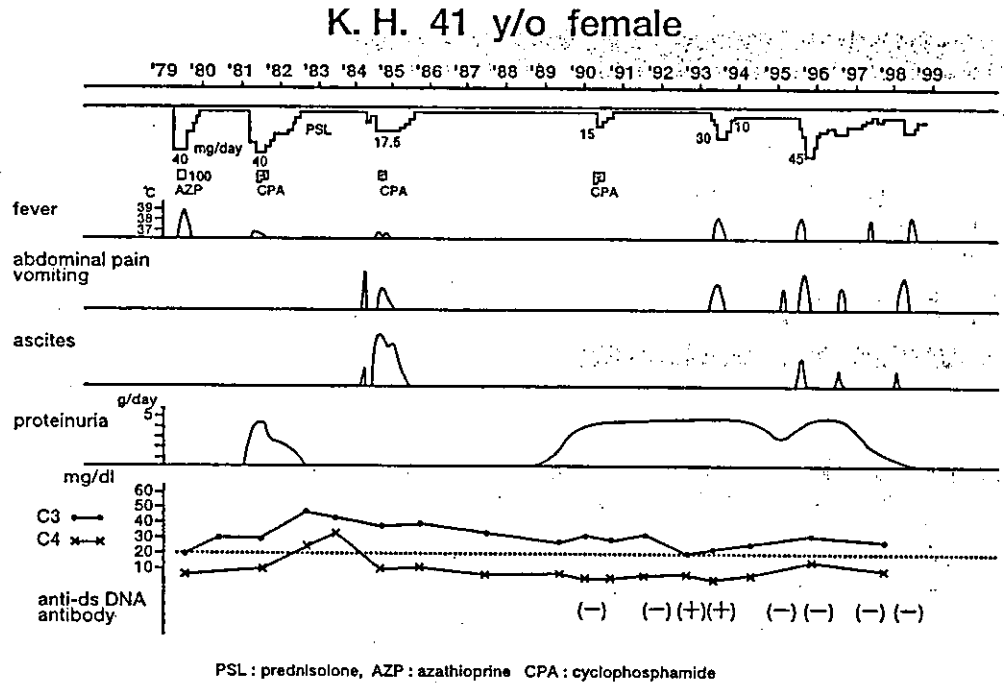
On physical examination she had a slight fever of 37.2°C, but other vital signs were normal. Chest and cardiovascular examination revealed no abnormalities. Abdominal examination showed that her bowel was silent and that tenderness of the left and middle lower abdomen was noted.

Laboratory testing showed an erythrocyte sedimentation rate (ESR) of 19 mm/h (normal <15), no abnormality of blood cell counts, urinalysis or stool test; FDP was 1382 ng/ml (normal <100) and CRP

Fig. 1 The clinical course of the present case. The patient suffered from recurrent bouts of fever, abdominal pain, vomiting and ascites, and her US and CT showed marked edematous thickening of the wall of the small intestine. She was diagnosed as having lupus enteritis, and the dose of PSL was increased each time, resulting in a rapid improvement of her GI symptoms within a few days



PSL : prednisolone, AZP : azathioprine   CPA : cyclophosphamide

1.95 mg/dl (normal < 0.15). Serum complement levels were decreased (C3 30 mg/dl, C4 16 mg/dl, and CH50 25.4 U/ml; normal range 60–80, 20–35, 30–40, respectively). FANA was positive (speckled pattern, titer of 1:320). Anti-U1 RNP and anti-SS-A/Ro antibodies were positive, although anti-ds DNA antibody was negative. Abdominal US and CT showed an 'accordion-like appearance' and a 'target-like appearance', respectively, demonstrating marked edematous thickening of the wall of the small intestine (Fig. 2).

The patient was diagnosed as having lupus enteritis and peritonitis, and the dose of PSL was increased to 20 mg daily, resulting in a rapid improvement of her GI

Fig. 2 Abdominal US and CT. Left: US reveals small intestinal wall thickening, showing an 'accordion-like appearance'. Right: CT demonstrates marked intestinal wall thickening, showing a 'target-like appearance' (arrow)
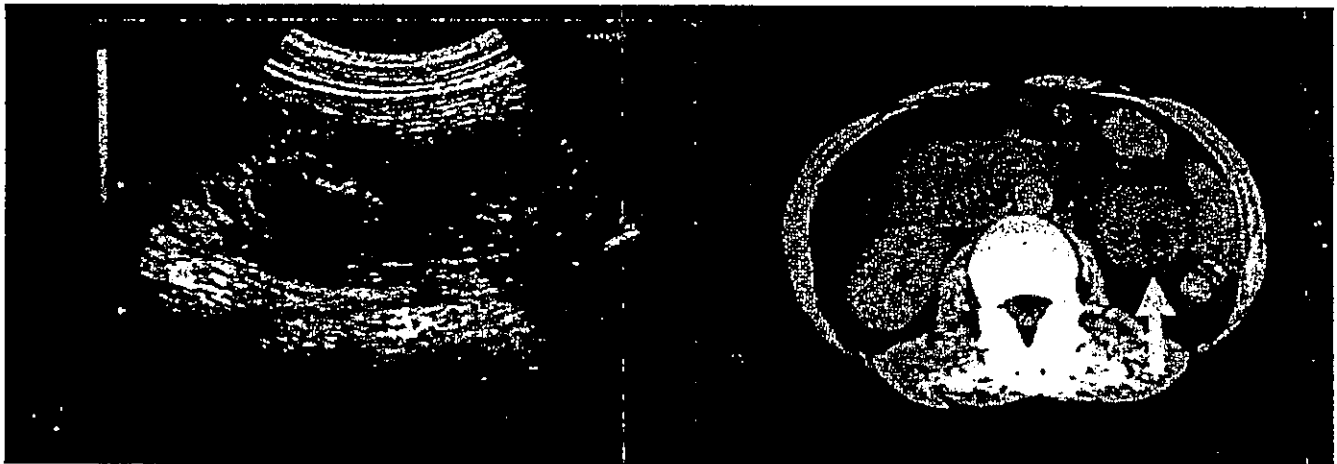
symptoms within a few days. Follow-up US performed 1 week after treatment revealed disappearance of the thickening of the intestinal wall. She was discharged in a good condition.

## Discussion

SLE is a chronic inflammatory disease that affects the skin, joints, kidneys, the central nervous system, and other organs. GI symptoms have been reported to be common in SLE patients [5, 6, 7]. Non-specific symptoms such as anorexia, nausea, vomiting and diarrhea may be seen in approximately 30%–50% of patients [2, 3, 4]. Abdominal pain and tenderness can also be the first and the most common symptom of lupus-induced GI involvement. Although it was reported that lupus enteritis was the most common cause of abdominal pain in SLE [4] and sometimes critical, it is usually

Table 1 Three cases of lupus enteritis in our department

| Case | 1 | 2 | Present case |
|---|---|---|---|
| Age, sex | 36, F | 57, F | 41, F |
| Duration from diagnosis | 11y | 12y | 15y |
| Symptoms | | | |
| Abdominal pain | (+) | (+) | (+) |
| Vomiting | (+) | (+) | (+) |
| Ascites | (+) | (+) | (+) |
| Activity of SLE | Fever | Fever | Fever |
| | ESR↑ | ESR↑ | ESR↑ |
| | Complement↓ | Complement↓ | Complement↓ |
| | Facial erythema | WBC↓ | |
| | Arthralgia | | |
| Avascular necrosis | (+) | (+) | (+) |
| Autoantibody | Anti-U1 RNP(+) | Anti-U1 RNP (+) | Anti-U1 RNP (+) |

difficult to determine the cause of pain, because a variety of sources is implicated and corticosteroid therapy may often affect the pathological findings from biopsy specimens.

In previous reports, intestinal symptoms have been described as a peritoneal irritation in association with lupus peritonitis. It has been noted that ascites occurring with abdominal pain can be found together with abdominal vasculitis [8]. Lupus enteritis is defined as 'enteritis specific (or proper) to SLE, unrelated to other causes'. In the present case it should be noted that the enhanced CT of the abdomen showed marked wall thickening of the small intestine. The findings of their cross-sections were compatible with the 'target-like appearance' reported by Tsushima et al. [9]. Moreover, US demonstrated edematous thickening of the small intestine, where the submucosal layer is shown as a prominent hypoechoic area and kerckring folds with submucosal edema resembled an accordion, called an 'accordion-like appearance' reported by Shirato et al. [10]. It is also noteworthy that the disappearance of GI tract wall thickening was observed in the follow-up US, indicating a good response to corticosteroid therapy. Therefore, these characteristic findings on CT as well as US are likely to be useful both in the diagnosis of lupus enteritis and in monitoring the efficacy of the treatment [9]. However, further examinations of other clinical features, radiographic or endoscopic manifestations would be required for the differential diagnosis of other conditions showing diffuse thickening of the bowel wall, including ischemic enteritis, amyloidosis, infectious enteritis, radiation-induced enteritis, Crohn's disease, angioneurotic edema and ileus [10].

Chung et al. reported a Japanese woman with lupus enteritis in Canada, whose abdominal CT showed thickened loops of small intestine similar to our case [11]. However, the racial difference in the prevalence and features of lupus enteritis are still unknown, as most previous cases with this disease have been included in lupus peritonitis or cystitis with gastroenteral manifestations. Therefore, further analysis of a large cohort of patients focusing on lupus enteritis will be required for our understanding of the pathogenetic mechanism of this disease.

The pathogenesis of this condition is unknown. However, it is speculated that vascular lesions in SLE, especially immune complex-mediated vasculitis, may lead to ischemia of the bowel wall followed by mucosal edema [8, 9, 10, 11, 12, 13, 14, 15]. Table 1 summarizes the clinical and laboratory features of three SLE patients with associated lupus enteritis in our patient cohort. All patients presented with lupus enteritis over the following more than 10 years after the initial diagnosis of SLE. All had GI symptoms such as abdominal pain, vomiting and ascites, with marked wall thickening of the small intestine revealed by US, and they were all treated with PSL. Interestingly, all patients were found to have anti-U1 RNP antibody and were suffering from aseptic necrosis of the femoral head that might be associated with vasculitis.

In conclusion, abdominal US and CT procedures have been shown to be useful in assessing the presence of lupus enteritis and the prompt initiation of treatment.

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# Autoantibodies from primary biliary cirrhosis patients with anti-p95c antibodies bind to recombinant p97/VCP and inhibit *in vitro* nuclear envelope assembly

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## SUMMARY

We have reported previously that p95c, a novel 95-kDa cytosolic protein, was the target of autoantibodies in sera of patients with autoimmune hepatic diseases. We studied 30 sera that were shown previously to immunoprecipitate a 95 kDa protein from [$^{35}$S]-methionine-labelled HeLa lysates and had a specific precipitin band in immunodiffusion. Thirteen sera were available to test the ability of p95c antibodies to inhibit nuclear envelope assembly in an *in vitro* assay in which confocal fluorescence microscopy was also used to identify the stages at which nuclear assembly was inhibited. The percentage inhibition of nuclear envelope assembly of the 13 sera ranged from 7% to 99% and nuclear envelope assembly and the swelling of nucleus was inhibited at several stages. The percentage inhibition of nuclear assembly was correlated with the titre of anti-p95c as determined by immunodiffusion. To confirm the identity of this autoantigen, we used a full-length cDNA of the p97/valosin-containing protein (VCP) to produce a radiolabelled recombinant protein that was then used in an immunoprecipitation (IP) assay. Our study demonstrated that 12 of the 13 (93%) human sera with antibodies to p95c immunoprecipitated recombinant p97/VCP. Because p95c and p97 have similar molecular masses and cell localization, and because the majority of sera bind recombinant p97/VCP and anti-p95c antibodies inhibit nuclear assembly, this is compelling evidence that p95c and p97/VCP are identical.

**Keywords** autoantibody  conformational epitope  nuclear envelope assembly  p95c  p97/VCP  primary biliary cirrhosis

## INTRODUCTION

Patients with autoimmune liver diseases such as primary biliary cirrhosis (PBC), autoimmune hepatitis (AIH), autoimmune cholangiopathy (AIC) and primary sclerosing cholangitis (PSC) produce autoantibodies that differ from those found in patients with systemic rheumatic diseases such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), systemic sclerosis (SSc) and Sjögren's syndrome (SjS) [1–3]. In particular, anti-mitochondrial antibodies (AMA) have been reported in 85–90% of patients with PBC and these are among the most prevalent autoantibodies found in any autoimmune disease [4–6]. Autoantibodies that bind to components of the nuclear envelope, such as anti-gp210 and anti-p62 complex, are also important markers for the diagnosis of

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PBC patients with and without AMA, and for monitoring the progression of disease [7–9]. Other studies have shown that anticentromere antibodies, especially anticentromere B antibody, anti-SP100 and antibodies to high mobility group (HMG) proteins 1 and 2 may also be useful for the diagnosis of PBC [10–14]. Anti-liver kidney microsome (LKM) antibody and peripheral antineutrophil cytoplasmic antibodies (p-ANCA) are valuable for the diagnosis of type 2 AIH [15,16] and PSC [17].

In 1998, we reported a novel antibody directed against a conformational epitope on a 95-kDa protein in patients with autoimmune hepatic diseases [18]. This antibody was found in 12% of PBC and 9·7% of AIH patients, but was not detected in other autoimmune conditions without hepatic involvement. Interestingly, unlike LKM and AMA and many other autoantigens, this antigen was not detected by immunoblot. Double immunodiffusion that used antigens extracted from rat liver homogenates showed a specific precipitin line that was different from other known immune precipitin systems [18]. Based on

immunoprecipitation of extracts of metabolically labelled HeLa cells, the molecular mass of this autoantigen was estimated to be 95 kDa.

Recently, p97/VCP (valosin-containing protein) was characterized and found to play an important role in nuclear envelope assembly and the formation of the endoplasmic reticulum and Golgi apparatus during the final stage of mitosis [19,20]. Of interest, antibodies to p97/VCP inhibited nuclear reassembly *in vitro* [21]. Based on studies and paradigms of other autoantibodies that bind to and inhibit functional domains or active sites of the cognate antigens [2], we reasoned that if autoantibodies to p95 and p97/VCP were identical that they too would reduce its biological activity and inhibit nuclear assembly. In this study, we have sought to determine whether the cognate antigen of anti-p95c and p97/VCP are identical by investigating the ability of the autoantibody to inhibit nuclear envelope assembly and to immunoprecipitate recombinant p97/VCP.

## MATERIALS AND METHODS

### Patients and sera

Thirty sera with antibodies to p95c were identified by immunodiffusion in a serum bank established in the Health Sciences Research Institute. The diagnosis of the patients was established according to published clinical parameters and histological features of liver biopsies [22,23]. Sufficient amounts of sera from 13 patients were available for the inhibition of nuclear envelope assembly assay and to identify anti-p95c to anti-p97/VCP by immunoprecipitation (described below). A prototype serum (I) with antibodies to p95c and normal human serum were used as controls to study the steps of nuclear assembly inhibition during the cell cycle by confocal immunofluorescence microscopy.

### Indirect immunofluorescence

Antinuclear antibody (ANA) and AMA were detected by indirect immunofluorescence, as described in detail elsewhere [24]. Briefly, HEp-2 slides and cryostat sectioned rat kidney and stomach (Fluoro AID 1 test, MBL Inc., Japan) were used for ANA and AMA, respectively. The sera were incubated on the substrates and after excess antibody was washed away and they were then incubated with polyvalent anti-human immunoglobulin conjugated to fluorescein isothiocyanate. The slides were read on a fluorescence microscope (Nippon Optico, Japan).

### Double immunodiffusion

The Ouchterlony double immunodiffusion (ID) method was used to demonstrate the identity of precipitin reactions between soluble antigen and serum antibodies. The antigen source was prepared from rat liver mitochondrial, microsomal and supernatant fractions as described previously [18]. The mitochondrial fraction was sonicated (Tosho Electric Company, Japan) for 45 s at full power to release antigens and the protein concentration of the soluble antigens determined as described previously [18]. Sixty mg/ml of the microsomal fraction was then used as the antigen source for the detection of anti-LKM 1 and anti-p95c antibodies. Conventional antimitochondrial antibodies (i.e. antibodies to pyruvate dehydrogenase complex) and antibodies to nuclear antigens, such as anti-U1RNP, Sjögren's syndrome antigen A (SS-A)/

Ro and anti-Sm, were not detected under these experimental conditions [25].

### In vitro transcription/translation and immunoprecipitation

The cDNA representing the full-length valosin-containing protein (p97/VCP: Accession number CAA78412; a gift from Dr Graham Warren, Yale University, New Haven, CT, USA) was used as a template for *in vitro* transcription and translation (TnT, Promega, Madison, WI, USA) in the presence of [35S]-methionine as described previously [26,27]. TnT reactions were conducted at 30°C for 1·5–2 h and the presence of translation products was confirmed by subjecting 2–5 μl samples to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and analysis by autoradiography. The *in vitro* translated products were then used as the antigen source. IP reactions were prepared by combining 100 μl 10% protein A-Sepharose beads (Sigma, catalogue no. P-3391), 10 μl human serum, 500 μl NET2 buffer (50 mM Tris-HCl, pH 7·4, 150 mM NaCl, 5 mM EDTA, 0·5% Nonidet P-40, 0·5% deoxycholic acid, 0·1% SDS, 0·02% sodium azide) and 5–10 μl of labelled recombinant protein obtained from the TnT reaction described above. After 1 h of incubation at 4–8°C, the Sepharose beads were washed five times in NET2, and the proteins eluted in 10 μl of sample buffer. The proteins were analysed by 10% or 12·5% SDS-PAGE as described previously [26].

### Nuclear assembly assays

Demembranated sperm chromatin was prepared as described [28] and stored at −80°C at a concentration of 40 000 units/μl. *Xenopus* sp. eggs were collected, the jelly layer removed and then lysed to prepare an interphase extract [29]. The nuclear envelope assembly assays were then performed essentially as described by Smythe and Newport [30]. Briefly, the *Xenopus* egg extracts, cytosol and membrane fractions were supplemented with an ATP regenerating system (10 mM phosphocreatine, 2 mM ATP (pH 7·0), 5 μg/ml creatine kinase), and then mixed with demembranated sperm chromatin. The standard reaction mixture consisted of 10 000 units of chromatin and 10 μl crude extract or 10 μl cytosol + 1 μl membrane. After incubation at room temperature (23°C) for 1·5 h, a 2 μl aliquot of the reaction mixture was removed and diluted with 2 μl of Hoechst in dihexylocarbocyanine iodine (DHCC) buffer (15 mM PIPES-KOH, pH 7·4, 0·2 M sucrose, 7 mM MgCl₂, 80 mM KCl, 15 mM NaCl, 5 mM EDTA) containing 20 mg/ml bis-benzimide DNA dye (Hoechst 33342; Calbiochem-Novabichem), a lipid dye, 3,3′-DHCC (Aldrich, Japan), and 3·7% formaldehyde on a glass slide. The sample was mounted with a cover-slip and examined under a 100x objective lens on a phase contrast and Axioplan fluorescence microscope (Carl Zeiss) fitted with exciter barrier reflector combinations appropriate for the fluorescent dyes described above.

For confocal microscopy, DNA was visualized by staining the preparations with propidium iodide and DHCC. Images were recorded with a Radiance 2000 confocal fluorescence system (Bio-Rad, Tokyo) mounted on a Nikon E600 fluorescence microscope (Nikon, Tokyo). The rate of inhibition of nuclear assembly was calculated by applying the formula: corrected inhibition rate of nuclear assembly (%) = (inhibition rate of nuclear assembly obtained from adding patient's serum (%) − inhibition rate of nuclear assembly obtained from adding normal healthy serum (%)/nuclear assembly obtained from adding normal healthy serum (%)

## RESULTS

The diagnosis of the 30 patients that IP the p95c protein included 23 with PBC and seven with AIH (Table 1). Twenty-four of the 30 (80%) patients were female. Twelve patients presented with other concurrent diseases: eight with SjS, two with Hashimoto's thyroiditis, one with RA and one with dilated cardiomyopathy. Within the group of eight SjS patients, four had an overlap syndrome manifested as SLE/SjS, SSc/SjS, mixed connective tissue disease (MCTD)/SjS or RA/SjS. Among the 13 PBC patients that had a liver biopsy, two cases were classified as Scheuer stage 4, but the remaining cases were classified as either stage 1 or 2.

Antimitochondrial antibodies (AMA) were detected in 21 (70%) of the sera with titres ranging from 1/20 to 1/640. A positive ANA was observed in 18 patients with titres that ranged from 1/40 to 1/20 480. Eight patients had antibodies to SS-A, 5 had anti-U1RNP and one with a PBC/SjS/SSc overlap syndrome had antitopoisomerase 1.

### Inhibition of in vitro nuclear envelope assembly

The 13 sera (PBC 10, AIH 3) with p95c antibodies demonstrated 7–99% inhibition of the envelope assembly in the *in vitro* assay (Table 1). When the data are plotted as percentage of inhibition rates (Fig. 1), the degree of inhibition was correlated with the titre of anti-p95c. In separate experiments, crude *Xenopus* egg extracts were pre-incubated with buffer alone, control normal serum and a prototype serum (IK). The degree and stage at which nuclear assembly was inhibited was examined by adding *Xenopus* sperm chromatin to the reaction. Pre-incubation with buffer or normal serum yielded continuous nuclear rim-staining with the phospholipid stain DHCC, indicating the presence of assembled chromatin (Fig. 2). On the other hand, pre-incubation with the prototype serum yielded discontinuous lipid-staining of the nuclear rims with some areas well covered by membrane while others were not (Fig. 2). When the surface of the chromatin was viewed under higher magnification, the nuclear envelope was not fully enclosed and the lipid-staining was reticulated.

**Table 1.** Clinical and serological features of 30 patients with antibodies to p95c

| No. | Sex | Liver biopsy | Primary diagnosis* | Secondary diagnosis | AMA titre** | ANA titre** | ANA specificity | Anti-p95c ID** | Anti-VCP IP | INA % |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | M | + | PBC:S1-2 | SjS | 160 | 160 | U1RNP/SS-A | 128 | + + | 82 |
| 2 | F | n.d. | AIH | SjS | – | 80 | U1RNP/SS-A | 1 | + + + | 67 |
| 3 | F | n.d. | AIH | SLE/SjS | – | 640 | U1RNP/SS-A | 8 | + + | 29 |
| 4 | F | n.a. | PBC | – | 40 | – | – | 32 | + + + | 13 |
| 5 | F | n.a. | PBC | – | – | – | – | 1024 | + + + + | 88 |
| 6 | M | + | PBC:S1 | – | 40 | 80 | – | 256 | + + + + | 59 |
| 7 | M | n.d. | PBC | – | 40 | – | – | 64 | + + + + | 64 |
| 8 | F | n.d. | PBC | – | – | 40 | – | 16 | + + + | 13 |
| 9 | F | + | AIH | – | – | – | – | 16 | + | 7 |
| 10 | F | + | PBC:S2 | – | 40 | – | – | 64 | –/+ | 99 |
| 11 | F | + | PBC:S1 | Hashimoto | 320 | 80 | – | 4 | + | 15 |
| 12 | F | + | PBC:S1 | SjS | 80 | 20 | SS-A | 32 | + + | 23 |
| 13 | F | n.d. | PBC | – | 640 | – | – | 512 | + + + + | 83 |
| 14 | M | + | PBC:S1 | – | 80 | – | – | 16 | n.d. | n.d. |
| 15 | F | + | PBC:S1 | – | 160 | – | – | 256 | n.d. | n.d. |
| 16 | F | + | PBC:S2 | – | 160 | 320 | – | 2 | n.d. | n.d. |
| 17 | F | + | PBC:S2 | – | 320 | – | – | 16 | n.d. | n.d. |
| 18 | F | + | PBC:S4 | – | 640 | 20 | – | 16 | n.d. | n.d. |
| 19 | M | + | PBC:S4 | – | 640 | – | – | 8 | n.d. | n.d. |
| 20 | F | n.d. | PBC | – | 160 | 80 | – | 32 | n.d. | n.d. |
| 21 | F | n.d. | PBC | – | 640 | – | – | 128 | n.d. | n.d. |
| 22 | F | n.d. | PBC | – | 320 | – | – | 32 | n.d. | n.d. |
| 23 | F | + | PBC:S1 | Hashimoto | 320 | 80 | – | 128 | n.d. | n.d. |
| 24 | F | + | PBC:S1 | SjS/SSc | 20 | 320 | SS-A/Topo1 | 32 | n.d. | n.d. |
| 25 | F | n.d. | PBC | SjS/MCTD | – | 20480 | U1RNP/SS-A | 16 | n.d. | n.d. |
| 26 | M | n.d. | PBC | DCM | 320 | – | – | 32 | n.d. | n.d. |
| 27 | F | + | AIH | – | – | 160 | – | 256 | n.d. | n.d. |
| 28 | F | + | AIH | SjS | 320 | 320 | U1RNP/SS-A | 8 | n.d. | n.d. |
| 29 | F | n.d. | AIH | RA | – | 160 | – | 64 | n.d. | n.d. |
| 30 | F | n.d. | AIH | SjS/RA | – | 160 | SS-A | 64 | n.d. | n.d. |

*S indicates Scheuer's staging of liver pathology [22,23]. **AMA, ANA and p95c titres are the reciprocal of number shown; + indicates the semiquantitative reaction with recombinant VCP as determined by the autoradiogram of immunoprecipitated [³⁵S]-labelled protein. AIH, autoimmune hepatitis; AMA, antimitochondrial antibodies; ANA, antinuclear antibodies; DCM, dilated cardiomyopathy; ID, immunodiffusion; INA, inhibition of nuclear assembly; IP, immunoprecipitation; MCTD, mixed connective tissue disease; n.a., result not available; n.d., not done; PBC, primary biliary cirrhosis; RA, rheumatoid arthritis; SjS, Sjögren's syndrome; SLE, systemic lupus erythematosus; SS-A, Sjögren's syndrome antigen A; Topc1, topoisomerase 1; U1RNP, U1 ribonucleoprotein; VCP, valosin-containing protein.

*Immunoprecipitation of recombinant p97/VCP*

The same 13 sera were then used in an IP assay that employed radiolabelled recombinant p97/VCP produced in the rabbit reticulocyte lysate system (Table 1). Twelve (93%) of the sera immunoprecipitated the ~97 kDa recombinant protein, but normal human sera and PBC sera with antimitochondrial antibodies, but no antibodies to p95c, did not (Fig. 3). One serum (no. 10: Table 1, Fig. 3) showed an equivocal IP result.

## DISCUSSION

This study provides compelling evidence that the previously described p95c autoantigen and p97/VCP are the same proteins. This conclusion is based on a number of remarkable similarities between p95c and p97/VCP. First, the molecular masses and cellular localization in the cytosol are nearly identical. Secondly, all sera with anti-p95c antibodies have been shown to inhibit nuclear envelope assembly. Thirdly, all but one of the available 13 sera that had anti-p95 antibodies and inhibited nuclear assembly immunoprecipitated the recombinant p97/VCP protein.

We have shown previously that anti-p95c antibodies that were found in the sera of patients with PBC and AIH were demonstrated easily by immunodiffusion and immunoprecipitation but



**Fig. 1.** Relationship between percentage inhibition of nuclear assembly and titres of anti-p95c antibodies in the sera of PBC patients. Sera with low titre (<1 : 128) anti-p95c antibodies exhibit less inhibition of nuclear assembly whereas sera with high titres (>1/128) show the most marked inhibition of nuclear assembly.

could not be detected by conventional immunoblotting techniques [18]. This is in contrast to most autoantibodies found in other autoimmune diseases that usually show reactivity to the cognate antigens by immunoblotting [3,31]. Based on these observations, we suggested that the epitope of the p95c autoantigen was conformational [18].

In the present study, all but one of the sera with anti-p95 antibodies bound to the recombinant p97/VCP protein in an IP assay. In this assay, certain conformational epitopes are probably present, although it is likely that post-translational modifications, which are features of the native protein, are not represented fully in the recombinant protein produced in the rabbit reticulocyte lysate system. The requirement for certain post-translational modifications may explain why the one serum did not immunoprecipitate the recombinant VCP. To gain further insight, studies are under way to map the linear and conformational epitopes on p97/VCP.

When we considered possible autoantigenic targets of the anti-p95c sera, two novel antigens in the cytosol with molecular masses each of 97 kDa came to our attention. The first was $\beta$-karyopherin (importin-$\beta$), which plays a key role in nuclear import [16]. The other was p97/VCP, which plays an important role in various membrane fusions such as Golgi and nuclear envelope assembly, and ubiquitin-dependent protein degradation [32,33]. This protein is a member of a family of AAA-ATPases, some of which (i.e. $F_0$-ATPase) are located in the inner mitochondrial membrane [17] and others (i.e. $F_1$-ATPase) localized to the matrix space [34,35]. The p97/VCP complex and N-ethylmaleidemide sensitive fusion protein (NSF) share certain similarities in that they both form ring-shaped homo-hexamers (in contrast to hetero-hexamers of $F_1$-ATPase) and are involved in biogenesis and functional activities of the Golgi and nuclear membranes [33,36]. The function of p97 can be inhibited by $\alpha$-SNAP, a component of the NSF pathway, and the function of NSF can be inhibited by p47, also a component of the p97 pathway [32]. The mechanism of nuclear inhibition is thought to involve competition between $\alpha$-SNAP and p47 to bind syntaxin 5, a common component of the functional p97 and NSF pathways [32].

In our study the inhibition of nuclear assembly was generally correlated with the titre of anti-p95c antibody as determined by ID, but there was a less clear-cut correlation between the nuclear inhibition assay and the semiquantitative assessment of the TnT IP results (Table 1). Therefore, it has yet to be shown conclusively that anti-p95c antibodies are indeed the factors that inhibit the
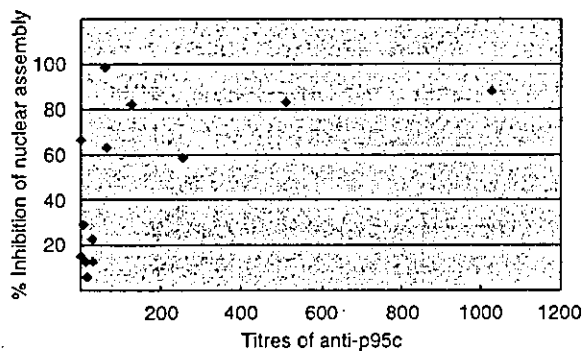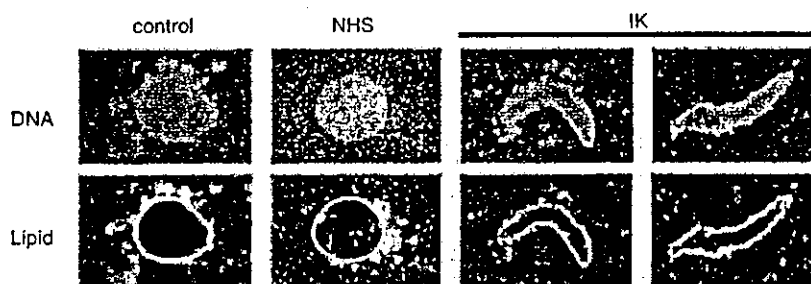


**Fig. 2.** Confocal immunofluorescent microscopy of the nuclear reassembly assay. Nuclear envelope assembly was inhibited by the index serum (IK) but not by phosphate buffered saline or normal healthy serum. Nuclear assembly was judged to have occurred when the length of the long axis divided by the short axis was less than 2.
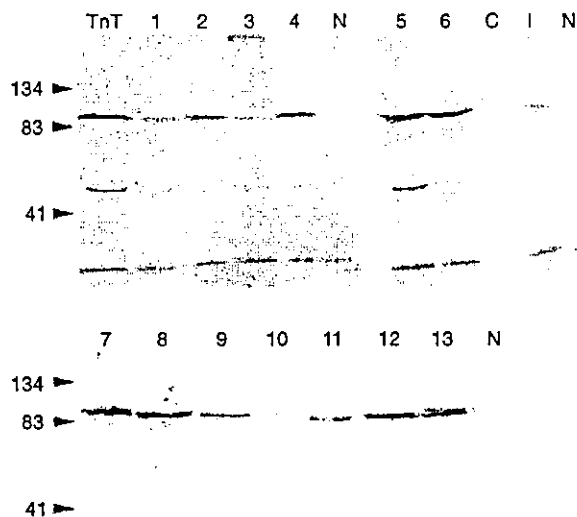
nuclear assembly. A number of observations support our conclusion. First, like our observations, Hetzer and his colleagues demonstrated that anti-p97/VCP antibody inhibits nuclear envelope assembly in a *Xenopus* egg extract in the *in vitro* system [21]. Secondly, we have shown previously that AMA and anti-gp210 antibodies, which are found in sera from patients with PBC [5], and anti-U1RNP/Sm, anti-SS-A/Ro, anti-SS-B/La, which are found in sera from patients with SLE or SjS [2,3], did not inhibit nuclear assembly in the *in vitro* assay (data not shown). However, it remains to be determined if autoimmune sera with antibodies directed against nuclear envelope proteins such as lamin B receptor (LBR), LAP2 (lamina associated polypeptide 2), lamin A/C and lamin B might inhibit nuclear assembly. According to a recent report, some human sera might contain antibodies to other antigens, such as glyceraldehydes-3-phosphate, that also participate in nuclear assembly [28].

Hetzer *et al.* has reported that several steps are involved in the process of nuclear assembly and, specifically, p97–p47 is required for nuclear envelope expansion in the final steps of nuclear envelope fusion [21]. In our study, confocal immunofluorescence microscopy showed that inhibition of nuclear assembly occurred at more than one stage because some areas of chromatin were well covered by the nuclear envelope while others were not. Thus, anti-p95c antibodies may be directed against domains of p97 that are critical in different steps of nuclear envelope assembly. The

antigenic domain(s) bound by anti-p95c is not known; studies are under way to define the primary conformational and linear epitopes. The assumption that human anti-p95c antibodies bind to the active site of p95/VCP and inhibit its function is based on studies of other human autoantibodies that have been shown to bind functional sites of proteins and inhibit their biological activities [37,38].

In summary, we provide evidence that the previously described p95c autoantigen and p97/VCP are identical proteins. To maintain understanding between disciplines we propose that anti-p95c antibodies be referred to as anti-p97/VCP in the future. Anti-p97/VCP was found in approximately 12·5% of patients with PBC and in 9·7% with AIH [18]. In contrast to the prevalence of AMA [5], the prevalence of anti-p95c antibody in PBC is relatively low. Therefore, the diagnostic importance of anti-p95c antibody occurs frequently in AMA-negative PBC and in ANA-negative AIH. In our study, many of the PBC or AIH patients with this antibody had overlap conditions, particularly SjS. Prospective studies of SjS patients would be important to determine if anti-p97/VCP antibodies antedate the appearance of autoimmune liver disease.

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