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EDITORIAL

The definition of autoimmune disease: are Koch's postulates applicable?

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To date about 60 to 80 diseases have a proven or strongly suspected autoimmune aetiology. Nevertheless, it remains a matter of debate how to prove that a given disease is indeed an autoimmune disease. While some people claim that every disease is autoimmune until proven otherwise, others advocate that every autoimmune disease is a chronic hypersensitivity response to an infectious agent.
This issue of The Netherlands Journal of Medicine contains two papers that deal with autoimmune disease: a review on several aspects of autoimmunity in the context of Addison's disease,[1] and a case report on a patient with thymoma and four different autoimmune diseases.[2] These papers have given rise to re-evaluation of the currently available criteria a disease has to fulfil to be called an autoimmune disease. We will apply these criteria to a couple of diseases covering the spectrum of diseases from proven to disputed with respect to being autoimmune in nature, i.e. myasthenia gravis (MG), multiple sclerosis (MS), Wegener's granulomatosis (WG), and celiac disease (CD). We will show that the criteria are far too stringent for many diseases that are generally covered in the currently available textbooks on autoimmune diseases.[3,4]

It is well accepted that a disease can be classified as autoimmune if one shows that an adaptive immune response to a self-antigen causes the observed pathology. Koch's postulates for the role of micro-organisms in infectious diseases have thus been adjusted as follows:

1. the specific adaptive immune response is directed to the affected organ or tissue;
2. autoreactive T cells and/or autoantibodies are present in the affected organ or tissue;
3. autoreactive T cells and/or autoantibodies can transfer the disease to healthy individuals or animals;
4. immunisation with the autoantigen induces the disease in animal models;
5. elimination or suppression of the autoimmune response prevents disease progression or even ameliorates the clinical manifestation.

Myasthenia gravis (MG), a prototypic antibody-mediated autoimmune disease, is characterised by a humoral immune response against the acetylcholine receptor (AChR) on the post-synaptic membrane.[5] The autoantibodies cause the functional blockade and loss of AChR at the neuromuscular junction, resulting in muscular weakness. Passive transfer of anti-AChR antibodies to animals, or via the placenta to the neonate, reproduces the clinical manifestations of MG in the recipient, and immunisation of rodents with AChR induces experimental autoimmune MG. Finally, elimination of autoantibodies by plasmapheresis is beneficial, but long-term immunosuppression is required for down-modulation of the autoimmune response. Hereby MG is one of the few autoimmune diseases that completely fulfil the criteria according to Koch's postulates.

Multiple sclerosis (MS) is considered a T-cell-mediated autoimmune disease, although more recently a role for the humoral immune response has been attributed to a subgroup of MS patients. MS is a chronic inflammatory demyelinating disease affecting the central nervous system.[6] Sites of active demyelination are characterised by inflammatory infiltrates composed of CD4 T cells, activated macrophages, and small numbers of B cells. The T cells are directed to a whole array of myelin-associated autoantigens, including myelin basic protein, proteolipoprotein, and myelin oligodendrocyte glycoprotein. Adoptive transfer of T-cell-mediated autoimmune disease, however, is difficult

to establish: T cells do not cross the placenta and transfer of human T cells to animal models is hampered by the self-MHC restriction of the respective T cells. Nevertheless, the clinical manifestations of MS can be reproduced in animals upon immunisation with myelin-associated antigens and T cells can adoptively transfer disease to syngeneic recipients. Finally, immune suppressive agents are used as treatment for relapses and/or acute MS exacerbations, but the beneficial effects of immunomodulatory agents for the long-term control of MS are not yet well established.

Wegener's granulomatosis (WG) is a small-vessel vasculitis affecting the kidneys, the upper and lower airways, and other organ systems.[7] WG is usually associated with anti-neutrophil cytoplasmic autoantibodies (ANCA) directed against proteinase-3 (PR3). Although circulating autoantibodies are detected in WG patients, the renal lesions are characterised as pauci-immune crescentic glomerulonephritis, indicating that autoantibodies, and/or immune complexes, are absent in the affected organ. Apparently, the autoantibodies are not present in the affected kidneys. Nevertheless, rises in levels of PR3-ANCA often precede disease activity, suggesting a role for PR3-ANCA in the pathophysiology of WG. PR3 is stored in intracellular granules of neutrophils. It has been suggested that during infection, neutrophils become 'primed' or pre-activated, resulting in surface expression of PR3. This enables binding of PR3-ANCA with PR3. This interaction firstly leads to neutrophil degranulation and release of free radicals, and subsequently results in endothelial cell damage, which is characteristic of vasculitis. Since human PR3-ANCA are not cross-reactive with rodent PR3, passive transfer studies cannot reveal the possible pathogenetic involvement of these antibodies. Since WG is a granulomatous disease, it was expected that autoreactive T cells were not only providing T-cell help for ANCA production, but were also involved in the production of cell-mediated tissue injury.[8] Indeed, Th1-like cells are localised in the upper airway granulomatous lesions, but the role of T cells in the pathophysiology has not yet been established. Moreover, there is no obvious or consistent HLA association, suggesting that the T-cell response may be heterogeneous. Since there is evidence that the majority of patients with WG are chronic nasal carriers of *Staphylococcus aureus* and since this carriage is associated with an increased frequency of relapses of WG, the lacking HLA association may be explained by a polyclonal T-cell activation, possibly by superantigens produced by *S. aureus*.[9] Therefore it is hypothesised that infections play a major role in the pathogenesis of WG, whereas involvement of the autoimmune nature of the disease remains hard to prove. Indeed, limited or loco-regional forms of WG can be treated with cotrimoxazole only. Nevertheless, the generalised forms of WG are usually treated by a combination of prednisolone and cyclophosphamide in order to induce disease remission.

Coeliac disease (CD) is characterised by hypersensitivity to gluten, a cereal grain storage protein.[10] The gluten-sensitive enteropathy results in weight loss, diarrhoea, nutritional deficiencies, and growth failure. The mucosal lesions consist of a T-cell infiltrate of the lamina propria, followed by crypt hyperplasia and villous atrophy. The T cells from CD lesions recognise deamidated epitopes of gliadin, the ethanol-soluble fraction of gluten, in the context of HLA-DQ2. Active CD is associated with the presence of circulating antibodies to gliadin as well as to the autoantigen tissue transglutaminase (tTG). Autoreactive T cells recognising tTG, however, have not yet been described. The presence of anti-tTG autoantibodies is considered to be secondary to the selective lack of T-cell tolerance for deamidated gliadin, combined with the inherent characteristics of tTG to catalyse post-translational modification (deamidation) of gliadin and thereby enable the formation of gliadin-tTG complexes. In the presence of these complexes tTG-specific B cells can be supported by gliadin-specific CD4 T cells and eventually produce the anti-tTG antibodies. Animal models for CD have not yet been described and since (auto)antibodies in CD are predominantly of the IgA isotype, cross-placental transfer to the neonate does not occur. Therefore, a possible pathophysiological function of antibodies to gliadin, and in particular of anti-tTG autoantibodies, has not been established. Finally, elimination of gluten from the diet results in complete resolution of the clinical symptoms as well as the disappearance of (auto)antibodies in the circulation.

From these examples we conclude that Koch's postulates are far too stringent as a definition for the wide array of diseases with potentially an autoimmune aetiology. Especially criterion 3, i.e. passive/adoptive transfer of the autoimmune component to animals, is often hampered by the poor recognition of the xenogeneic autoantigen by autoantibodies, as well as by the MHC-restricted antigen recognition of T cells. As starting point we would like to propose that every disease is an autoimmune disease as long as there is: a clear adaptive immune response to a self-antigen, and unequivocal involvement of this autoimmune response in the immunopathology of the disease (cf MG). For this second criterion it should be acceptable that the role of autoantibodies and/or autoreactive T cells in the immunopathology is demonstrated only in animal models (cf MS). If the immunopathology can only be explained theoretically and is not proven in human or experimental models (cf WG), there should be additional criteria: therapy based on the eradication or suppression of the autoimmune response should ameliorate the clinical manifestation or at least prevent progression of the disease,

and there should not be another well-defined cause that explains the whole spectrum of the disease. Obviously, the latter criterion should not exclude the involvement of a co-factor, like infections, as is the case in WG. Finally, CD does not fulfil these criteria since the aberrant T-cell response to the dietary gluten is sufficient to explain the enteropathy. On the other hand, CD also has several extra-intestinal manifestations like the blistering skin rash of dermatitis herpetiformis (DH). This is associated with granular IgA deposition in the dermal papillae. Since it is not to be suspected that gliadin is present in the skin, it is most likely that the IgA antibodies react with tTG in the extracellular matrix. If so, some of the extra-intestinal manifestations of CD, like DH, may be true autoimmune diseases that are secondary to the gluten-hypersensitivity.

Altogether, these examples illustrate that it is hard to give a definition that completely covers the ever-increasing number of diseases that are considered autoimmune in terms of pathogenesis. It should be kept in mind, however, that the label autoimmune disease is predominantly important as a working hypothesis for further research in terms of therapy and elucidation of the pathophysiology; definitions, and/or criteria, should be used as such.

REFERENCES

1. Martorell PM, Roep BO, Smit JWA. Autoimmunity in Addison's disease. Neth J Med 2002;60:269-75.
2. Asakawa H, Kashihara T, Fukuda H, Yamamoto M. A patient with thymoma and four different organ-specific autoimmune diseases. Neth J Med 2002;60:292-5.
3. Bona CA, Siminovitch KA, Zanetti, M, Theofilopoulos AN. The molecular pathology of autoimmune diseases. Chur, Switzerland: Harwood Academic Publishers, 1993.
4. Peter JB, Shoenfeld Y. Autoantibodies. Amsterdam: Elsevier Science, 1996.
5. Vincent A, Palace J, Hilton-Jones D. Myasthenia gravis. Lancet 2001;357:2122-8.
6. Compston A, Coles A. Multiple sclerosis. Lancet 2002;359:1221-31.
7. Yi ES, Colby TV. Wegener's granulomatosis. Semin Diagn Pathol 2001;18:34-46.
8. Clayton AR, Savage CO. What you should know about PR3-ANCA. Evidence for the role of T cells in the pathogenesis of systemic vasculitis. Arthritis Res 2000;2:260-2.
9. Popa ER, Stegeman CA, Kallenberg CG, Cohen Tervaert JW. Staphylococcus aureus and Wegener's granulomatosis. Arthritis Res 2002;4:77-9.
10. Farrell RJ, Kelly CP. Celiac sprue. N Engl J Med 2002;346:180-8.

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REVIEW

Autoimmunity in Addison's disease

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ABSTRACT

Addison's disease has a low incidence and is most frequently the result of an autoimmune disease in developed countries. Addison's disease can present as an isolated entity or in combination with other autoimmune diseases: Addison's disease can be part of the distinct polyglandular autoimmune syndromes APS I and II. Autoantibodies in patients with isolated Addison's disease are directed against the enzymes involved in steroid synthesis, P450c21, P450scc and P450c17. Addison's disease, both isolated and in the context of APS II, has been associated with the haplotype HLA-A1, -B8 and DR3. The value of the increased expression of these molecules on adrenocortical cells could point towards an infectious pathogenesis. Given the prevalence, up to 80%, of autoantibodies in Addison's disease as well as the high predictive value for developing the disease when antibodies are present (41% in three years), we advise screening high-risk populations, such as patients with other autoimmune endocrinopathies or their relatives for the presence of these antibodies. The adrenocortical function of patients positive for antibodies should be followed yearly.

INTRODUCTION

In this review different aspects of autoimmunity in the context of Addison's disease are discussed. Firstly, the different types of autoimmune adrenocortical insufficiency (isolated or associated with an autoimmune polyglandular syndrome) and their features are dealt with. Next, the focus is on the prevalence of adrenocortical antibodies as well as the antigens against which they are directed in the different types of autoimmune Addison's disease. Thirdly, the possible pathogenesis of the disease is considered. The article concludes by looking at the clinical implications of adrenocortical autoantibodies with respect to adrenal dysfunction and the value of screening in the clinical setting.

ADDISON'S DISEASE

Addison's disease is a pathological process of the adrenal cortex leading to insufficient production of glucocorticoids, mineralocorticoids and sex steroids.

The prevalence of Addison's disease in the general population is low, ranging from 30 to 60 cases per million,[1-5] although some authors report prevalences of up to 117 cases per million.[6]

Before, antibiotics were widely used, tuberculosis was the main cause of primary adrenocortical failure. However, with the introduction of effective antibiotic therapies against tuberculosis, autoimmune Addison's disease is now the prime cause, accounting for nearly 80% of the cases.

Addison's disease manifests when 90% of the adrenocortical gland has been destroyed, and thus can remain subclinical for a long time.

In the case of autoimmune Addison's disease, adrenocortical failure usually develops in five stages.[5-7] In stage 0 normal adrenocortical function is retained. In stage 1 high plasma renin activity (PRA) is observed, with normal or low aldosterone levels. In stage 2 the cortisol response to ACTH is impaired. In stage 3 elevated ACTH basal levels

are measured and in stage 4 clinically overt Addison's disease is present.
The diagnosis of the disease is discussed under the heading Clinical implications of adrenocortical autoantibodies for the diagnosis of Addison's disease. The current treatment of chronic Addison's disease falls beyond the scope of this article.

ASSOCIATED POLYENDOCRINE AUTOIMMUNE DISEASES

Autoimmune Addison's disease is often associated with other endocrine autoimmune diseases, most frequently with autoimmune thyroid disease, pernicious anaemia and diabetes mellitus.[8-10]
Three types of autoimmune polyendocrine syndromes can be distinguished, the characteristics of which are given in *table 1*. In autoimmune polyendocrine syndrome type I, (APS I, also known as Blizzard's syndrome or autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED)) Addison's disease is often associated with hypoparathyroidism and chronic mucocutaneus candidiasis. The diagnosis is established by the presence of at least two of these main disease components.
This rare autosomal recessive disease usually appears at a young age. Approximately half of the patients eventually develop all three major components. The incidence of APS I seems to be especially high in the Finnish,[11] Iranian Jewish[12] and Sardinian populations.[13] In contrast with APS II, most authors found that APS I had no association with the HLA DR3 gene.
The mode of inheritance is autosomal dominant, with incomplete penetrance.[1] The major features of this polyendocrinopathy are Addison's disease, autoimmune thyroid disease, and type 1 diabetes mellitus. As is the case in APS I, there is often gonadal failure. Other components, such as those APS I (alopecia, vitiligo, pernicious anaemia) may also be present, but are much less commonly associated with APS II.

Table 1
Autoimmune polyglandular syndromes

APS	DISEASE COMPONENTS	FREQUENCY OF THE DIFFERENT COMPONENTS[11,15]	P450 ANTIGENS MOST OFTEN INVOLVED
	Main disease components		
	Addison's disease	72%	
	Chronic mucocutaneous candidiasis	100%	
	Hypoparathyroidism	79%	
I	**Associated conditions**		
	Gonadal failure: - ovarian failure	60%	P450scc (67-100%)[10,13]
	- testicular failure	14%	P450c17 (0-41%)[13,19,20]
	Diabetes mellitus	12%	
	Chronic active hepatitis	12%	
	Alopecia	29%	
	Vitiligo	35%	
	Main disease components		
	Addison's disease	100%	
	Autoimmune thyroid disease	69%	
II	IDDM	52%	P450c21 (67-80%)[16,19,21]
	Gonadal failure	4%	P450scc (0-50%)[16,19]
	Associated conditions		
	Vitiligo	5%	
III	**Autoimmune thyroid disease**		
	IDDM		None

APS I: Ahonen et al.,[11] APS II: Neufeld et al.[15] and Uibo et al.[19] make a distinction between APS I patients with and without the adrenocortical insufficiency component. This percentage refers to APS I patients with Addison's disease. No data regarding the frequencies of the different disease components in APS III have been found.

APS II, or Schmidt's syndrome, is more common than APS I and has a later age of onset, usually between the ages of 20 and 40 years (peak incidence around 10 years).[14,15]
A third type of APS (APS III) consists of autoimmune thyroid disease and IDDM, without Addison's disease.[16]

AUTOIMMUNITY IN ADDISON'S DISEASE

Autoantibodies against adrenocortical antigens can often be detected in sera from patients with clinical autoimmune Addison's disease. The prevalence found in literature ranges from 25%[3] up to 84%.[1,8,9,16,17] The prevalence of these antibodies in the general population has been estimated at 1:10,000.[8,18] The most common adrenal antibodies found in patients with Addison's disease are directed against the P450 enzymes involved in steroid synthesis, 21-hydroxylase (P450c21), 17α-hydroxylase (P450c17) and cholesterol side chain-cleaving enzyme (P450scc). In immunofluorescence studies it has been shown that both P450c21 and P450scc antigens are found in all three layers of the adrenal cortex (*figure 1*), while sera reacting with the antigen P450c17 do not stain the outermost zona glomerulosa.[19,20] The antigens P450c17 and P450scc are found in all steroid-producing cells, such as gonadal (both antigens) and placental tissue (only P450scc). This correlates with the finding that reactivity against these antigens also occurs in these organs in the presence of these antibodies. This could explain the relatively common presence of gonadal failure in APS I and APS II patients. P450c21 is only present in the adrenal glands.
The prevalence of each of these antibodies usually depends on the type of autoimmune Addison's disease (isolated or associated with a polyglandular syndrome).
In the isolated form of Addison's disease the antigen most often involved is P450c21. The prevalence of autoantibodies in patients with this type of Addison's was found to vary from 20 to 100%.[9,16,19-22] This variability in prevalence can be due to several factors. Different detection methods were used in the different studies, some using Western blotting techniques, others using immunofluorescence or radiobinding assays. These last two methods were found having the highest prevalences. Antibodies against P450scc are also present in these patients although in a much smaller proportion than P450c21.[16] P450c17 seems to be the least common target antigen in isolated autoimmune Addison's.[3,9,16,19]
In the case of APS I, the literature is not unanimous as to which of these three types of antibodies is predominant. Some authors report relatively high prevalences of antibodies against P450c21 in APS I patients with Addison's disease (42%),[19] while others have found this antigen to be the least immunogenic.[13,20] There seems to be a general consensus that P450scc and P450c17 are the antigens most often involved. Some authors claim the first one to be the main antigen[13,19,20,23] while others have found antibodies against P450c17 more common.[3] Antibodies against the hepatic cytochrome antigen P4501A2 were also frequently observed in these patients. This antigen is different from those found in autoimmune and viral hepatitis, and could

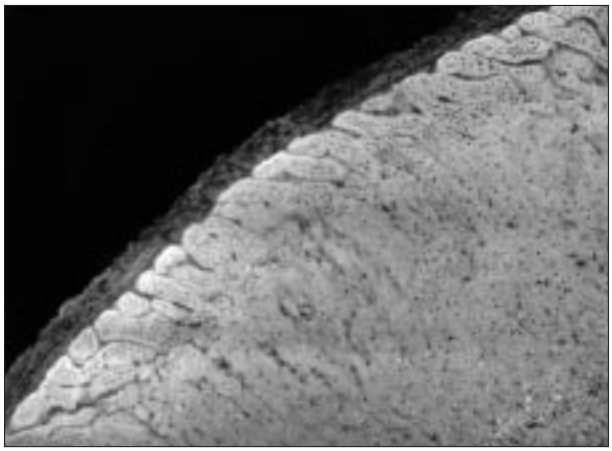

Figure 1a
(100 x amplification)

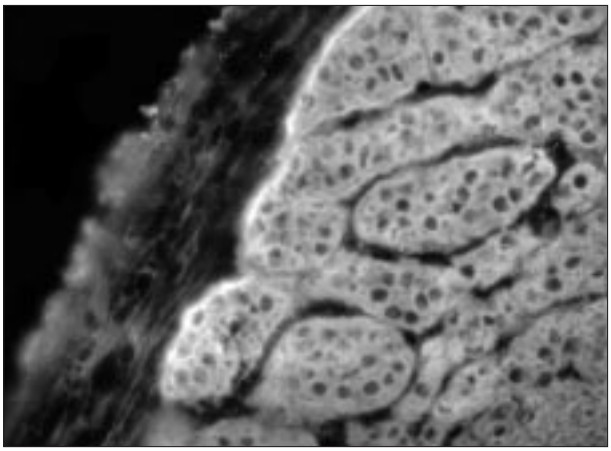

Figure 1b
(400 x amplification)

Immunofluorescence pattern of adrenocortical antibodies on monkey adrenal tissue
Monkey adrenal tissue (Scimedx, Denville, USA) was incubated with patients' sera and stained with antihuman IgG fluorescein isothyacyanate gammaglobuline (FITC, Scimedx, Denville, USA).

be used to identify APS I patients at risk of developing chronic active hepatitis, as fulminant hepatic failure is a serious complication of APS I.

As in the case of isolated Addison's disease, P450c21 is the predominant target antigen in APS II.[16,19,21] Antibodies against P450scc are also present and P450c17 appears to be the least immunogenic of the three antigens.[16,20]

In APS III, P450 enzymes appear to play a minor role.[16]

Adrenal autoantibodies are generally of the IgG class, and less frequently of the IgA and the IgM class.[4,7,18]

Antibodies directed against antigens other than P450 cytochrome enzymes have been found in some patients with isolated Addison's disease. Wulffraat *et al.*[5] found antibodies of the IgG type directed against the ACTH receptor on adrenal cells, which blocked ACTH-induced cortisol secretion as well as ACTH-induced DNA synthesis in these cells. This was the case in 74% and 80%, respectively, of the Addisonian patients studied, and was found in none of the healthy control subjects. This blocking effect was lost at higher titres of IgG concentration. However, these antibodies were also found in some patients with other adrenal disorders (Cushing's disease, adrenal nodules). Weetman, however, observed no clear blocking effect from such antibodies in Addisonian patients.[1] The reason for this discrepancy in results is not clear.

PATHOGENESIS

In a study of a murine model,[24] autoimmune adrenalitis was induced in mice by immunising them with a mixture of adrenal extract and *Klebsiella* O3 lipopolysaccharide as adjuvant. Histologically, extensive lymphocytic infiltration of the adrenal cortex was seen. The adrenal medulla remained unaffected in all cases.

In humans, the pathological picture is similar. In early stages of the disease the glands may be enlarged with extensive lymphocytic infiltration.[14] In a later stage the cortex of the adrenal gland becomes atrophic. The cortex is characterised by a strong mononuclear infiltration (mainly lymphocytes, but also plasma cells and macrophages).

The mechanism by which the adrenal cortex is destroyed in autoimmune Addison's disease is not yet clear. The antigens most often involved, P450 cytochrome enzymes (P450c21, P450c17 and P450scc), are intracellular and localised in the endoplasmatic reticulum, and thus no sensitisation against them could take place prior to the destruction of the tissue since plasma membranes are impermeable to immunoglobulins. According to some studies, these adrenal antigens are also represented on the plasma membrane.[25] Therefore, the question remains if the autoantibodies are the cause of the tissue destruction or actually a result of it.

Antibodies may destroy cells by activating the cytolytic complement cascade (leading to lysis of the cell) or by initiating antibody-dependent cell-mediated immunity (leading to phagocytosis). Complement fixing autoantibodies have been found in a number of Addisonian patient cohorts.[7,18,26] In one study,[4] patients with adrenal cortex autoantibodies all had complement fixing antibodies against adrenal cells. All had C3 and C4 fixing antibodies, whereas seven of the nine cases also had C5-C9 fixing antibodies, which have a lytic effect on cells. The four patients who developed overt Addison's disease during follow-up all had C5-C9 fixing antibodies. Betterle *et al.* therefore postulate complement fixing autoantibodies, and C5-C9 fixing antibodies in particular, to be a marker for patients at risk of developing Addison's disease.

Analogous with other endocrine autoimmune disease such as hypothyroidism or IDDM, another possible mechanism would be antibody-mediated cytotoxic cellular response by T lymphocytes as reviewed by Isselbacher *et al.*[27]

Several T-cell abnormalities are associated with Addison's disease. An increased percentage of activated HLA-DR positive T lymphocytes have been found in some studies with Addisonian patients.[26] Another study showed increased T-cell sensitisation to a heterogeneous variety of adrenal antigens more frequently than antibodies in Addisonian patients.[2] Arulanantham *et al.* have found hypergammaglobulinaemia, selective IgA deficiency and defective T-cell suppressor activity in some APS I patients.[28] These studies indicate that several components of the immune system, including T cells, autoantibodies and complement, are involved in the pathogenesis of adrenocortical destruction.

Addison's disease is associated with certain HLA polymorphisms. This is also the case with autoimmune hypothyroidism and IDDM. Addison's disease in the isolated form and in the context of APS II has been associated with HLA-A1, -B8 and possibly -DR3.[1,10,29] HLA-DR4 was also found by Maclaren and Riley to be more prevalent amongst these patients,[10] but these results have not been confirmed since. Some HLA-DR genes (DR2, DR5 and DR7) appear to protect against Addison's disease.[10] However, the strongest association remains with HLA-DR3.

No clear HLA association has been found in the case of APS I. HLA-DR3 was found by most authors to have no association with APS I.[10,30] Others have found HLA-A28 to be positively associated with this disease. This link was, nevertheless, weaker than that found between HLA-DR3 and HLA-B8 and Addison's disease in the isolated form and in APS II.[30]

The role of MHC class II molecules on adrenal cells remains unclear. These molecules were thought to be present only on antigen presenting (APC) cells and capillary endothelium. They play an important role in the regulation of an immune response by CD4+ cells. The presence of these molecules on APCs is a condition for the successful presentation of antigens to CD4+ cells. The adrenal cortex,

and particularly the zona reticularis, has also been found to express MHC class II molecules under normal conditions.[29,31,32] In autoimmune Addison's disease, an increased expression of these molecules is observed.[29] This could facilitate the activation of T lymphocytes against adrenal cells. It has been postulated that HLA-DR expression could be the result of previous infectious episodes, and of the IFN-γ produced as a result by activated T lymphocytes,[33] as has been found in thyrocytes in autoimmune hypothyroidism.[34] This would enhance the expression of HLA-DR molecules on cells and could induce T cells to react with autoantigens. The idea of infectious processes underlying autoimmune diseases has long been suggested.

However, the consequence of T-cell interaction with HLA on the target (adrenocortical cells) will be determined by the costimulation with other membrane proteins. In the absence of costimulation or in case of expression of B7.2 (CD86) on the target, interaction with CTLA-4 will inhibit rather than amplify the T-cell response. Therefore, hyperexpression of HLA class I or aberrant expression of HLA class II on adrenocortical cells could be an immune escape mechanism to avoid tissue destruction.

In this context, it is of interest that genetic CTLA-4 polymorphisms have been found to be associated with the development of Addison's disease.[35,36]

CLINICAL IMPLICATIONS OF ADRENOCORTICAL AUTOANTIBODIES FOR THE DIAGNOSIS OF ADDISON'S DISEASE

An important question is whether the presence of autoantibodies can predict the chance of developing Addison's disease, and whether the levels correlate with the severity of the disease.

A strong positive correlation between the presence of autoantibodies aimed at adrenal cortex cells (particularly the P450 enzyme antigens) and the subsequent development of overt adrenal impairment has been found.

Betterle *et al.*[4] found during follow-up that four out of nine patients with autoantibodies developed clinical Addison's disease. An additional patient had an inadequate response to ACTH stimulus. In another study, Betterle *et al.*[7] performed a longitudinal analysis of 15 patients positive for adrenocortical antibodies. Nine were initially at stage 0 of adrenocortical dysfunction, two at stage 1, two at stage 2 and two at stage 3. Follow-up varied from six months to ten years. Of the patients with a subclinical adrenal dysfunction when entering the study (two at stage 2 and two at stage 3) three developed clinically overt Addison's disease. Of the nine patients at stage 0 three developed clinical Addison's disease as well. Of the control patients two developed autoantibodies, of which one later developed Addison's disease. In conclusion, 41% of the patients with anti-adrenal antibodies developed Addison's disease during a mean observation time of 3.2 years. The yearly incidence of worsening of adrenal function was 19%. In another study,[18] five out of 30 patients with other endocrine disorders and with adrenal cortex autoantibodies developed Addison's disease. Interestingly, four of these five had complement fixing antibodies. Three of these cases had adrenal insufficiency on entering the study, while the other two developed Addison's disease during follow-up.

The results of these studies show that many of the patients positive for these autoantibodies later progress towards clinically overt disease. In all three of these studies, antibodies were detected using standard immunofluorescence methods. The populations used in these studies are, however, small.

Several authors suggested that titres of adrenal autoantibodies correlate positively with the chance of developing the clinical disease, specially in high-risk populations such as people with other organ-specific autoimmune diseases and their family relatives, as well as family relatives of Addisonian patients.[21,37,38]

The assessment of adrenal function in patients positive for autoantibodies includes basal cortisol and ACTH levels, rapid and prolonged ACTH stimulation tests, and PRA and aldosterone levels.

Although basal cortisol levels and ACTH levels have been used to assess adrenal function, these are generally insufficient to exclude adrenal dysfunction, unless the cortisol level found is <0.55 μmol/L.[39]

ACTH stimulation tests are often used to detect subclinical adrenocortical dysfunction with a high sensitivity.[40] In the past, a 250 μg dose of ACTH was used in the rapid ACTH stimulation test, but several studies have achieved better results using lower doses of 10 μg[41] or 1 μg.[42]

Some studies also report disappearance of the antibodies with increasing disease duration.[8,37,38] This disappearance of the antibodies sometimes goes hand in hand with recovery of the impaired adrenal function, and in some cases even with total remission of the disease.[37,38] The percentage of patients who underwent this recovery was 26% and 30% respectively for each of these studies. Other authors have not been able to find such a remission of antibody titre or of the actual disease.[43]

In conclusion, given the prevalence (up to 80%) of autoantibodies in Addison's disease, as well as the high predictive value for developing the disease when antibodies are present (40% in three years), we advise screening high-risk populations, such as patients with other autoimmune endocrinopathies or their relatives for the presence of these antibodies. When antibodies are present, an ACTH test should yearly be performed, unless fasting cortisol levels are >0.55 μmol/L.

REFERENCES

1. Weetman AP. Autoimmunity to steroid producing cells and familial polyendocrine autoimmunity. Bailliere's Clin Endocrinol Metab 1995;9:157-74.
2. Freeman M, Weetman AP. T and B cell reactivity to adrenal antigens in autoimmune Addison's disease. Clin Exp Immunol 1992;88:275-9.
3. Muir A, Schatz DA, Maclaren NK. Autoimmune Addison's disease. Springer Semin Immunopathol 1993;14:275-84.
4. Betterle C, Zanchetta R, Trevisan A, et al. Complement fixing adrenal autoantibodies as a marker for predicting onset of idiopathic Addison's disease. Lancet 1993;1:1238-41.
5. Wulffraat NM, Drexhage HA, Bottazzo GF, Wiersinga WM, Jeucken P, Gaag R van der. Immunoglobulins of patients with idiopathic Addison's disease block the in vitro action of adrenocorticotropin. J Clin Endocrinol Metab 1989;69:231-8.
6. Laureti S, Vecchi L, Santeusanio F, Falorni F. Is the prevalence of Addison's disease underestimated? J Clin Endocrinol Metab 1998;84:1762.
7. Betterle C, Scalici C, Presotto F, et al. The natural history of adrenal function in autoimmune patients with adrenal autoantibodies. J Endocrinol 1988;117:467-75.
8. Sotsiu F, Bottazzo GF, Doniach D. Immunofluorescence studies on autoantibodies to steroid-producing cells and to germline cells in endocrine disease and infertility. Clin Exp Immunol 1980;39:97-111.
9. Söderbergh A, Winqvist O, Norheim I, et al. Adrenal autoantibodies and organ specific autoimmunity in patients with Addison's disease. Clin Endocrinol 1996;45:453-60.
10. Maclaren NK, Riley WJ. Inherited susceptibility to autoimmune Addison's disease is linked to human leukocyte antigens-DR3 and/or DR4, except when associated with type I autoimmune polyglandular syndrome. J Clin Endocrinol Metab 1986;62:455-9.
11. Ahonen P, Myllärniemi S, Sipilä I, Perheentupa J. Clinical variation of autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) in a series of 68 patients. N Engl J Med 1990;322:1829-36.
12. Zlotogora J, Shapiro MS. Polyglandular autoimmune syndrome type I among Iranian Jews. J Med Genet 1992;29:824-6.
13. Clemente MG, Obermayer-Straub P, Meloni A, et al. Cytochrome P450 1A2 is a hepatic autoantigen in autoimmune polyglandular syndrome type I. J Clin Endocrinol Metab 1997;82:1353-61.
14. Orth DN, Kovacks WJ, Debold CR. Wilson & Foster. Williams textbook of Endocrinology. 8th ed. Philadelphia: WB Saunders Company, 1992.
15. Neufeld M, Maclaren NK, Blizzard RM. Two types of autoimmune Addison's disease associated with different polyglandular autoimmune (PGA) syndromes. Medicine 1981;60:355-62.
16. Carmo Silva R do, Kater CE, Dib SA, et al. Autoantibodies against recombinant human steroidogenic enzymes 21-hydroxylase, side-chain cleavage and 17a-hydroxylase in Addison's disease and autoimmune polyendocrine syndrome type II. Eur J Endocrinol 2000;142:187-94.
17. Chen S, Sawicka J, Prentice L, et al. Analysis of autoantibodies epitopes on steroid 21-hydroxylase using a panel of monoclonal antibodies. J Clin Endocrinol Metab 1998;83:2977-85.
18. Scherbaum WA, Berg PA. Development of adrenocortical failure in non-addisonian patients with antibodies to adrenal cortex. Clin Endocrinol 1982;16:345-52.
19. Uibo R, Aavik E, Peterson P, et al. Autoantibodies to cytochrome P450 enzymes P450scc, P450c17, and P450c21 in autoimmune polyglandular disease types I and II and in isolated Addison's disease. J Clin Endocrinol Metab 1994;78:323-8.
20. Winqvist O, Gustafsson J, Rorsman F, Karlsson A, Kämpe O. Two different cytochrome P450 enzymes are the adrenal antigens in autoimmune polyendocrine syndrome type I and Addison's disease. J Clin Invest 1993;92:2377-85.
21. Falorni A, Nikoshkov A, Laureti S, et al. High diagnostic accuracy for idiopathic Addison's disease with a sensitive radiobinding assay for autoantibodies against recombinant human 21-hydroxylase. J Clin Endocrinol Metab 1995;80:2752-5.
22. Falorni A, Laureti S, Nikoshkov A, et al. 21-hydroxylase autoantibodies in adult patients with endocrine autoimmune diseases are highly specific for Adisson's disease. Clin Exp Immunol 1997;107:341-6.
23. Winqvist O, Karlsson FA, Kämpe O. 21-hydroxylase, a major autoantigen in idiopathic Adisson's disease. Lancet 1992;339:1559-62.
24. Fujii Y, Kato N, Kito J, Asai J, Yokochi T. Experimental autoimmune adrenalitis: a murine model for Adisson's disease. Autoimmunity 1992;12:47-52.
25. Khoury EL, Hammond L, Bottazzo GF, Doniach D. Surface-reactive antibodies to human adrenal cells in Addison's disease. Clin Exp Immunol 1981;45:48-55.
26. Rabinowe SL, Jackson RA, Dluhy RG, Williams GH. Ia-positive T lymphocytes in recently diagnosed idiopathic Addison's disease. Am J Med 1984;77:597-601.
27. Isselbacher KJ, Braunwald E, Wilson J, et al. Harrison's textbook of internal medicine. 13th ed. New York: Mc Graw Hill, 1994.
28. Arulanantham K, Dwyer JM, Genel M. Evidence for defective immunoregulation in the syndrome of familial candidiasis endocrinopathy. N Engl J Med 1979;300:164-8.
29. Jackson R, McNicol AM, Farquharson M, Foulis AK. Class II MHC expression in normal adrenal cortex and cortical cells in autoimmune Addison's disease. J Pathol 1988;155:113-20.
30. Ahonen P, Koskimies S, Lokki ML, Tiilikainen A, Perheentupa J. The expression of autoimmune polyglandular disease type I appears associated with several HLA-A antigens but not with HLA-DR. J Clin Endocrinol Metab 1988;66:1152-7.
31. Khoury EL, Greenspan JS, Greenspan FS. Adrenocortical cells of the zona reticularis normally express HLA-DR antigenic determinants. Am J Pathol 1987;127:580-91.
32. McNicol AM. Class II MHC antigen expression in adrenal cortex. Lancet 1986;2:1282.
33. Bottazzo GF, Pujol Borrell R, Hanafusa T, Feldmann M. Hypothesis: Role of aberrant HLA-DR expression and antigen presentation in induction of endocrine autoimmunity. Lancet 1983;2:1115-9.
34. Hanafusa T, Pujol Borrell R, Chiovato L, Russell RCG, Doniach D, Bottazzo GF. Aberrant expression of HLA-DR antigen on thyrocytes in Graves' disease: relevance for autoimmunity. Lancet 1983;2:1111-5.
35. Donner H, Braun J, Seidl C, et al. Codon 17 polymorphism of the cytotoxic T lymphocyte antigen 4 gene in Hashimoto's thyroiditis and Addison's disease. J Clin Endocrinol Metab 1997;82:4130-2.

36. Kemp EH, Ajjan RA, Husebye ES, et al. A cytotoxic T lymphocyte antigen-4 (CTLA-4) gene polymorphism is associated with autoimmune Addison's disease in English patients. Clin Endocrinol 1998;49:609-13.
37. Laureti S, Bellis A de, Muccitelli VI, et al. Levels of adrenocortical autoantibodies correlate with the degree of adrenal dysfunction in subjects with preclinical Addison's disease. J Clin Endocrinol Metab 1998;83:3507-11.
38. Bellis A de, Bizzarro A, Rossi R, et al. Remission of subclinical adrenocortical failure in subjects with adrenal autoantibodies. J Clin Endocrinol Metab 1993;76:1002-7.
39. Abdu TAM, Elhadd TA, Neary R, Clayton RN. Comparison of the low dose short synachten test (1 μg), the conventional dose short synachten test (250 μg), and the insulin tolerance test for assessment of the hypothalamo-pituitary-adrenal axis in patients with pituitary disease. J Clin Endocrinol Metab 1999;84:838-43.
40. Oelkers W, Diederich S, Bähr V. Diagnosis and therapy surveillance in Adisson's disease: rapid adrenocorticotropin (ACTH) test and measurement of plasma ACTH, renin activity, and aldosterone. J Clin Endocrinol Metab 1992;75:259-64.
41. González González JG, Garza Hernández NE de la, Mancillas Adame LG, Montes Villareal J, Villareal Pérez. A high sensitivity test in the assessment of adrenocortical insufficiency: 10 μg vs 250 μg cosyntropin dose assessment of adrenocortical insufficiency. J Endocrinol 1998;159:275-80.
42. Laureti S, Arvat E, Candeloro P, et al. Low dose (1 μg) ACTH test in the evaluation of adrenal dysfunction in pre-clinical Adisson's disease. Clin Endocrinol 2000;53:107-15.
43. Boscaro M, Betterle C, Sonino N, Volpato M, Paoletta A, Fallo F. Early hypofunction in patients with organ specific autoantibodies and no clinical adrenal insufficiency. J Clin Endocrinol Metab 1994;79:452-5.

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Diuresis pattern, plasma vasopressin and blood pressure in healthy elderly persons with nocturia and nocturnal polyuria

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ABSTRACT

Background: Nocturia, a common symptom in the elderly, is often caused by increased urine production at night.

Methods: The present study comprised 17 men and six women aged 68.1 ± 4.7 (mean ± SD) years with nocturia (≥2 nocturnal voids) and nocturnal polyuria (nocturnal urinary output of ≥0.9 mL min^{-1}). A physical examination, measurements of recumbent blood pressure after a 15-minute rest, plasma AVP assay at noon and midnight, and urine collection performed during a 24-hour period.

Results: The daytime urine output was 1358 ± 664 mL, and the nocturnal urine output 796 ± 312 mL. The AVP level was lower at midnight than at noon in 17 persons, and higher at midnight in six persons. Blood pressure was 142.0 ± 15.7/87.4 ± 9.1 mmHg. Systolic (but not diastolic) blood pressure increased with decreasing nocturnal plasma AVP. Increasing nocturnal diuresis rate (r^2=0.26; p<0.01) but not plasma AVP was associated with increasing systolic blood pressure.

Conclusion: In elderly persons with nocturia and nocturnal polyuria, the plasma AVP is low and does not rise nocturnally. The systolic blood pressure is increased with increasing diuresis but unaffected by plasma AVP.

INTRODUCTION

Nocturia is a common symptom in the elderly, with a profound influence on health and the quality of life.[1] In many cases nocturia is caused by a nocturnal overproduction of urine due to the absence of an increase in the plasma level of arginine vasopressin (AVP) at night, or to a low level of AVP throughout the whole 24-hour period.[2-5] This condition has been termed the Nocturnal Polyuria Syndrome (NPS).[6] Nocturnal polyuria commonly occurs in elderly men with persistent nocturia after prostatic resection or in association with other lower urinary tract symptoms (LUTS).[4,7] Nighttime incontinence among nursing home residents and elderly persons with impaired health is often caused by an increased urine production at night.[5]
In an earlier study it was found that 29% of the men and 24% of women with a mean age of 74 ± 6 years had two or more nocturnal micturition episodes.[1] Most cases of nocturia among the elderly are not explained by LUTS.[7] The aim of the present report was to analyse the relationship between nocturnal polyuria, AVP and blood pressure in a group of healthy elderly men and women with nocturia but without LUTS.

MATERIALS AND METHODS

All persons aged 60 to 74 years and resident in two municipalities (Strömsund and Hammerdal) in the county of Jämtland, Sweden were contacted by post and were asked to answer a questionnaire concerning health and micturition habits. A reminder questionnaire was sent out to those who did not respond within one month.

The respondents were invited to proceed in the study if:
- they were healthy, i.e. had no diseases or medications with a possible influence on the AVP system;
- they had no symptoms or signs of any disease of this system at a thorough physical examination and after an extensive laboratory work-up;
- they were non-smokers and not misusing alcohol;
- they had nocturia (≥2 nocturnal voids) and nocturnal polyuria (a nocturnal diuresis rate of ≥0.9 mL min^{-1}).[8-10]

The participants were asked to live as usual with regard to eating and drinking, physical activity, rest and sleep. They completed a questionnaire on current medical symptoms and a thorough physical examination was performed, including measurements of recumbent blood pressure after 15-minutes rest, electrocardiography (ECG) and plasma AVP assay at noon and at midnight. Urine collection was performed throughout a 24-hour period, from rising in the morning to bedtime, and from bedtime to rising in the morning, and voiding measurements were made during the daytime (defined as time out of bed) and at night (defined as time in bed). In the daytime the investigations were performed at Strömsund Hospital, while at night the subjects were visited by trained nurses in their homes. Blood samples were drawn by direct venipuncture at 12 noon and 12 midnight. The midnight samples were taken with the subject in a recumbent position, while at noon the subjects sat comfortably for sampling. Samples for AVP measurement were sent to Ferring Pharmaceuticals, Malmö, Sweden. The laboratory procedure has been described previously.[11] In the numerical analysis undetectable AVP was given an arbitrary value of 0.25 pg mL^{-1}.

Statistical methods

Values are presented as mean ± standard deviation (SD) unless otherwise stated and were calculated according to standard methods. Student's t-test was used to compare two numerical variables. For comparison of pairs of numerical data, regression analysis was used, while forward stepwise regression analysis was performed (StatView SE Graphics for Macintosh) for the multivariate analysis.

Ethics

The study was approved by the Ethics Committee of the University of Umeå and conducted in accordance with the rules of the Declaration of Helsinki. Each person gave his/her informed consent of participation (oral and signed).

RESULTS

The target group for selection consisted of 1438 individuals, 43% of whom were men. The first questionnaire was completed by 882 persons. On the second occasion, 231 persons replied (total response rate = 77.4%). Among the respondents, 246 reported having two or more micturitions per night, of whom 150 persons considered themselves to be healthy.

After exclusions for contraindicating illnesses, concomitant medication and smoking, 64 persons remained. Fifty persons agreed to undergo a clinical examination. After exclusions based on the results of the examination, 23 persons (17 men and 6 women) remained and formed the study group.

Their age was 67.8 ± 4.7 years, height 174.7 ± 8.6 cm and weight 78.3 ± 11.7 kg. Their blood pressure was 142.0 ± 15.7/87.4 ± 9.1 mmHg.

Diuresis

In the whole group the daytime urine output was 1358 ± 664 mL, the nocturnal urine output was 796 ± 312 mL and the total 24-hour urine output was 2154 ± 712 mL. Accordingly, the diuresis rates were 1.4 ± 0.7, 1.6 ± 0.7 and 1.5 ± 0.5 mL min^{-1}, respectively. In 16 subjects the diuresis rate was higher at night than in the daytime.

The 24-hour diuresis was 2848 ± 732 mL 24 h^{-1} in subjects without detectable AVP at midnight and 1910 ± 534 mL 24 h^{-1} in subjects with detectable AVP ($p<0.01$). The diuresis rates in these two groups were 2.0 ± 0.5 and 1.3 ± 0.4 mL min^{-1}, respectively ($p<0.01$). The daytime diuresis was 2081 ± 835 mL in persons without detectable AVP at midnight and 1103 ± 350 mL in those with detectable AVP ($p<0.001$). There was no difference in nocturnal diuresis between subjects with and without detectable AVP at midnight.

There was no correlation between daytime and nocturnal diuresis.

Vasopressin levels and diuresis

The plasma AVP concentrations at noon and at midnight were 1.0 ± 0.6 and 0.8 ± 0.5 pg mL^{-1}, respectively. In two samples at noon and in six samples at midnight the AVP level was undetectable (<0.5 pg mL^{-1}). The AVP level was lower at midnight (N-) than at noon in 17 subjects, and higher at midnight (N+) in six subjects. Plasma AVP showed no relation to age. The daytime AVP level increased with increasing nocturnal AVP ($r=0.68$; $r^2=0.45$; $p<0.001$).

The 24-hour urine excretion in N- subjects was 30.7 ± 9.6 mL/kg^{-1} body weight and in N+ subjects 20.6 ± 2.9 mL/kg^{-1}. N- subjects had a higher diuresis rate both in the daytime and at night than N+ subjects (*figure 1*).

A higher nocturnal AVP level was associated with lower 24-hour urine output (*figure 2*). A stepwise regression analysis with 24-hour urine output as the dependent variable and plasma AVP at midnight and at noon as explanatory variables revealed a decrease in 24-hour urine output with increasing AVP at midnight ($r=0.60$; $r^2 = 0.36$; $p<0.01$), while plasma AVP at noon was deleted by the model.

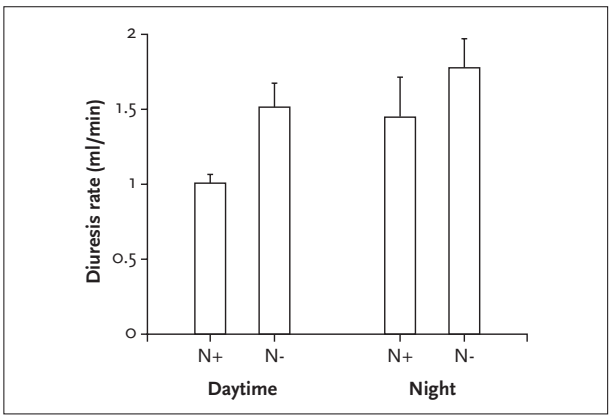

Figure 1
The daytime and nighttime urine output in subjects with higher plasma AVP at night than in the daytime (N+) and in those with lower plasma AVP at night than in the daytime (N-) (mean ± SEM)

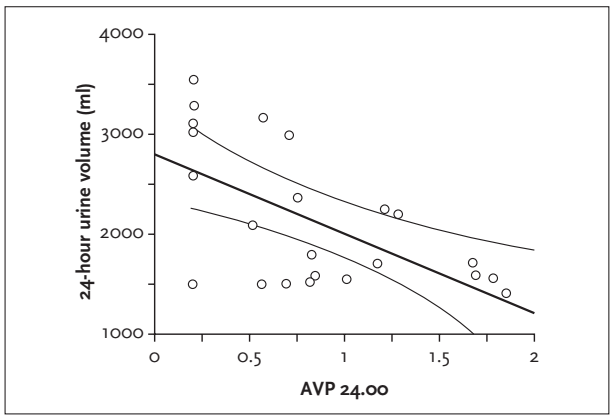

Figure 2
The relationship between plasma AVP at midnight and 24-hour urine output (regression line ± 95% confidence limits for mean 24-hour diuresis; r^2=0.35; p<0.05)

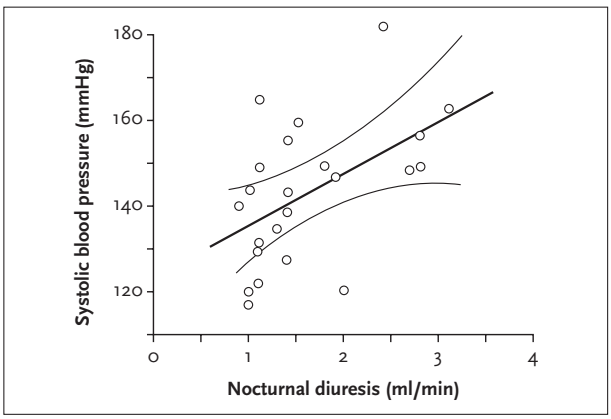

Figure 3
The relationship between nocturnal diuresis and systolic blood pressure (regression line ± 95% confidence limits for systolic blood pressure; r^2=0.20; p<0.05)

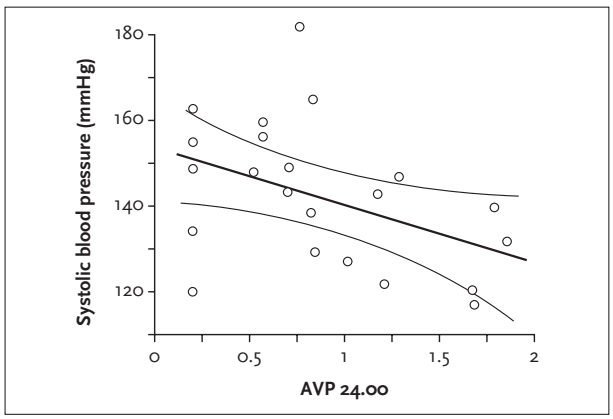

Figure 4
The relationship between plasma AVP at midnight and systolic blood pressure (regression line ± 95% confidence limits for systolic blood pressure; r^2=0.20; p<0.05)

Blood pressure, diuresis and vasopressin levels

The blood pressure was 142.6 ± 16.0/87.2 ± 9.3 mmHg. A higher nocturnal diuresis rate was associated with higher systolic blood pressure than a lower diuresis rate (*figure 3*). This was true for both men (r=0.48; r^2= 0.25; p<0.05) and women (r=0.85; r^2= 0.72; p<0.05). Systolic blood pressure showed no relation to the daytime diuresis rate. There was no relation between diastolic blood pressure and either nocturnal or daytime diuresis rate.

The systolic (but not diastolic) blood pressure increased with decreasing nocturnal AVP level (*figure 4*). A stepwise regression analysis with systolic blood pressure as the dependent variable and nocturnal diuresis rate and plasma AVP at noon and at midnight as explanatory variables revealed an increase in systolic blood pressure with increasing nocturnal diuresis (r=0.50; r^2= 0.25; p<0.05), while plasma AVP at noon and at midnight was deleted by the model.

DISCUSSION

Nocturia due to overproduction of urine is common in elderly persons.[10,12-14] Frequent voiding episodes at night cause sleep disturbance and daytime tiredness, leading to impaired well-being and poor daytime performance.[1] In

elderly persons in poor health and with difficulties in locomotion, incontinence is a common consequence of nocturnal polyuria.[13]

In general the present study group was healthy and free from diseases or symptoms of the urogenital tract. The criterion for nocturnal polyuria, a nocturnal diuresis rate of ≥0.9 mL min^{-1}, was chosen to include both severely polyuric subjects and subjects with an assumed normally functioning nocturnal AVP secretion and, thus, near normal urinary output.[7,9,10] By studying subjects with this range of urine output, it should be possible to analyse the 24-hour distribution of the urinary output and plasma AVP secretion in different degrees of nocturnal polyuria.

In the present study the urine output was high both at night and during the daytime in persons with a lower nocturnal than daytime AVP (*figure 1*). Their nocturnal diuresis was, in accordance with the inclusion criteria, higher than could be expected in an unselected group of persons of the same age.[10] However, also the daytime diuresis was higher in persons with lower AVP at night than in the daytime.

The total 24-hour urine volume was higher in the present study group than in previously reported studies among healthy elderly persons.[7,9] Although the nocturnal diuresis rate was higher than that in the daytime in two-thirds of the study subjects, the mean nocturnal diuresis rate in the whole group was only moderately increased as compared with that in the daytime (*figure 1*).

The plasma AVP levels in this studied group of polyuric persons were in general lower both at night and in the daytime in comparison with those in the non-polyuric group.[3,15] In a study on AVP in healthy persons with a mean age of 78 ± 3 years, Johnson *et al.* found a mean daytime AVP level of 4.7 ± 0.7 pg mL^{-1}, and in an earlier study of healthy elderly persons without nocturnal voiding episodes we found a mean midnight AVP level of 7.3 ± 10.2 pmol L^{-1} in the men and 1.3 ± 1.2 pmol L^{-1} in the women, and at noon 3.2 ± 3.9 pmol L^{-1} and 1.3 ± 1.0 pmol L^{-1} in the men and women, respectively.[3,15] On average, the subjects of the present study were about five years younger than our previous group. The plasma AVP showed an age-related increase, but it is not probable that more than a minor part of the difference in AVP is explained by age.[15]

The 24-hour urine output was about 1000 ml higher in persons without detectable AVP at midnight than in those with detectable AVP. Increasing nocturnal plasma AVP levels were associated with a decreasing 24-hour urine output (*figure 2*). In two-thirds of the cases the AVP level at midnight was lower (N-) than that at noon (N+). The N- group showed higher urine output figures both at night and in the daytime than the N+ group. Matthiesen *et al.* found that compared with non-polyuric persons, polyuric persons had a slightly increased daytime urine output and an increased 24-hour urine volume.[16] The daytime urine output showed a more pronounced increase in the present study.

One surprising finding was that there was no relation between the daytime and nocturnal urine output. This is in contrast to results reported by Matthiesen *et al.* concerning elderly men who suffered from nocturnal polyuria and lower urinary tract symptoms (LUTS).[16] The discrepancy between the results may be explained by differences in the inclusion criteria between the two studies. In the present study persons who were free from LUTS, and thus had pure polyuria, were enrolled. An increase in the nocturnal diuresis rate was associated with an increase in systolic blood pressure (*figure 3*). Such a relationship has previously been observed among men.[16] The results in the women in the present study were in accordance with those in the men, but this finding should be interpreted with caution as the groups, especially the group of women, were very small. This result may be in line with the observation by Bulpitt *et al.* that among elderly hypertensives nocturia is the most frequent complaint in persons with isolated systolic hypertension (68% in both sexes).[17] A decrease in nocturnal plasma AVP was also associated with an increase in systolic blood pressure (*figure 4*). When the influence of the nocturnal diuresis rate and nocturnal plasma AVP were analysed together, however, only the former was associated with systolic blood pressure. This is in accordance with a recently suggested hypothesis that urodilatin, rather than AVP, plays an important role in the occurrence of nocturnal polyuria in elderly hypertensives, and that 'nocturnal polyuria and hypertension are manifestations of the same pathophysiological process'.[18,19] High AVP levels have been observed particularly in persons with secondary hypertension.[20] However, Kawano *et al.* summarise their findings in a study of the interaction between AVP and blood pressure by stating: 'Our results suggest that AVP does not play an important role in mild essential hypertension'.[21]

From the present study it was concluded that nocturnal polyuria occurs in persons with normal blood pressure and that the nocturnal AVP level is not related to the blood pressure level in normotensive persons.

In summary, in elderly persons with nocturia and nocturnal polyuria the plasma AVP level is low and does not rise nocturnally. Their 24-hour diuresis is increased and the urine output shows no circadian rhythm. The systolic blood pressure is increased with increasing diuresis, but is unaffected by plasma AVP.

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REFERENCES

1. Asplund R, Åberg H. Health of the elderly with regard to sleep and nocturnal micturition. Scand J Prim Health Care 1992;10:98-104.
2. Asplund R, Åberg H. Diurnal rhythm of antidiuretic hormone in elderly subjects with nocturia. Med Sci Res 1991;19:765-6.
3. Asplund R, Åberg H. Diurnal variation in the levels of antidiuretic hormone in the elderly. J Int Med 1991;229:131-4.
4. Bodo G, Gontero P, Casetta G, et al. Circadian antidiuretic hormone variation in elderly men complaining of persistent nocturia after urinary flow obstruction removal. Scand J Urol Nephrol 1998;32:320-4.
5. Ouslander JG, Nasr SZ, Miller M, et al. Arginine vasopressin levels in nursing home residents with nighttime urinary incontinence. J Am Geriatr Soc 1998;46:1274-9.
6. Asplund R. The nocturnal polyuria syndrome (NPS). Gen Pharm 1995;26:1203-9.
7. Asplund R. Micturition habits and diuresis in relation to sleep and well-being in elderly subjects with emphasis on antidiuretic hormone thesis. Stockholm, 1992.
8. Asplund R, Sundberg B, Bengtsson P. Desmopressin for the treatment of nocturnal polyuria in elderly subjects: a dose titration study. Br J Urol 1998;82:642-6.
9. Kirkland JL, Lye M, Lewy DW, Banerjee AK. Patterns of urine flow and electrolyte excretion in healthy elderly people. Br Med J 1983;287:1665-6.
10. Asplund R, Åberg H. Micturition habits of older people. Voiding frequency and urine volumes. Scand J Urol Nephrol 1992;26:345-9.
11. Asplund R, Åberg H, Wetterberg L. The seasonal interrelationship between melatonin, vasopressin and osmolality in elderly subjects. J Pin Res 1998;25:67-72.
12. Weiss JP, Blaivas JG, Stember DS, Brooks MM. Nocturia in adults: etiology and classification. Neurourol Urodyn 1998;17:467-72.
13. Donahue JL, Lowenthal DT. Nocturnal polyuria in the elderly person. Am J Med Sci 1997;314:232-8.
14. Madersbacher S, Pycha A, Klingler CH, Schatzl G, Marberger M. The International Prostate Symptom score in both sexes: a urodynamics-based comparison. Neurourol Urodyn 1999;18:173-82.
15. Johnson AG, Crawford GA, Kelly D, Nguyen TV, Gyory AZ. Arginine vasopressin and osmolality in the elderly. J Am Geriatr Soc 1994;42:399-404.
16. Matthiesen TB, Rittig S, Norgaard JP, Pedersen EB, Djurhuus JC. Nocturnal polyuria and natriuresis in male patients with nocturia and lower urinary tract symptoms. J Urol 1996;156:1292-9.
17. Bulpitt CJ, Fletcher AE, Thijs L, et al. Symptoms reported by elderly patients with isolated systolic hypertension: baseline data from the SYST-EUR trial. Systolic Hypertension in Europe. Age Ageing 1999;28:15-22.
18. McKeigue PM, Reynard JM. Relation of nocturnal polyuria of the elderly to essential hypertension. Lancet 2000;355:486-8.
19. Meyer M, Richter R, Brunkhorst R, et al. Urodilatin is involved in sodium homeostasis and exerts sodium-state-dependent natriuretic and diuretic effects. Am J Physiol 1996;271:F489-97.
20. Jensen LW, Pedersen EB. Nocturnal blood pressure and relation to vasoactive hormones and renal function in hypertension and chronic renal failure. Blood Press 1997;6:332-42.
21. Kawano Y, Matsuoka H, Nishikimi T, Takishita S, Omae T. The role of vasopressin in essential hypertension. Plasma levels and effects of the V1 receptor antagonist OPC-21268 during different dietary sodium intakes. Am J Hypertens 1997;10:1240-4.

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Contact tracing using DNA fingerprinting in an asylum seeker with pulmonary tuberculosis

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ABSTRACT

Background: The diagnosis of tuberculosis in asylum seekers is followed by contact tracing, which is routinely performed by the Municipal Health Service (MHS). We investigated cases of tuberculosis whose symptoms became apparent after closure of regular contact tracing.

Methods: Analysis of data from the DNA Fingerprinting Surveillance Project on all *Mycobacterium tuberculosis* isolates and contact tracing instances.

Results: Four additional cases of tuberculosis were detected, caused by bacteria of identical DNA fingerprints. No further contacts with a bacteriologically confirmed form of tuberculosis were found around these four new patients.

Conclusion: DNA fingerprinting contributed to tracing instances of late manifestations of tuberculosis transmission.

INTRODUCTION

In the period 1993 to 1998, an average of 32,986 asylum seekers per year entered the Netherlands.[1] The annual percentage of screening for tuberculosis by chest X-ray on entry varied from 90 to 98%. Pulmonary tuberculosis was diagnosed in an average of 93 asylum seekers per year, which is a detection rate of almost 300 per 100,000 of the population. In 37 persons, the diagnosis was confirmed bacteriologically; i.e. a prevalence rate of 124 per 100,000.[1] Since 1993, all *M. tuberculosis* cultures have been sent to the National Institute of Public Health and the Environment (RIVM) for DNA fingerprinting.[2] This technique provides insight into which tuberculosis patients in the Netherlands have been infected by the same *M. tuberculosis* strain. This is of importance for source case finding and contact tracing. It has also led to new insights into the epidemiology of tuberculosis in broader terms.

Tracing of close contacts is effective in identifying further diseased individuals at an early stage. In asylum patients with tuberculosis, contact tracing is performed in the usual way according to the ring principle.[3,4] First, the close contacts who share the same facilities or have been involved in the same procedures from the native country onwards, are traced and examined. If the infection rate within this first ring is high, it is decided to extend contact tracing to the second ring of shelter employees or visitors or even residents who have moved to other shelters for asylum seekers. However, on the basis of contact tracing it is unknown how many cases of tuberculosis occur in which symptoms became apparent after closure of the regular contact tracing. The DNA Fingerprinting Surveillance Project was structurally analysed to examine such transmission from an asylum seeker.

PATIENTS AND METHODS

DNA typing of *M. tuberculosis* isolates was performed by the IS*6110* Restriction Fragment Length Polymorphism (RFLP) technique. Different *M. tuberculosis* strains contain a varying number of IS*6110* insertion elements, and/or vary in the genomic sites where IS*6110* elements are inserted. In RFLP typing, restriction fragments are

visualised that contain IS*6110* DNA.
Patients infected by the same *M. tuberculosis* strains (100% identical DNA fingerprints) belong to one cluster.[2] Such a cluster may be subdivided into groups of patients who are epidemiologically linked. To establish such a link a nurse from the Royal Netherlands Tuberculosis Association (KNCV) contacts a TB nurse at the respective MHS where the patients with 100% identical DNA fingerprints have been found. This is done in the framework of the nationwide DNA Fingerprinting Surveillance Project.[5] To make an epidemiological link, consultation takes place between the TB nurse from the MHS and the individual patients, under strict privacy regulations. Potential epidemiological links are assessed by the TB nurse through evidence of direct or highly probable contact between patients; e.g.: do they know each other very well, or have they been living in the same area in the infectious period of one of them?

RESULTS

In November 1993, an infectious form of pulmonary tuberculosis was diagnosed in an aspirant asylum seeker from Algeria who was staying at the Temporary Shelter (POC) 'Waalhaven'. Examination took place within the framework of the compulsory screening for tuberculosis, applicable to asylum seekers after entering the Netherlands. Because the ventilation facilities at the Waalhaven POC were absolutely inadequate, a high rate of transmission was considered.[3] Contact tracing was performed by the MHS according to the usual ring principle.[4] Contact tracing around the persons who belonged to the first ring of the asylum seeker revealed an infection rate of 60% (n=5). Therefore, it was decided to extend the contact tracing to the second ring. The second ring comprised 105 employees/visitors and 110 asylum seekers residing at the Waalhaven POC.[3] Eleven employees/visitors had a positive Mantoux test. This meant an infection rate of 10.5% (n=105). In the meantime, the 110 asylum seekers had been transferred to 16 other shelters.
With the cooperation of the affiliated MHSs, the respective persons could be approached personally. The asylum seekers had all received BCG vaccination in their countries of origin, which made detection of a latent tuberculosis infection by the Mantoux test impossible. Therefore, screening for tuberculosis was done by chest X-ray and on the basis of clinical symptoms. No cases of active tuberculosis were detected among the 110 asylum seekers.

After the regular contact tracing had been completed in February 1994, an infectious form of pulmonary tuberculosis was diagnosed in four of these 110 asylum seekers (one from Afghanistan, one from Bosnia and two from Somalia). Three of them had already contacted a physician on their own initiative because of tuberculosis symptoms. This happened in March 1994, April 1994 and September 1996, respectively. In the remaining asylum seeker, infectious tuberculosis was diagnosed in April 1994 within the framework of the six-monthly screening programme for tuberculosis that applies to asylum seekers and immigrants during their first two years in the Netherlands.[6] Screening was performed by chest X-ray.
RFLP typing by the RIVM revealed that these four patients were infected by *M. tuberculosis* bacteria with identical DNA fingerprints to those of the index patient who came from Algeria and had been a co-tenant at the Waalhaven POC in November 1993.
These five patients belonged to a cluster of 14 persons whose *M. tuberculosis* DNA fingerprints were identical. Three groups of patients could be distinguished within this cluster, comprising of five, three and six persons each; no epidemiological links could be established between the three different groups, and between the six patients of the remaining group 3.
Group 1 of the cluster comprised five persons: the asylum seeker from Algeria who was diagnosed with an infectious form of pulmonary tuberculosis at the Waalhaven POC, and the four asylum seekers from Afghanistan (1), Bosnia (1) and Somalia (2). These four asylum seekers were staying at the Waalhaven POC in November 1993 and belonged to the second ring of the patient from Algeria. In these four asylum seekers there were no signs of active tuberculosis in November 1993 or in February 1994.
In group 2 of the cluster, three patients had been in contact with each other: two were members of a family from Somalia and the remaining person was a Dutch nurse. The link between the nurse and her index patient (from Somalia) was made after it became known that the DNA fingerprints were identical; this was later on confirmed by anamnesis.
Group 3 of the cluster comprised six patients who had not shared accommodation or shelter facilities. They indicated that to their knowledge they had never met any of the other persons in the group or any of the other eight patients in the cluster with an identical DNA fingerprint. One patient came from Algeria, two from Iran, one from the Netherlands, one from Somalia and one from Syria.

DISCUSSION

In the Netherlands, the IS*6110* RFLP patterns of *M. tuberculosis* strains are highly polymorphic. However, an identical fingerprint does not form indisputable evidence that contact has taken place between patients in the same cluster, because there can be a non-identified index patient or an external source. The finding of identical DNA fingerprints in immigrants means that the patients must have had a

common source in the Netherlands or in their country of origin, or that they infected each other in their country of origin or in the Netherlands.
Regular contact tracing by the MHS is conducted according to the ring principle. If infections are found, for example, in co-tenants, i.e. the first ring, then contact tracing is extended to the next ring and so on until no more cases of infection are found.

On the basis of contact tracing by TB nurses from MHSs on 1673 patients over the period 1997 to 1999 an epidemiological link was suspected in 22% of the patients. This was before the results of RFLP typing were known.[7] A definite epidemiological link was made in 26% of the patients and a suspected link in 21% with the aid of the RFLP technique and supplementary anamnesis. Therefore, RFLP typing contributed significantly to demonstrating links that were not found by regular contact tracing.
These findings support the results of the present study. None of the four contacts with the index patient at the Waalhaven POC were detected by means of regular contact tracing in February 1994. Three out of these four patients consulted a physician because of symptoms. Patients who might have been exposed to infection and have therefore been invited for contact tracing belong to risk groups for tuberculosis. In persons known to have a positive Mantoux test and in persons who have received a BCG vaccination in the past, it is not possible to make a diagnosis of latent tuberculosis by means of a Mantoux test. A Mantoux test can also give a false-negative result. Three of the four contacts became ill within two years of infection. This rate is compatible with data in the literature on the latency of tuberculosis infections. Although the incubation period for tuberculosis may be lifelong, the majority of patients manifest active tuberculosis within two years of infection.[8] Endeavouring to achieve optimal compliance with the voluntary six-monthly screening for tuberculosis by means of a chest X-ray during the first two years of an asylum seeker's stay in the Netherlands is therefore highly desirable.[6] On the one hand, because the risk of developing active tuberculosis is the greatest in the first two to five years after immigration and on the other hand, because it is possible for a person to become infected while staying at a shelter for asylum seekers.[9] The risk of infection is the most pronounced when the ventilation facilities at a shelter are deficient and when a (too) large group of people, who have not been screened for tuberculosis, are sharing such accommodation. Regular contact tracing every three months after the last contact cannot always be performed, because it is common practice for asylum seekers to be transferred from one shelter to another.

From the evaluation of DNA fingerprinting programmes in San Francisco and New York it was expected in the Netherlands that patient clustering would re-open or extend contact tracing to detect previously unidentified infections. Five years after the start of the DNA surveillance programme, a re-opening of the contact tracing was only decided upon in 1% of the patients. A total number of 2640 contacts were examined of whom 75 (3%) appeared to be infected, and 12 (0.5%) had active tuberculosis. The ring principle for contact tracing, in practice in the Netherlands since the sixties, still is an efficient method of surveillance.[7]

On the basis of contact tracing it is very likely that one index patient caused a latent tuberculosis infection in 14 persons (positive Mantoux test, normal chest X-ray and no symptoms of active disease). Moreover, on the basis of 100% identical DNA fingerprints and anamnestic findings, it is very likely that the same index patient caused bacteriologically proven active tuberculosis in four other persons. At the time of regular contact tracing, none of these four asylum seekers had any signs of active tuberculosis. Three out of these four persons became ill within two years of infection and one became ill within three years. Three of the four patients were detected as a result of symptoms and one was detected by screening. The epidemiological link with the source case was found by DNA fingerprinting of the *M. tuberculosis* isolates. No further patients with a bacteriologically proven form of tuberculosis were found around these four new patients. DNA fingerprinting appears useful to detect late manifestation of transmission, long after contact tracing has been finalised.

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REFERENCES

1. Royal Netherlands Tuberculosis Association (KNVC). Index Tuberculosis 1998. The Hague, 2000.

2. Soolingen D van. Molecular epidemiology of tuberculosis and other mycobacterial infections; main methodologies and achievements. Review J Int Med 2001;249:1-26.
3. Loenhout-Rooyackers JH van. Risico van tuberculose bij inadequate opvang van aspirant-asielzoekers. Ned Tijdschr Geneeskd 1994;138:2496-500.
4. Veen J. Microepidemics of tuberculosis: the stone-in-the-pond principle. Tubercle and Lung Disease 1992;73:73-6.
5 Šebek M. DNA fingerprinting and contact investigation. Int J Tuberc Lung Dis 2000;4(suppl):S45-S8.
6. Bwire R, Nagelkerke N, Keizer ST, et al. Tuberculosis screening among immigrants in the Netherlands: what is its contribution to public health? Neth J Med 2000;56:63-71.
7. Šebek MMGG, Kalisvaart NA. Vijf jaar DNA-surveillance van *M. tuberculosis* in Nederland. Tegen de Tuberculose 2001;97:93-7.
8. Asbroek AHA ten, Borgdorff MW, Nagelkerke NJD, et al. Estimation of serial interval and incubation period of tuberculosis using DNA fingerprinting. Int J Tuberc Lung Dis 1999;3:414-20.
9. Menzies D. Tuberculosis crosses borders. Int J Tuberc Lung Dis 2000;4(suppl):S153-S9.

ABOUT THE COVER

Narcissus

Miranda Penning

This month's cover, entitled 'Narcissus', shows graphic art by Miranda Penning. The technique is the art of woodcarving. Miranda was born in Oosterhout, Noord-Brabant, the Netherlands in 1974. She graduated at the Academy of St. Joost in Breda and the Academy of Art in Arnhem.

Miranda received a number of assignments (e.g. from 'Royal Nederland' in 2000 and from 'De Blauwe Kamer'), participated in the Graphic fair in Essen, Germany (2001) and contributed to the Siemens Art Calendar (2002). She is inspired by the world's mythologies and transfers the contents of these stories to pictures. She focuses on a surrealistic world with structures of nature, such as rocks, trees, water and air. She depicts dreams and a symbolic and mythical animal world.

A limited edition of original prints (size 85 x 78 cm) of this month's cover is available at a price of € 300. You can order the print at Galerie Unita, Rijksstraatweg 109, 6573 CK Beek-Ubbergen, the Netherlands or by e-mail: galerie-unita@planet.nl.

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Regular alcohol intake and fibrinolysis

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ABSTRACT

Background: Light to moderate alcohol consumption is associated with a reduced risk of coronary heart disease. Stimulation of fibrinolysis has been suggested as one of the mechanisms involved. The present study analyses the effect of regular alcohol consumption on various parameters of fibrinolysis. The question whether the alcohol-induced plasma increase of plasminogen activator inhibitor (PAI-1) may originate from thrombocytes was also addressed.

Methods: Six healthy male volunteers consumed three glasses of red wine daily during two periods of a week, with a week of abstinence from alcohol in between. PAI-1 antigen and activity levels, t-PA antigen and activity levels and plasmin antiplasmin (PAP) complexes were measured on days 1, 3, 8, 15, 17 and 22 of the experiment period. On the first day, PAI-1 antigen and activity before and after alcohol consumption was also measured in platelet-rich plasma (prp).

Results: Although some slight shifts in the various parameters could be noticed during the drinking periods, all favouring impairment rather than stimulation, no significant effect of regular moderate alcohol use could be observed on fibrinolysis. Alcohol did not trigger a release of PAI-1 from platelets.

Conclusions: Regular moderate alcohol consumption has no significant effect on fibrinolysis. The alcohol-induced increase of plasma PAI-1 does not originate from thrombocytes. The cardioprotective effect of moderate alcohol consumption cannot be explained by a beneficial influence on fibrinolysis.

INTRODUCTION

Light to moderate alcohol consumption is associated with a reduced incidence of coronary heart disease.[1] However, the mechanisms involved in this cardioprotective effect are only partly understood and data from experimental studies are scarce. Alcohol causes a slight decrease in low-density lipoprotein-cholesterol (LDL-c) and a marked increase in high-density lipoprotein-cholesterol (HDL-c), which might explain 50 to 60% of its beneficial effect.[2] Apart from lipid metabolism, alcohol also influences the haemostatic balance.[3] Thrombocytes as well as components of the clotting system and fibrinolysis play a role in the process of atherosclerosis and the final occlusion of vessels leading to infarction. Alcohol is known to affect platelet function and to lower levels of fibrinogen, von Willebrand factor and factor VII.[4] It has also significant effects on the serum levels of two important components of the fibrinolytic system, tissue-type plasminogen activator (t-PA) and its inhibitor plasminogen activator inhibitor-1 (PAI-1).[5,6] Serum levels of these two components increase within hours after alcohol consumption with the rise of PAI-1 being more outspoken than that of t-PA, resulting in inhibition of fibrinolysis.

Although alcohol consumption shifts fibrinolysis in an unfavourable direction in the short term, this does not necessarily imply that such a shift also occurs in regular moderate drinking. Since the acute intake of alcohol causes a very profound increase of PAI-1, regular consumption may result in a gradual exhaustion of this component. Such exhaustion is not unthinkable, since blood platelets with their half-life time of seven days contain the majority of PAI-1 antigen concentration in blood.[7] If alcohol were to release PAI-1 from platelets, PAI-1 concentrations could be

expected to fall within a week of daily alcohol consumption. With t-PA concentrations being less affected, the balance may even gradually shift towards enhancement of fibrinolysis.
This hypothesis was addressed in the present study, which investigates the effect of daily moderate alcohol consumption on both the antigen and activity levels of PAI-1 and t-PA as well as on the production of plasmin, the end product of the fibrinolytic pathway. The question whether the alcohol-induced serum increase of PAI-1 originates from thrombocytes was also addressed.

MATERIALS AND METHODS

Subjects

Six male volunteers participated in the study (age 34 ± 6.1 years, BMI 24 ± 2). All individuals were healthy, non-smoking men taking no medication or supplementary vitamins. Their average alcohol consumption was two units of an alcoholic beverage per day. Before entering the study a routine blood profile was performed showing normal liver function tests and a normal lipid profile.

Study design

All volunteers gave informed consent to the study protocol, which was approved by the medical ethical committee of the Eemland Hospital, Amersfoort. All abstained from alcohol for a period of one week preceding the experiment. On the first day of the experiment three glasses of red wine (Diego de Almagro 1994, 12.5 vol. % alcohol) were consumed with a meal between 5 and 6 p.m. That first day blood samples were drawn from an antecubital vein with minimal venous stasis after 15 minutes in supine position at 3 p.m., 6 p.m., 8 p.m. and 11 p.m.
After that first day each volunteer consumed three glasses of red wine either at dinner or during the evening for a period of a week. After the first week of the experiment the volunteers abstained from alcohol for another week, while in the third week of the experiment the regimen of the first week was repeated. No other alcoholic beverages were allowed during the whole experiment. Apart from the first day, blood samples were taken on day 3, 8, 15, 17 and 22 at 8 a.m.

Methods

Blood samples were centrifuged within 30 minutes at 4 °C at 3000 rpm (1800 g). Plasma samples were snapfrozen and stored at -70 °C until assay. Platelet-rich plasma was prepared and lysis performed by snapfreezing and thawing. PAI-1 activity and t-PA activity were determined using chromogenic amydolytic assays (Biopool®). PAI-1 antigen and t-PA antigen were determined with enzyme immunoassays (Innotest®, Innogenetics). Plasmin-antiplasmin complexes were determined with an enzyme immunoassay by Enzygnost®, PAP micro, Boehringer, Germany.

Statistical analysis

Statistical calculations were performed with a non-parametric manoeuvre comparing measurements of the group in time with day 1.

RESULTS

Release of PAI-1 from platelets was analysed by measuring PAI-1 in platelet-rich plasma (prp) before and after alcohol consumption (three glasses of red wine = 36 g of alcohol). No significant decrease of this component could be observed after alcohol consumption indicating no depletion of PAI-1 from platelets (*table 1*). Since PAI-1 significantly increased in platelet-poor plasma after alcohol intake, PAI-1 must

Table 1
Plaminogen activator inhibitor (PAI-1) activity and antigen after the intake of three glasses of red wine at 5 p.m.

PARAMETER	PRP (PLATELET RICH PLASMA)		PPP (PLATELET POOR PLASMA)	
PAI-1 activity U/ml (n=6)	**Mean and SD**	**(median)**	**Mean and SD**	**(median)**
4 p.m.	20 ± 4	(19)	4 ± 6	(2)
6 p.m.	31 ± 10	(29)	20 ± 11	(18)
8 p.m.	27 ± 10	(25)	18 ± 11	(16)
11 p.m.	30 ± 13	(27)	21 ± 14	(21)
PAI-1 antigen μg/l (n=6)	**Mean and SD**	**(median)**	**Mean and SD**	**(median)**
4 p.m.	540 ± 88	(535)	25 ± 19	(19)
6 p.m.	576 ± 93	(565)	62 ± 30	(57)
8 p.m.	592 ± 74	(620)	72 ± 34	(65)
11 p.m.	573 ± 81	(580)	64 ± 35	(61)

originate from another source than platelets. In accordance with the observation that thrombocytes were not involved is the fact that platelet count, LDH and von Willebrand factor were also not affected by the consumption of three glasses of red wine (data not shown).
As shown in *table 2* no significant changes in the parameters of fibrinolysis measured (PAI-1 antigen, PAI-1 activity, t-PA antigen, t-PA activity and PAP complexes) could be observed during the weeks of alcohol intake (days 1-8 and 15-22). However, the small and non-significant shifts all point in the same direction, inhibition of fibrinolysis rather than stimulation. PAI-1 antigen and activity tend to rise, while t-PA activity and PAP complexes show the tendency to drop. These shifts return to baseline values during the week of abstinence.

Table 2
Parameters of fibrinolysis during two weeks of daily moderate alcohol consumption (days 1-8 and 15-22) and one week of abstinence (days 8-15)

PARAMETER AT 8 A.M.	MEAN AND SD	MEDIAN
PAI-1 activity U/ml (n=6)		
Day 1	16 ± 16	13
Day 3	18 ± 14	16
Day 8	15 ± 7	15
Day 15	18 ± 25	6
Day 17	20 ± 24	7
Day 22	12 ± 9	9
PAI-1 antigen :g/l (n=6)		
Day 1	59 ± 56	49
Day 3	66 ± 46	63
Day 8	64 ± 26	63
Day 15	67 ± 83	33
Day 17	76 ± 74	41
Day 22	54 ± 40	45
t-PA activity U/ml (n=6)		
Day 1	0.7 ± 0.4	0.85
Day 3	0.6 ± 0.3	0.6
Day 8	0.6 ± 0.2	0.6
Day 15	0.9 ± 0.6	1.1
Day 17	0.6 ± 0.3	0.8
Day 22	0.7 ± 0.3	0.6
t-PA antigen :g/l (n=6)		
Day 1	4.2 ± 1.5	4.7
Day 3	5.3 ± 1.1	5.1
Day 8	5.5 ± 1.0	5.6
Day 15	4.5 ± 1.2	4.4
Day 17	5.4 ± 1.1	5.6
Day 22	5.8 ± 2.3	5.1
PAP compl. :g/l (n=6)		
Day 1	388 ± 131	398
Day 3	313 ± 93	330
Day 8	295 ± 59	310
Day 15	421 ± 152	485
Day 17	328 ± 159	285
Day 22	344 ± 96	325

DISCUSSION

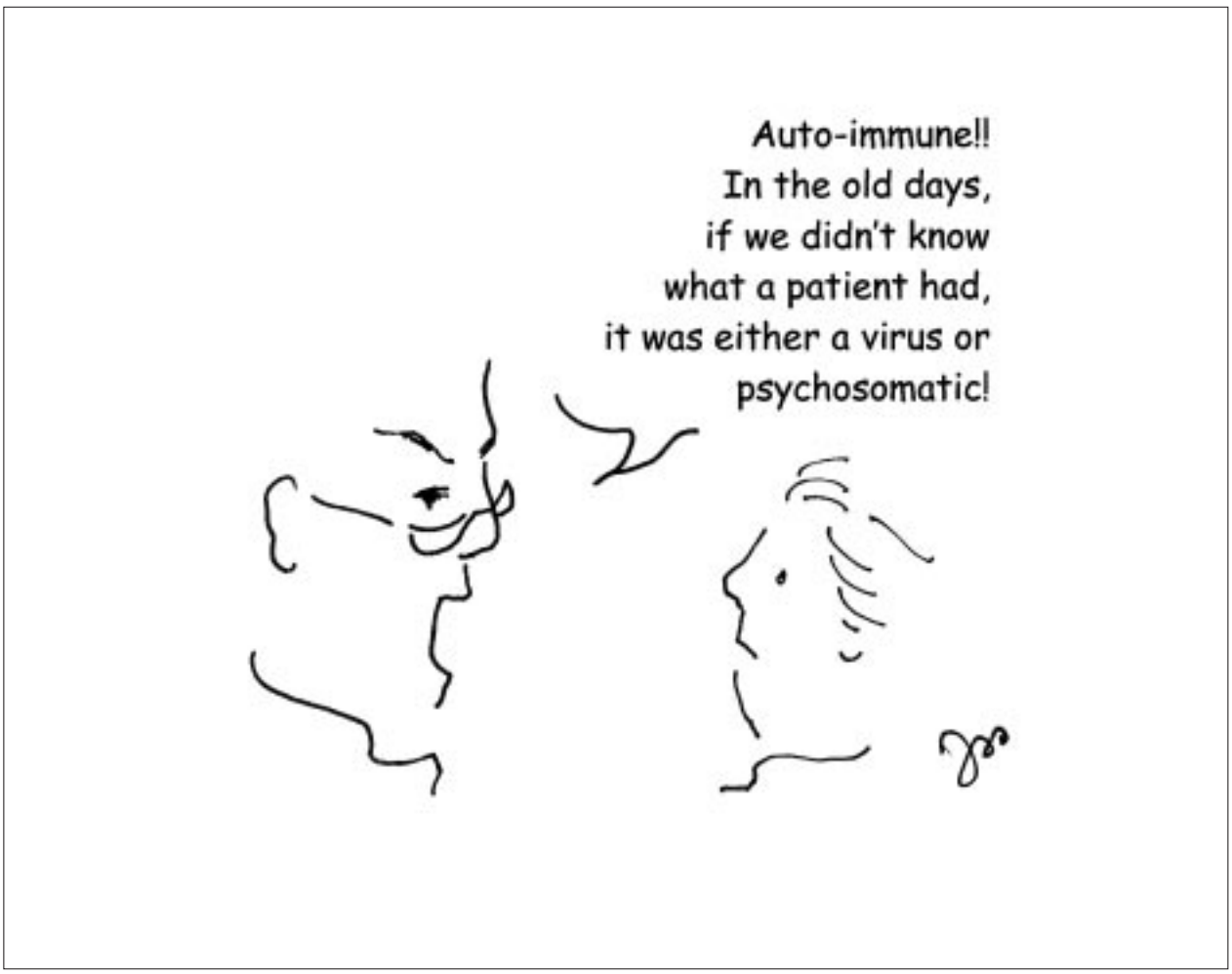
Alcohol has an acute inhibitory effect on fibrinolysis that, in the case of excessive drinking, may interfere with the circadian rhythm of the fibrinolytic system and contribute to a higher risk for acute cardiac events.[6] Since this effect has been shown for red wine, beer as well as spirits, it should be attributed to the alcohol component and not to other substances.[5] It has been suggested that a more chronic moderate drinking pattern stimulates fibrinolysis, a hypothesis mainly based on a rise of t-PA antigen.[8] This would be true if t-PA activity were to increase as well, which happens if the counteracting component, PAI-1, does not change or decreases. The present study shows no significant effect of regular and moderate drinking on fibrinolysis although there are small shifts that all point to inhibition rather than stimulation of fibrinolysis. PAI-1 antigen and its activity tend to rise while t-PA activity and PAP complexes tend to drop. All parameters return to normal baseline values within a week of total abstinence from alcohol. It cannot be excluded that prolongation of the drinking periods would result in a significant inhibition. Since thrombocytes are a source of PAI-1 and alcohol is known to affect certain platelet functions, chronic alcohol consumption might stimulate fibrinolysis by depleting platelets from their PAI-1 content gradually leading to lower PAI-1 concentrations in the circulation.
However, our study shows that the PAI-1 content of platelets is not affected by alcohol consumption and that no exhaustion of this inhibitor of fibrinolysis occurs. Our experimental data are therefore in accordance with a recent observational analysis from the Framingham Offspring Cohort.[4] In that 20-year observational study an alcohol intake of 7 to 21 drinks weekly, generally considered as moderate drinking, was associated with impaired fibrinolytic potential, reflected by higher levels of the antigens of PAI-1 and t-PA. In contrast to platelets, the endothelium is a more likely source from which both PAI-1 and t-PA are released after alcohol intake. Up-regulation of the production of these components by endothelial cells has been demonstrated during alcohol challenge.[9] Because of the extent and production capacity of this organ, exhaustion of PAI-1 is unlikely even in cases of chronic excessive alcohol abuse.

In fact, high levels of PAI-1 and return to normal levels have been described in chronic alcoholics at admission and during their stay in hospital.[10]

In conclusion, regular moderate alcohol consumption is not associated with significant changes in fibrinolysis. Therefore, the cardioprotective effect of this type of drinking cannot be attributed to a favourable effect on this part of the haemostatic balance.

REFERENCES

1. Rimm EB, Klatsky A, Grobbee D, Stampfer MJ. Review of moderate alcohol consumption and reduced risk of coronary heart disease; is the effect due to beer, wine or spirits? Br Med J 1996;312:731-6.
2. Suh I, Shaten J, Cutler JA, Kuller LH. Alcohol use and mortality from coronary heart disease: the role of high-density lipoprotein cholesterol. Ann Intern Med 1992;116:881-7.
3. Booyse FM, Parks DA. Moderate wine and alcohol consumption: beneficial effects on cardiovascular disease. Thromb Haemost 2001;86:517-28.
4. Mukamal KJ, Jadhav PP, D'Agostino RB, et al. Alcohol consumption and hemostatic factors, analysis of the Framingham offspring cohort. Circulation 2001;104:1367-73.
5. Hendriks HFJ, Veenstra J, Velthuis-te Wierik EJM, Schaafsma G, Kluft C. Effect of moderate dose of alcohol with evening meal on fibrinolytic factors. Br Med J 1994;303:1003-6.
6. Wiel A van de, Golde PM van, Kraaijenhagen RJ, Borne PAK von dem, Bouma BN, Hart HCh. Acute inhibitory effect of alcohol on fibrinolysis. Eur J Clin Invest 2001;31:164-70.
7. Booth NA, Simpson AJ, Croll A, Bennett B, McGregor IR. Plasminogen activator inhibitor (PAI-1) in plasma and platelets. Br J Haem 1988;70:327-33.
8. Ridker PM, Vaughan DE, Stampfer MJ, Glynn RJ, Hennekens CH. Association of moderate alcohol consumption and plasma concentration of endogenous tissue-type plasminogen activator. JAMA 1994;272:929-33.
9. Booyse FM, Aikens ML, Grenett HE. Endothelial cell fibrinolysis: transcriptional regulation of fibrinolytic protein gene expression (t-PA, u-PA and PAI-1) by low alcohol. Alcohol Clin Exp Res 1999;23:1119-24.
10. Delahousse B, Maillot F, Gabriel I, Schellenber F, Lamisse F, Gruel Y. Increased plasma fibrinolysis and tissue-type plasminogen activator/tissue-type plasminogen activator inhibitor ratios after ethanol withdrawal in chronic alcoholics. Blood Coagul Fibrinolysis 2001;12:59-66.



Van Golde, et al. Regular alcohol intake and fibrinolysis.

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CASE REPORT

Severe neutropenia due to naproxen therapy in rheumatoid arthritis: a case report and review of literature

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ABSTRACT

Rheumatoid arthritis is a chronic inflammatory disease that primarily affects the joints and is often treated with non-steroidal anti-inflammatory drugs (NSAIDs) on demand and disease-modifying antirheumatic drugs (DMARDs), with a relatively low risk of side effects. Although an infrequent side effect, neutropenia has been described as a sequel of NSAIDs. We report a case of neutropenia proven (by rechallenge) to be due to naproxen therapy. The literature on neutropenia during treatment with NSAIDs and DMARDs is briefly reviewed.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disease resulting in inflammation primarily of joints, but possibly involving extra-articular sites as well. Because of the severity and chronicity of rheumatoid inflammation, disease-modifying antirheumatic drugs (DMARDs) are prescribed in RA. DMARDs bear a relatively low and acceptable risk of evoking side effects. At the onset of DMARD therapy non-steroidal anti-inflammatory drugs (NSAIDs) are often prescribed to directly reduce the painful inflammation. Neutropenia has been reported during the treatment of RA, due to NSAIDs, and/or DMARDs, and/or due to the disease itself. A patient is reported in whom neutropenia occurred, which was proven to be due to naproxen following a thorough analysis including rechallenge.

CASE REPORT

A 53-year-old man was admitted because of active RA. His medical history revealed a mild chronic obstructive pulmonary disease (COPD) and an IgM-rheumatoid factor (RF) positive RA that had started two months before. At that time several treatment modalities were discussed, and the NSAID naproxen 500 mg b.i.d. combined with the DMARD leflunomide (100 mg daily for three days, followed by 20 mg daily) were started. Due to a misunderstanding the patient accidentally stopped the leflunomide after 30 days. At the seventh week of treatment, his white blood cell count (WBC) had fallen to 2.2 x 10^9/l. (normal range 4.5 to 10 x 10^9/l). The course of the haemoglobin, thrombocytes and WBC is depicted in *figure 1*. He was then prompted to stop all medication. An active polyarthritis recurred within one week. Because of inflammatory pain, naproxen was restarted three days prior to admission. On admission, his WBC was 5.0 x 10^9/l. Therapy was started with methotrexate (MTX) plus folic acid (prophylactically) instead of leflunomide, which was held responsible for the neutropenia. Naproxen was continued. Five days later the WBC again dropped to 2.1 x 10^9/l. Vitamin B12 and folate levels were normal. Bone marrow aspiration showed normal granulopoiesis. Hepatosplenomegaly was excluded because abdominal ultrasonography was normal. As inadequate production and excessive destruction were ruled out, it was concluded that the leucopenia was drug-related. However, it was unclear which medication had actually caused it. Therefore, all medication was stopped immediately. Within a week the WBC normalised to 4.0 x 10^9/l. The potential causes of the second neutropenia were MTX, naproxen, or possibly naproxen combined with low blood levels of leflunomide. The neutropenia occurred one day

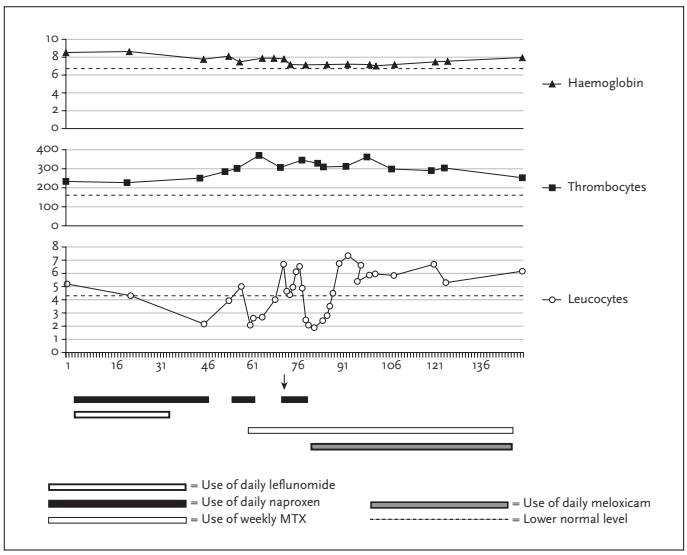

Figure 1
Time course of haemoglobin, thrombocytes and leucocytes
Day 1 is start of leflunomide and naproxen. The black arrow indicates the start of the rechallenge

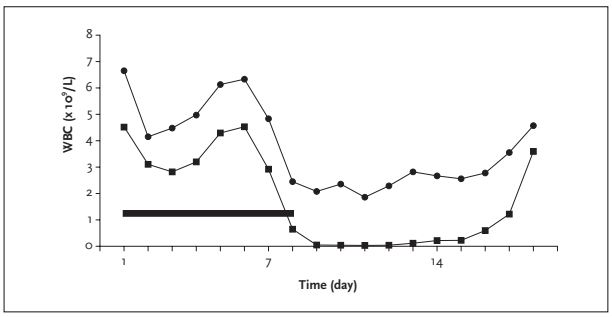

Figure 2
Counts of leucocytes (circle) and neutrophils (square) during rechallenge with naproxen (black box)

after the first application of MTX and after the rechallenge the WBC remained normal despite continuing MTX, excluding MTX as causative drug. Although leflunomide was suspected to be the neutropenic agent in the first episode, during the second episode there was no evidence as to what had caused the neutropenia. After performing a literature search, we concluded that epidemiologically the odds that naproxen had caused these neutropenias were very low. After informed consent was obtained, the patient was rechallenged with naproxen under close clinical control. Naproxen was restarted at 500 mg b.i.d., and MTX plus folic acid was continued. The course of the WBC and differentiation during rechallenge is depicted in *figure 2*. The naproxen was stopped permanently when after one week the WBC again dropped to 2.5 x 10^9/l with 0.6 x 10^9/l neutrophils. Interaction with low levels of leflunomide is unlikely given the half-life of leflunomide (approximately two weeks) and the time of rechallenge (70 days after the first neutropenia). The patient developed a fever without a clear focus, for which a course of the antibiotic cefuroxim was given. Nine days later the WBC had returned to normal values again. DMARD and NSAID therapy were re-evaluated: MTX with folic acid supplementation was continued, propionic acid derivatives including naproxen were prohibited, and meloxicam, a preferential COX-2 selective NSAID was given. Follow-up was uneventful with this medication regimen and remission was reached without further irregularities.

DISCUSSION

Neutropenia may occur secondary to inadequate granulocyte production, excessive granulocyte destruction, or sometimes as a chronic, usually benign condition. After thorough analysis, it was concluded that the leucopenia was drug-related in our patient.

Various side effects have been reported as being secondary to DMARDs and there are major differences between the various DMARDs and the organ systems involved (*table I*). Although data on these side effects are reported in percentages, odds ratios and incidence per 100 patient years and therefore not easily comparable, DMARDs seem to cause blood dyscrasias (especially neutropenia) more frequently than NSAIDs (*table I*). Neutropenia is most frequently observed during azathioprine and intramuscular gold therapy. In the case of azathioprine an incidence of 2.0 to 4.5 per 100 patient years was found.[1-5] An incidence of 0.4 to 3.5 per 100 patient years was reported for intramuscular gold.[2,4,6] MTX (particularly with folic acid supplementation) and sulphasalazine (SSZ) have less often been associated with neutropenia. Stewart *et al.* found that leucopenia developed in three out of 200 patients who were on low-dose MTX plus folic acid supplementation,[7] while others reported an incidence 0.5 to 2.0 per 100 patient years.[2-4,6] The risk of neutropenia secondary to SSZ is 0 to 5.6%.[3,8-11] Neutropenia is relatively uncommon with hydroxychloroquine[4] and d-penicillamine[2,3] treatment. Data on leflunomide, a novel isoxazol DMARD, are scarce. It has been prescribed in about 76,000 patients of whom only eight developed a mild leucopenia.[12]
Blood dyscrasias, such as agranulocytosis, aplastic anaemia and thrombocytopenia, have been described during the application of NSAIDs in the past.[13] Strom *et al.* found that the relative risk of developing neutropenia during NSAID therapy was 4.2 (95% CI 2.0 - 8.7).[14] Given the low incidence rate, for every one million patients exposed to NSAIDs during a full year, 145 extra cases of neutropenia were to be expected. Usually, these blood dyscrasias are caused by the pyrazolidinediones, and to a lesser extent by indomethacin. The arylpropionic acids, including naproxen, are commonly regarded as the safest among the NSAIDs with respect to blood dyscrasias. Although

Table 1

Neutropenia due to medication used in rheumatoid arthritis and eponyms of well-known side effects

	NEUTROPENIA		CLINICAL SIDE EFFECTS	REFERENCE FOR NEUTROPENIA
	%	Incidence/100 patient years	Odds ratio	
DMARDs				
Azathioprine	0 - 18.1	2.0 - 4.5	L, B	1-5
Gold i.m.	0 - 10.3	0.4 - 3.5	S, R, B	2, 4, 6
Methotrexate	0 - 6.4	0.5 - 2.0	G, L, P, B	2-4, 6-7
Sulphasalazine	0 - 5.6	1.5	G, L, B, S	3, 8-11
Hydroxychloroquine	0 - 8.3		E	4
d-Penicillamine	0 - 6.9	0.6 - 0.8	S, R, B	2-3
Leflunomide	0.01		L, G	12
NSAIDs		4.2	G, R	14

Clinical side effects: P = pulmonary, B = blood, L = liver, S = skin, G = gastrointestinal, E = eye, R = renal. Data taken from literature are given in percentage, incidence and odds ratio and therefore are not directly comparable.

extensively prescribed worldwide, naproxen has been associated with severe neutropenia only very rarely.[14-17] One patient who developed a neutropenia after naproxen was replaced by ibuprofen has been reported.[17] In the Netherlands, neutropenia due to naproxen has never been reported to the Dutch pharmacovigilance foundation LAREB (personal communication).

In our patient, leflunomide was initially suspected to be the causative drug. After the second episode naproxen was incriminated as the causative agent. This was confirmed following rechallenge.

In a chronic disorder such as rheumatoid arthritis, it is of eminent importance to ascertain which medication causes the side effect encountered, since RA patients will often have to continue medication for many years thereafter. In our patient it is clear that a propionic acid derivative will bear a risk. In the hypothetical case of MTX failure this patient may well be willing to take leflunomide again in the future.

REFERENCES

1. Singh G, Fries JF, Spitz P, Williams CA. Toxic effects of azathioprine in rheumatoid arthritis. A national post-marketing perspective. Arthritis Rheumatism 1989;32:837-43.
2. Fries JF, Williams CA, Ramey D, Bloch DA. The relative toxicity of disease-modifying antirheumatic drugs. Arthritis Rheum 1993;36:297-306.
3. Grove ML, Hassell AB, Hay EM, Shadforth MF. Adverse reactions to disease-modifying anti-rheumatic drugs in clinical practice. Q J Med 2001;94:309-19.
4. Singh G, Fries JF, Williams CA, Zatarain E, Spitz P, Bloch DA. Toxicity profiles of disease modifying antirheumatic drugs in rheumatoid arthritis. J Rheumatol 1991;18:188-94.
5. Whisnant JK, Pelkey J. Rheumatoid arthritis: treatment with azathioprine (Imuran®). Clinical side effects and laboratory abnormalities. Ann Rheum Dis 1982;41(suppl 1):S44-S7.
6. Jaarsveld CHM van, Jahangier ZN, Jacobs JWG, et al. Toxicity of antirheumatic drugs in a randomized clinical trial of early rheumatoid arthritis. Rheumatol 2000;39:1374-82.
7. Stewart KA, Mackenzie AH, Clough JD, Wilke WS. Folate supplementation in methotrexate-treated rheumatoid arthritis patients. Sem Arthr Rheumatism 1991;20:332-8.
8. Jick H, Myers MW, Dean AD. The risk of sulfasalazine- and mesalazine-associated blood disorders. Pharmacotherapy 1995;15:176-81.
9. Amos RS, Bax DE. Leucopenia in rheumatoid arthritis: relationship to gold or sulphasalazine therapy. Br J Rheumatol 1988;27:465-8.
10. Marabani M, Madhok R, Capell HA, Hunter JA. Leucopenia during sulphasalazine treatment for rheumatoid arthritis. Ann Rheum Dis 1989;48:505-7.
11. Amos RS, Pullar T, Bax DE, Situnayake D, Capell HA, McConkey B. Sulphasalazine for rheumatoid arthritis: toxicity in 774 patients monitored for one to 11 years. Br Med J 1986;293:420-3.
12. Prakash A, Jarvis B. Leflunomide: a review of its use in active rheumatoid arthritis. Drugs 1999;58:1137-64.
13. Biscarini L, Vrhovac B. Anti-inflammatory analgesics and drugs used in gout. In: Meyler's side effects of drugs. Dukes MNG, editor. An encyclopaedia of adverse reactions and interactions. 13th ed. Amsterdam: Elsevier, 1996. p. 204-32.
14. Strom BL, Carson JL, Schinnar R, Snyder ES, Shaw M, Lundin E Jr. Nonsteroidal anti-inflammatory drugs and neutropenia. Arch Intern Med 1993;153:2119-24.
15. Berry H, Nicholls A. An open study of 1.5 g naproxen daily in osteoarthritis. Curr Med Res Opin 1985;9:366-70.
16. Nygard N, Starkebaum G. Naproxen and agranulocytosis. JAMA 1987;257:1732.
17. Jick H, Derby LE, Garia Rodriguez LA, Jick SS, Dean AD. Nonsteroidal antiinflammatory drugs and certain rare, serious adverse advents: a cohort study. Pharmacotherapy 1993;13:212-7.

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CASE REPORT

A patient with thymoma and four different organ-specific autoimmune diseases

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Abstract

This is the first report of a patient with four organ-specific autoimmune diseases; myasthenia gravis, type 1 diabetes mellitus, autoimmune hepatitis and Hashimoto's thyroiditis. The clinical history suggests a relationship with a non-removed thymoma. Not only the thymoma seems to have triggered these four diseases, the dramatic progressive course with an active autoimmune hepatitis and high concentrations of multiple autoantibodies was probably also associated with non-removal of the thymoma. Thymectomy should be performed in myasthenia gravis patients with thymoma and associated autoimmune diseases.

INTRODUCTION

Myasthenia gravis (MG) is an autoimmune disease caused by the binding of antibody to acetylcholine receptors on the muscle membrane. Patients with MG often have circulating antibodies to a variety of tissue constituents and several studies have shown an increased occurrence of other autoimmune diseases in MG patients.[1,2] For example, thyroid diseases were observed in more than 5% of MG patients.[1,2] However, the occurrence of MG and type 1 diabetes mellitus (DM) is very rare.[3-5]. The association of MG and autoimmune hepatitis is also very rare.[6,7] On the other hand, autoimmune thyroiditis and type 1 DM are closely associated in Japan. A survey showed that the prevalence of autoimmune thyroiditis was 22% at onset of type 1 DM and 28% after three years.[8] According to the nation-wide survey in Japan, hypothyroidism, which is usually due to Hashimoto's thyroiditis, was seen in 12% of autoimmune hepatitis patients.[9] However, a patient with MG, Hashimoto's thyroiditis, type 1 DM and autoimmune hepatitis has not been reported. These four conditions are organ-specific autoimmune disease. Patients with MG often also have thymoma. We report here a case with thymoma, MG, Hashimoto's thyroiditis, type 1 DM and autoimmune hepatitis.

CASE REPORT

A 77-year-old female visited the Ophthalmology Department in our hospital because of recent blepharooptosis in May 1997. Her edrophonium chloride (Tensilon) test was positive and acetylcholine receptor antibody titre was high; 64.1 nmol/l (upper limit of normal: >0.3, RIA). She was diagnosed with MG and pyridostigmine bromide (Mestinon) was administered at 180 mg a day. The administration improved her symptoms, but in December 1998, she experienced general fatigue, thirst and pollakisuria. Her fasting plasma glucose level was 310 mg/dl, her HbA_{1c} was 13.3%, thus she was diagnosed with diabetes mellitus and admitted to our hospital. There was no known family history of diabetes mellitus and autoimmune diseases. Her family included a younger sister, her two children and four grandchildren who were all clinically unaffected. Her parents were not consanguineous; they had died of old age. Her other laboratory data were as follows: urinary C-peptide 2.7-2.8 μg/day (normal: 43-146); antiglutamic acid decarboxylase (GAD) antibody titre 17,000 U/ml (normal: >1.5, RIA); islet cell antibody (ICA) positive; percent of anti-insulin binding antibody 6.8% (normal: <10); level of aspartate aminotransferase

(ASAT) 22 IU/L (7-38); alanine aminotransferase (ALAT) 26 IU/L (5-35); alkaline phosphatase (AP) 145 IU/L (40-250); total bilirubin 0.73 mg/dl (0.22-0.30); IgG 1586 mg/dl (800-1800); IgA 483 mg/dl (90-380); IgM 139 mg/dl (60-300); TSH 30.4 μIU/ml (0.24-3.7); free T3 1.8 pg/ml (2.4-4.3); free T4 0.6 ng/dl (0.9-1.8); thyroid test (antithyroglobulin antibody titre) x 102,400 (normal: x >100, gelatin particle agglutination); microsome test (antithyroid peroxidase antibody titre) x 409,600 (normal: x >100, gelatin particle agglutination).

Physical examination showed emaciation; BMI 17.5 kg/m^2 and multi-nodular goitre. Insulin treatment was started at once and improved her condition.

Chest computed tomography revealed an anterior mediastinal tumour of 5 cm in diameter, which suggested thymoma. She had undergone thoracoplasty at the age of 33 because of pulmonary tuberculosis: her left lung volume had decreased and her right lung had expanded. The anterior mediastinal tumour was deviated to the left and an aspiration biopsy was performed. The biopsy specimen showed epithelial components in lymphoid tissues, which were compatible with thymoma (*figure 1*). The patient refused thymectomy for her thymoma. Pyridostigmine bromide was administered, together with insulin. At this time, she was diagnosed with MG, type I DM and Hashimoto's thyroiditis with thymoma.

In August 1999, laboratory data showed liver dysfunction: ASAT 89 IU/L; ALAT 97 IU/L. In September, the data indicated that the dysfunction had deteriorated and, in October, she was readmitted. Physical examination showed icterus. Her laboratory data were as follows: level of ASAT 392 IU/L; ALAT 280 IU/L; AP 335 IU/L; total bilirubin 6.33 mg/dl; direct bilirubin 3.87 mg/dl (0.05-0.30); IgG 3134 mg/dl; IgA 1176 mg/dl; IgM 232 mg/dl; anti-GAD antibody titre 25900 U/ml; percent of anti-insulin binding antibody 25.7%; anti-DNA antibody titre 1280 (normal: x >80, passive haemagglutination test); anti-ss-DNA IgG antibody 30.3 AU/ml (normal: >25.0, ELISA); anti-ds-DNA IgG antibody negative; anti-RNP antibody negative; antinuclear antibody negative; antimitochondrial antibody negative; anti-smooth muscle antibody negative; anti-liver/kidney microsome (LKM) 1 antibody negative; anti-acetylcholine receptor antibody 108 nmol/l. Common viral aetiologies were excluded (hepatitis A, B, and C, and Epstein-Barr virus). She had not been receiving any other drugs. Abdominal CT and ultrasound showed no dilatation of intrahepatic bile duct. Therefore, we suspected autoimmune hepatitis and a laparoscopy for a biopsy was scheduled. However, the laparoscopy was abandoned because of the appearance of ascites. The administration of prednisolone 40 mg per day was started. However, total bilirubin and ammonia levels increased and prothrombin time and a test for vitamin k-dependent 'clotting' factors deteriorated. A plasma exchange was started. Finally plasmapheresis was performed four times and immunoabsorption was performed twice. Although her condition improved transiently, finally she died. The findings of necropsied liver specimens were compatible with autoimmune hepatitis (*figure 2*): connective tissue replacing the lost parenchyma, periportal necrosis and fibrosis extending far beyond the portal region and linking with an adjacent portal tract forming a bridging necrosis. Many of inflammatory cells had infiltrated the portal areas. They were mainly lymphocytes and 10 to 15% of them were plasma cells. Contrary to viral hepatitis, plasma cells were present in a significant number. According to the international scoring system (probably autoimmune hepatitis: 10-15, definite autoimmune hepatitis: <15) for the diagnosis of autoimmune hepatitis,[10] the patient's score was 15 (probably autoimmune hepatitis).

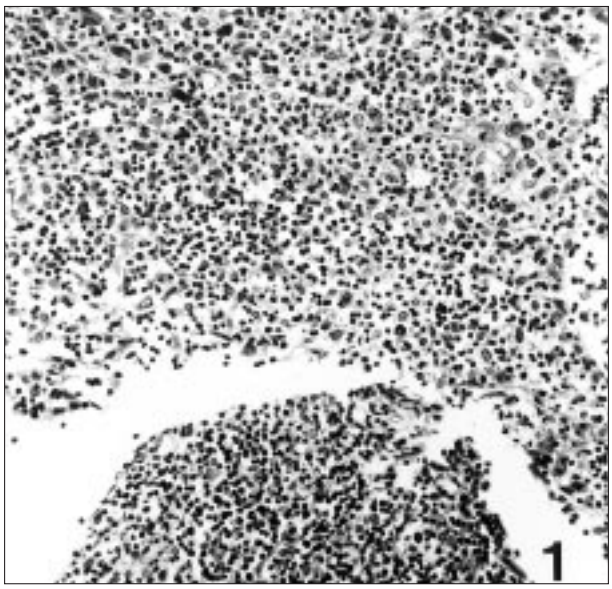


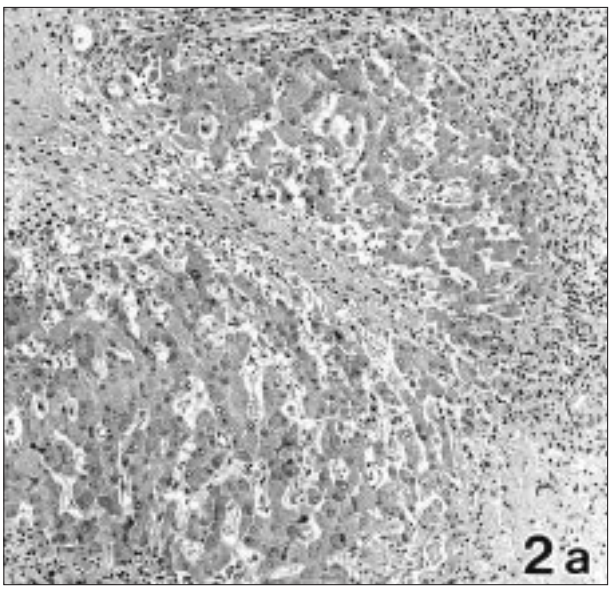
Figure 1
Epithelial components in lymphoid tissues were observed

DISCUSSION

Four autoimmune diseases occurring in the same patient suggests a pathogenic interrelationship between them. MG is induced by the secretion of antiacetylcholine receptor antibody and tests performed at the first visit to Itami City Hospital showed a high titre of antiacetylcholine receptor antibody in this patient. Type 1 DM is well-known to be induced by an autoimmune mechanism and the positive ICA and a high titre of anti-GAD antibody in this case indicate an autoimmune mechanism. Autoimmune hepatitis has been proposed as a distinct disease entity from primary biliary cirrhosis, without antimitochondrial

antibody but with a high titre of antinuclear antibody in the serum.[10] Our case showed negative antinuclear antibody and positive DNA antibody. Autoimmune hepatitis generally progresses slowly. However, in this subject the disease progressed rapidly and resulted in her death. Thus, we speculated that the massive production of autoantibody by the thymoma might explain this different clinical behaviour.
In Japan the major associated autoimmune diseases in autoimmune hepatitis are rheumatoid arthritis, Sjogren's syndrome and Hashimoto's thyroiditis.[9] All patients complicated with other autoimmune diseases were female. Our patient had Hashimoto's thyroiditis. A high titre on thyroid test and microsome test, and the mild hypothyroidism in this patient indicated Hashimoto's thyroiditis. These facts suggest that the production of various antibodies, triggered by thymoma, may have induced the occurrence of these four different autoimmune diseases.
Thymomas are associated with the highest frequency of paraneoplastic autoimmune disease, of which MG is the most common disease. Although various autoantibodies in patients with thymoma are detected, the antigens that these antibodies recognise are not always present in the thymomas.[11] It has been proposed that thymomas may generate autoantigen-specific T cells by a process of abnormal positive or negative T-cell selection and that these T cells may leave the thymoma to induce autoimmune diseases.[11] In our case different autoantigen-specific T cells might have been produced one after another in the thymoma and moved to the periphery to activate B cells, resulting in antibody production. Initially acetylcholine receptor antibody and anti-GAD antibody, which are related to muscle and neurons, were produced and later the other antibodies were also produced.
Polyglandular autoimmune (PGA) syndrome is well-known as a combination of autoimmune diseases.[12] Type 1 PGA consists of Addison's disease, hypoparathyroidism and chronic mucocutaneous candidiasis. Type II PGA commonly consists of Addison's disease, type 1 DM and autoimmune thyroiditis, but no hypoparathyroidism or candidiasis. Type III PGA consists of type 1 DM, autoimmune thyroiditis and pernicious anaemia, but no Addison's disease. Our case does not fit into any of the known PGA syndromes.
Thymectomy should be performed in MG patients with thymoma and associated autoimmune diseases. Since our patient refused the operation, it is unclear whether a thymectomy would have prevented the progression of autoimmune hepatitis. However, in this patient, the IgG level at second admission had increased twofold. We speculate that this increase may have induced autoimmune hepatitis. Thymectomy may have been useful in preventing the progression of autoimmune hepatitis.

In this case, we speculate that thymoma may have induced MG, type 1 DM and autoimmune hepatitis, although the timing of the occurrence of Hashimoto's thyroiditis was obscure. Further investigation into the mechanisms causing these four diseases is required.



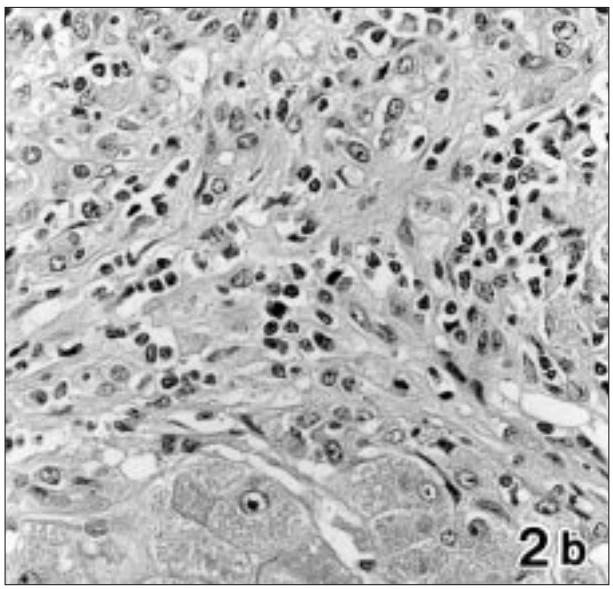


Figure 2

Necropsied specimen

Connective tissue replaces the lost parenchyma. Periportal necrosis and fibrosis extend far beyond the portal region and link with an adjacent portal tract forming a bridging necrosis.

Higher magnification of necropsied specimen

A lot of inflammatory cells infiltrate within the portal areas. They are mainly lymphocytes and 10 to 15% of them are plasma cells. Contrary to viral hepatitis, plasma cells are present in a significant number (x 260).

REFERENCES

1. Christensen PB, Jensen TS, Tsiropoulos I, et al. Associated autoimmune diseases in myasthenia gravis. Acta Neurol Scand 1995;91:192-5.
2. Monden Y, Uyama T, Nakahara K, et al. Clinical characteristics and prognosis of myasthenia graves with other autoimmune disease. Ann Thorac Surg 1986;41:189-92.
3. Green ST, Ng JP, Chan-Lam D. Insulin-dependent diabetes mellitus, myasthenia gravis, pernicious anaemia, autoimmune thyroiditis and autoimmune adrenalitis in a single patient. Scot Med J 1988;33:213-4.
4. Paolisso G, Varricchio M, Donofrio F. Thymectomy and azathioprine in the treatment of type 1 (insulin-dependent) diabetes mellitus. Diabetes Metab 1990;16:524-6.
5. Karp M, Kott E, Shapira M, et al. Unusual association of insulin-dependent diabetes mellitus with congenital myasthenia gravis and autoimmune thyroid disease. Isr J Med Sci 1984;20:1078-81.
6. Han YS, Kim BH, Kim TH, et al. Autoimmune hepatitis in a patient with myasthenia gravis and thymoma - a report on the first case in Korea. Korean J Intern Med 2000;15:151-5.
7. Ko KF, Ho T, Chan KW. Autoimmune chronic active hepatitis and polymyositis in a patient with myasthenia gravis and thymoma. J Neurol Neurosurg Psychiatry 1995;59:558-9.
8. Akamine H, Komiya I, Shimabukuro T, et al. High prevalence of GAD65 (and IA-2) antibodies in Japanese IDDM patients by a new immunoprecipitation assay based on recombinant human GAD65. Diabet Med 1997;14:778-84.
9. Onji M, Nonaka T, Horiike N, Moriwaki H, Muto Y, Ohta Y. Present status of autoimmune hepatitis in Japan. Gastroenterol Jpn 1993;28 (suppl 4):S134-S8.
10. Johnson PJ, McFarlane IG. Meeting report: International Autoimmune Hepatitis Group. Hepatology 1993;18:998-1005.
11. Vincent A, Willcox N. The role of T-cells in the initiation of autoantibody responses in thymoma patients. Pathol Res Pract 1999;195:535-40.
12. Neufeld M, Maclaren NK, Blizzard RM. Two types of autoimmune Addison's disease associated with different polyglandular autoimmune (PGA) syndromes. Medicine 1981;60:355-62.

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TYPE 1 DIABETES: HOW TO RESIST A FATAL ATTRACTION

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Introduction

Type 1 (insulin-dependent) diabetes is a T cell-mediated autoimmune disease in which the insulin-producing pancreatic β-cells are destroyed.[1] The disease has a strong genetic predisposition, but the events that could lead to autoimmunity and disease are still largely unknown. Until recently, the disease was considered to be incurable and the β-cell destruction irreversible. Against all odds, it has now been demonstrated beyond doubt that type 1 diabetes in humans can be cured though replacement of β-cells and effective immunotherapy, while β-cell neogenesis from autologous stem cells is possible. Furthermore, the critical components of the immune system involved in the pathogenesis are being unravelled. The pressure is on to translate these developments into clinical practice to the population of type 1 diabetic patients at large. Yet, significant problems need to be overcome to achieve this goal.

Pathogenesis

The hallmark in the pathogenesis of type 1 diabetes is the infiltration of the islets of Langerhans with mononuclear cells including T cells, B cells, macrophages and dendritic cells. With the disappearance of β-cells no insulitis remains, which indicates that the inflammatory process is β-cell driven.[2] Autoantibodies directed against β-cells have been described that precede the clinical manifestation of the disease. Yet, these autoantibodies are neither required nor sufficient. The same applies to B cells, as was recently demonstrated by the development of type 1 diabetes in a patients with severe inherited B cell deficiency (XLA).[3] The targets of islet autoantibodies are diverse. Most of these targets are neither β-cell nor disease specific, but islet cells autoantibodies serve as best predictor of preclinical type 1 diabetes. The critical involvement of T cells has been demonstrated by the recurrence of β-cell destruction after transplantation of a pancreas segment between identical twins to a diabetic recipient, the delay of β-cell destruction by cyclosporin A and the adoptive transfer of diabetes with bone marrow from a diabetic donor to an immunodeficient recipient. The last-mentioned has only been described if T cells were not depleted from the graft. The reason why T cells become autodestructive is an enigma. However, we were recently able to demonstrate that an important β-cell autoantigen (GAD65) bears sequence homology with cytomegalovirus protein that causes cross-reactivity of T cells from a prediabetic subject between virus and islets of Langerhans.[4] This molecular mimicry may be one of many ways in which tolerance to islet antigens is broken.

Cure

Beta-cell replacement in type 1 diabetic patients could be restored by implantation with allogeneic islets of Langerhans provided the initial cause of the disease is adequately dealt with. This was demonstrated in a clinical trial in which islet allografts were transplanted in type 1 diabetic patients that had previously received a kidney graft.[5] In this study of seven long-term type 1 diabetic patients receiving an implant of highly purified human islet preparations, it was demonstrated that successful islet graft function beyond one year was accompanied by absence of T cell auto- and alloreactivity to islets in three patients. In four patients, loss of β-cell function was associated with appearance of auto- or alloreactivity, suggesting a complex reaction between the graft and the immunosuppressed immune system in the recipient. All successful restorations of insulin production occurred following T-cell depletion with ATG, which suggests that a T cell-directed immunotherapy is critical to achieve a cure. This study further demonstrates that T-cell reactivities in peripheral blood can indeed be used to monitor immune mechanisms, which influence survival of β-cell allografts in diabetic patients. Allogeneic pancreas islet transplantation can restore insulin production in C-peptide negative type 1 diabetic patients, but persistence of β-cell function depends on control of recurrent autoreactivity and allograft rejection. Induction and maintenance of tolerance towards the islet allograft is therefore a critical component to achieve successful restoration of insulin production. Until recently, islet and pancreas transplantation was limited to patients that required suppressive medication because of implantation of a kidney (due to diabetic nephropathy), since life-long immune suppression is critical. With the development of

novel drugs it may become possible to offer islet implantation alone by rendering β-cells resistant to immune attack while other immune responses are not compromised. This would enable islet reconstitution in type 1 diabetic patients prior to development of complications.

Neogenesis

Another major problem is the availability of sufficient numbers of β-cells to restore insulin-independence. A promising development that creates novel opportunities for β-cell generation and reconstitution relates to β-cell neogenesis from precursor stem cells. This possibility arises because important gene products driving this type of endocrine differentiation have been identified (e.g. PDX-1, HES-1). Potentially, this enables the generation of autologous β-cells from type 1 diabetic patients that require less severe immune suppression to prevent recurrence of autoimmunity. In mice, islets grown from stem cells isolated from the pancreatic duct could reverse hyperglycaemia. Yet, perhaps the biggest challenge remains: suppression or eliminattion of the autoimmune process to allow new β-cells to survive.

References

1. Roep BO, Arden SD, Vries RRP de, Hutton JC. T cell clones from a type I diabetes patient respond to insulin secretory granule proteins. Nature (London) 1990;345:632-4.
2. Roep BO, Kallan AA, Hazenbos WLW, et al. T-cell reactivity to 38 kDa insulin-secretory-granule protein in recent onset type 1 diabetes. Lancet 1991;337:1439-41.
3. Martin S, Wolf-Eichbaum D, Duinkerken G, et al. Development of type 1 diabetes despite severe hereditary B lymphocyte deficiency. New Engl J Med 2001;345:1036-40.
4. Hiemstra HS, Schloot NC, Veelen PA van, et al. Cytomegalovirus in autoimmunity: T-cell crossreactivity to viral antigen and autoantigen GAD65. Proc Natl Acad Sci USA 2001;98:3988-91.
5. Roep BO, Stobbe I, Duinkerken G, et al. Auto- and alloimmune responses to human islet allografts transplanted to insulin-dependent diabetes mellitus patients. Diabetes 1999;48:484-90.

CRP AND CARDIOVASCULAR DISEASE: LINKED BY COMPLEMENT?

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Introduction

Assessment of the risk for cardiovascular events in healthy persons or patients with existing cardiovascular disease will help to select persons that should benefit from appropriate intervention. However, cardiovascular events still often occur in persons without 'traditional' risk markers such as smoking, diabetes mellitus, hyperlipidaemia, hypertension. Hence, identification of novel risk markers is urgently needed. During the last decade a number of studies have shown an association between plasma levels of the acute phase protein C-reactive protein (CRP) and the risk for cardiovascular events.[1] Essentially these studies indicate that high normal levels of CRP in apparently healthy persons or patients with stable angina pectoris are associated with a three- to fivefold increased risk for cardiovascular events, as compared with persons with low-normal levels. Most studies agree that the risk predicted by plasma CRP is independent of other risk markers. In addition, CRP levels in patients with unstable angina pectoris constitute the best plasma parameter predicting progression to infarction. Finally, the course of CRP following myocardial infarction is associated the risk for short-term mortality.

CRP

CRP is a member of the so-called pentraxin protein family, which is characterised by its members consisting of pentamers of one peptide chain. In man, CRP is the prototype of the acute phase proteins. CRP levels in normal persons are below 5 mg per L, which may increase up to 100-fold during acute phase reactions. This increased synthesis is stimulated by cytokines such as interleukin-6. Although the protein was discovered more than 70 years ago because of its property to bind to C-polysaccharide of pneumococci, its function is still unknown. Among the functions attributed to CRP are activation of complement upon binding to a ligand, and binding to phospholipids. We have shown that in man CRP also activates complement *in vivo*.[2] Furthermore, we have postulated that CRP in cooperation with another acute phase protein, secretory phospholipase A2 (sPLA2), can bind to injured cells suggesting not only that CRP may contribute to the clearance of dead cells and cell debris in a complement-dependent fashion, but also that under some conditions it may cause irreversible damage to reversibly injured cells by mobilising a complement attack.[3]

CRP and cardiovascular disease: explanations in the literature

There is now ample evidence that arteriosclerosis is at least in part an inflammatory disease.[4] Hence, the associations between CRP and cardiovascular events often have been considered to reflect that CRP, by virtue of its acute phase behaviour, is an indirect parameter for the degree of inflammation ensuing in atherosclerotic lesions or in the infarcted myocardium. Alternatively, CRP levels have been claimed to reflect an infection of the vessels by micro-organisms, for example *C. Pneumoniae*.

CRP and cardiovascular disease: a hypothesis

In vitro and *in vivo* studies have shown that CRP has the ability to activate complement via the classical pathway. Therefore an intriguing possibility is that CRP can bind to jeopardised cardiomyocytes and stimulate complement activation. The triggering event for the cells in binding CRP likely is a flip-flop of the membrane (exchange of phospholipids from the inner and outer leaflets of the cell) due to decreasing intracellular ATP. Flip-flopped cells, in the presence of sPLA2, which by hydrolysing phospholipids renders their phosphorylcholine groups more accessible, then bind CRP, which in turn starts to activate complement. Indeed, immunohistochemical studies show the colocalisation of CRP and activated complement fragments in infarcted, but not in normal myocardium of patients who had died from AMI.[5] Interestingly deposition of CRP was preceded by binding of sPLA2,[6] and coincided with the binding of β_2-glycoprotein I,[7] a protein with affinity for phosphatidylserine. These observations fully support the hypothesis. These results strongly implicate CRP as a main activator of complement in human AMI. Whether this supposed role of CRP contributes to infarction size is not known.
Human CRP administered to rats undergoing myocardial infarction significantly enhances infarct size by activation of complement,[8] suggesting that in human AMI indeed CRP and complement enhance infarction size. Attenuation of CRP-mediated complement activation thus provides an attractive therapeutic option for patients with myocardial infarction. Initial clinical trials seem to support this idea.

Conclusions

CRP levels in plasma constitute a novel and independent cardiovascular risk marker. A possible explanation for this association is that CRP actively participates in the local inflammatory reactions ensuing in the ischaemic myocardium, causing additional injury by activating complement. Future studies should reveal whether CRP-mediated complement activation constitutes a novel target for therapy in cardiovascular disease.

References

1. Lagrand WK, Visser CA, Hermens WT, et al. C-reactive protein as a cardiovascular risk factor: more than an epiphenomenon? Circulation 1999;100:96-102.
2. Wolbink GJ, Brouwer MC, Buysmann S, Berge IJM ten, Hack CE. CRP-mediated activation of complement in vivo: assessment by measuring circulating complement-C-reactive protein complexes. J Immunol 1996;157:473-9.
3. Hack CE, Wolbink GJ, Schalkwijk C, Speijer H, Hermens WT, Bosch H van den. A role for secretory phospholipase A2 and C-reactive protein in the removal of injured cells. Immunol Today 1997;18:111-5.
4. Ross R. Atherosclerosis - an inflammatory disease. N Engl J Med 1999;340:115-26.
5. Lagrand WK, Niessen HW, Wolbink GJ, et al. C-reactive protein colocalizes with complement in human hearts during acute myocardial infarction. Circulation 1997;95:97-103.
6. Nijmeijer R, Lagrand WK, Baidoshvili A, et al. Secretory type II phospholipase A(2) binds to ischemic myocardium during myocardial infarction in humans. Cardiovasc Res 2002;53:138-46.
7. Niessen HW, Lagrand WK, Rensink HJ, Meijer CJ, Aarden L, Hack CE. Apolipoprotein H, a new mediator in the inflammatory changes ensuring in jeopardised human myocardium. J Clin Pathol 2000;53:63-7.
8. Griselli M, Herbert J, Hutchinson WL, et al. C-reactive protein and complement are important mediators of tissue damage in acute myocardial infarction. J Exp Med 1999;190:1733-40.

TREATMENT OF AUTOIMMUNE DISEASES AND VASCULITIDES WITH IMMUNOSUPPRESSIVE DRUGS

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The arsenal of immunosuppressive drugs available to the clinic has increased. Thanks to different mechanisms of action, combinations of drugs working synergistically or additively may be selected.[1,2] Given a different profile of side effects, acute toxicity may be avoided by use of drug combinations where lower dosages of each can be selected. However, long-term hazards such as opportunistic infections and increased incidence of malignancies remain a major concern.
Corticosteroids have both immunosuppressive and anti-inflammatory properties. A main target of the action of corticosteroids is the transcription factor NF-6B. By preventing activation of this transcription factor, corticosteroids inhibit the transcription of several cytokine genes. As a consequence, the function of T lymphocytes, monocytes and macrophages is inhibited. Moreover, corticosteroids exert a profound influence on the traffic of mononuclear cells. Immediately following administration of a single dose, a profound depletion of both T lymphocytes and monocytes from the peripheral blood compartment is observed. Most likely, these cells are temporarily sequestered in lymphoid organs such as bone marrow and spleen. In that way their encounter of antigen in lymph nodes and tissues is prohibited. Calcineurin-inhibitors *cyclosporin* and *tacrolimus* prevent the dephosphorylation of NF-AT_c. Since phosphorylated NFAT cannot enter the nucleus, the initiation of transcription of the most important cytokine IL2 is inhibited, which will lead to inability of T cells to go into proliferation. The antiproliferative drugs *methotrexate, azathioprine* and *mycophenolic acid* interfere with nucleotide synthesis,

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leading to inhibition of DNA and RNA synthesis. Mycophenolic acid is rather selective in its action on lymphocytes because these cells do not have a salvage pathway for purine synthesis. The new drug *rapamycine (sirolimus)* is structurally related to tacrolimus, but has a different mechanism of action. It interferes with the intracellular signalling pathway that is initiated after binding of IL2 to its IL2 receptor. Thereby, it prevents cell progression from G1 to S phase of the cell cycle. This effect is probably related to its suppression of protein synthesis by inhibiting a kinase that increases the protein synthetic activity of the S6 ribosomal protein. The alkylating agent *cyclophosphamide* is a nitrogen mustard derivate that disrupts nucleic acid replication by alkylation and cross-linking of DNA strands. In that way, it interferes with the function of actively replicating T and B lymphocytes, which makes it to an inhibitor of the specific immune response. *Monoclonal antibodies* directed against the CD3 antigen on T lymphocytes inhibit reactivity of these T lymphocytes to antigen because of modulation of the CD3-TCR complex. Whether their ability to induce a profound depletion of CD3 positive T lymphocytes from the peripheral blood compartment is needed for their immunosuppressive action, is not known. IL2R-monoclonal antibodies are directed against the α-chain of the IL2R (CD25) and are effective against activated T lymphocytes only. They induce an effective blockade of IL2 receptors for about 30 days, thereby preventing full activation of T cells after they have encountered their antigen. Monoclonal antibodies and the TNF-receptor IgG1 fusion protein, each directed against TNFα, may prevent the tissue damaging actions of TNFα, which plays a role not only in the inductive phase, but also in the effector and inflammatory phase of the immune response.

In conclusion, a number of non-specific immunosuppressive drugs are available and of great value in medicine. However, because of their non-specific mechanism of action, they may induce several adverse effects. The ultimate goal of clinical immunosuppression, to achieve long-term unresponsiveness to a specific antigen without impairing the response to other antigens, i.e. tolerance, is still far beyond the horizon. Yet, considerable progress has been made in the last few years. Studies on the induction of antigen specific non-reactivity, i.e. tolerance, have focussed on inhibition of the 'second signal', provided by interactions via costimulatory molecules. It became apparent that some of the conventional immunosuppressive drugs may inhibit the induction of tolerance, in which process apoptosis plays an important role.[3] In contrast, anergy and/or immunoregulatory mechanisms appear to be crucial in the maintenance of tolerance.

In parallel with the extension in the arsenal of immunosuppressive drugs, studies have been performed or are in progress testing some of them in the treatment of autoimmune diseases and vasculitides.[4,5] For example, the effect of mycophenolate mofetil on SLE disease activity looks promising.[6] Interesting data have been published from a phase I clinical trial on immunosuppressive efficacy of CTLA4Ig on psoriatic skin disease.[7] Study protocols which are evaluating the optimal drug treatment for vasculitides such as Wegener's disease will be discussed.

References

1. Berge RJM ten, Surachno S, Wilmink JM, Schellekens PThA. Immunosuppressive drugs. In: Bovill JG, Howie MB, editors. Clinical Pharmacology for Anaesthesists. London: WB Saunders, 1999. p. 343-50.
2. Denton MD, Magee CC, Sayegh MH. Immunosuppressive strategies in transplantation. Lancet 1999;353:1083-91.
3. Li Y, Li XC, Zheng XX, Wells AD, Turka LA, Strom TB. Blocking both signal 1 and signal 2 of T-cell activation prevents apoptosis of alloreactive T cells and induction of peripheral allograft tolerance. Nature Medicine 1999;5:1298-302.
4. Langford CA, Klippel JH, Balow JE, James SP, Sneller MC. Use of cytotoxic agents and cyclosporin in the treatment of autoimmune disease. Part 1: rheumatologic and renal diseases. Ann Intern Med 1998;128:1021-8.
5. Langford CA, Klippel JH, Balow JE, James SP, Sneller MC. Use of cytotoxic agents and cyclosporin in the treatment of autoimmune disease. Part 2: inflammatory bowel disease, systemic vasculitis and therapeutic toxicity. Ann Intern Med 1998;129:49-58.
6. Chan TM, Li FK, Tang CS, et al. Efficacy of mycophenolate mofetil in patients with diffuse proliferative lupus nephritis. Hong Kong-Guangzhou Nephrology Study Group. N Engl J Med 2000;343:1156-62.
7. Sayegh MH. Finally, CTLA4Ig graduates to the clinic. J Clin Invest 1999;103:1223-5.

GENE THERAPY OF SEVERE COMBINED IMMUNODEFICIENCIES: FROM MICE TO HUMANS

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Primary immunodeficiencies (PID) include approximately 80 diseases that disturb development and function of the immune system. The most severe forms of PID are severe combined immunodeficiencies (SCID), which are characterised by a profound block in T-cell differentiation and thus, by absence of mature T cells. Patients with SCID suffer from recurrent infections, failure to thrive, and die during the first year of life. Haematopoietic stem cell transplantation (HSCT) is the treatment of choice for SCID, which is curative for those patients who have an HLA-identical donor. In the absence of this ideal donor haploidentical T-depleted HSCT has been performed but the

survival rate is around 60% due to the clinical consequences of acute graft-versus-host disease (GVHD) as well as the delay to immune function development.

Autologous transplantation of genetically modified bone marrow cells therefore appeared a reasonable therapeutic alternative to allogeneic HSCT.

Correction of an inherited disorder by gene therapy involves understanding the pathogenesis of the disease. In recent years, numerous disease-causing mutations have been identified, knockout mouse models have been produced. These models, in which pathological features mimic human SCID diseases, offered the opportunity to gain new insights into the pathogenesis of the disease and to evaluate new therapeutic approaches. One of the most frequent form of SCID in man, the X-linked SCID or SCID-X1 results from defects in the common γ chain (γ c) which participated in the receptors for IL-2, IL-4, IL-7, IL-9, IL-15, and likely IL-21. The molecular basis of this SCID-X1 highlights the role played by some cytokines in promoting survival and/or proliferation of early thymocytes. SCID-X1 is characterised by the complete absence of mature T and natural killer cells, whereas B cells are present in normal or increased numbers. The blockade in T cell development occurs very early and patient's thymuses are almost completely devoid of T cells. The γ c mutations result in either the absence of the γ c sub-unit or the expression of truncated protein that cannot associate with the Jak-3 tyrosine kinase and thus do not transduce growth signals. Mutations in Jak-3 cause a phenotype indistinguishable from patients with SCID-X1. The defect in γ c and Jak-3 appears more severe in humans than in mice. γ c-deficient mice are characterised by the development and accumulation of mature, dysfunctional T cells, while the B-cell development is markedly diminished. These mice lack NK cells and gut-associated intraepithelial lymphocytes (IELs). Despite the difference between humans and mice, this model is valuable to study the feasibility of *in vivo* retroviral gene transfer to correct the defects in lymphoid development and T-cell function. Murine- γ c or human- γ c gene transfer into γ c(-) bone marrow cells, led after transplantation to substantial lymphoid reconstitution of T, NK, and B cells. γ c-transduced lymphocytes are functional as demonstrated by their responsiveness to γ c-dependent cytokines and by their capacity to generate a cooperative (B-T cell) immune response following antigen immunisation.[1-3] Bunting and collaborators observed similar results in their mouse model of Jak-3 deficiency.[4] Transplanted mice displayed significant correction of both cellular and humoral immunity and also circulating immunoglobulin (Ig) levels.[5] In addition, it was found that transgene expression was much more frequent in lymphoid than in myeloid cells, thus providing *in vivo* evidence for a selective advantage conferred to transduced lymphoid cells. This naturally occurring selective advantage for Jak-3 expressing lymphoid progenitors has been confirmed by this group since they observed nearly the same immune reconstitution using lethally irradiated mice or unablated hosts.[5]

The selective advantage conferred to transduced progenitor cells accounts for clinical benefit obtained in four SCID-X1 with γ c deficiency following γ c gene transfer into CD34+ cells using a MFG vector. Evidence for complete correction of the immune system has been provided, T-lymphocyte counts reached normal values for age between three months and five months post-therapy. These T cells were shown to express the γ c transgene, to display a polyclonal repertoire, and to proliferate to mitogens and antigens after immunisation. Even, the fraction of transduced B cells is very low (1%), protective titres of specific antibodies to polioviruses, tetanus and diphtheria toxoids were detected in patients' sera. In all patients, γ c+ NK cells have also developed. These children are doing well, free of infections without any therapy with follow-up ranged between two years and one year.[6] The significant correction of γ c-deficient SCID phenotype suggests that human SCID conditions represent appropriate diseases for treatment by *ex vivo* gene transfer. SCID characterised by a deficiency in the early steps of V(D)J recombination and caused by mutations in either the Rag-1 or Rag-2 encoding genes (without increased radio-sensitivity) are the next candidates. Affected children, lack circulating T- and B-lymphocytes. A selective advantage of correct precursor cells on endogenous Rag-/- cells is also expected (although it might be less strong, as Rag-1 and Rag-2 are expressed at a later stage during T- and B-lymphocyte development). Another advantage is that constitutive expression of one of the two Rag proteins should not be harmful since concomitant expression of both genes is required for the recombination activity.

As a logical step towards gene therapy clinical trial, a preclinical model attempted to correct the immunological defect in Rag-1 and Rag-2 deficient mice models. Modified retroviral vectors (MND) encoding the Rag-1 or Rag-2 cDNA transgenes were built. Sca-1$^+$ selected bone marrow cells were transduced and transplanted into sublethaly irradiated Rag-2-/- mice. Treated mice have been analysed two to six months after transplantation, both peripheral T- and B-cell numbers as well as seric IgM and IgG were significantly increased. Long-term follow-up and secondary transplants will be required to determine the duration of the correction, TCR/BCR diversity and its corollary: the capacity of these mice to generate a full response to a live pathogen as well as the toxicity of transgene expression. Immune reconstitution after gene transfer of Rag-1 deficient mice is currently being studied with the double aim of proving the feasibility and the lack of toxicity of the ectopic expression of this catalytically active protein.

In conclusion, beside the efficacy criteria, animal models can reply to safety issues regarding potential toxicity of

gene therapy protocols, particularly in those diseases where gene expression should be tightly regulated. This is the case for the X-linked hyper-IgM syndrome, characterised by failure of immunoglobulin isotype switching and severe defects of cell-mediated immunity. This disease results from a deficiency in CD40 ligand. Brenners'group showed that constitutive low-level expression of CD40L in CD40L-/- treated mice induce T-lympho proliferative disorders.[7] The analysis of the *in vivo* consequences of constitutive gene expression may allow to circumscribe applications of gene therapy for some diseases in which effective regulation of the transgene is not required.

References

1. Soudais C, Tusjino S, Sharara L, et al. Stable and functional lymphoid reconstitution corni-non cytokine receptor gamma chain deficient mice by retroviral-mediated gene transfer. Blood 2000;95:3071-7.
2. Otsu M, Sugamura K, Candotti F, et al. Stem cell gene therapy for X-linked severe combined immunodeficiency mice expressing a truncated common gamma chain. Cold String Harborg Meeting on gene therapy abstract. 2000 Sep 25-29.
3. Lo M, Bloom ML, Imada K, et al. Restoration of lymphoid populations in a murine model of X-linked severe combined immunodeficiency by a gene-therapy approach. Blood 1999;94:3027-36.
4. Bunting KD, Sangster MY, Jhle JN, et al. Restoration of lymphocyte function in Janus Kinase 3 – deficient mice by retroviral – mediated gene transfer. Nature Medicine 1998;4:58-64.
5. Bunting KD, Lu T, Kelly PF, Sorrentino PB. Self-selection by genetically modified committed lymphocyte precursors reverses the phenotype of JAK3-deficient mice without myeloablation. Hum Gene Therapy 2000;11:2353-4.
6. Cavazzana-Calvo M, Hacein-Bey S, Saint Basile G de, et al. Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. Science 2000;288:669-72.
7. Brown M, Topham DJ, Sangster MY, Zhao J, et al. Thymic lymphoproliferative disease after successful correction of CD40 ligand deficiency by gene transfer in mice. Nature Medicine 1998;4:1253-60.

AUTOIMMUNE HEPATITIS: CLINICAL AND IMMUNOLOGICAL FEATURES

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Introduction

Autoimmune hepatitis (AIH) is a chronic necro-inflammatory disease of the liver that, if left untreated, leads to cirrhosis, liver failure, and death. Landmark studies conducted in the late 60s and early 70s have unequivocably shown that prompt treatment with corticosteroids (with or without azathioprine) effectively induces remission of the disease and almost normalises life expectancy. Paradoxically, the effectiveness of this treatment has been a barrier for the development of new regimens, and the progress achieved in immunosuppressive treatment of other conditions, e.g. solid organ transplants, has hardly changed the treatment of AIH. Here, we will briefly review topics that are of interest from clinical and immunological points of view.

Diagnostic problems

There is no single diagnostic marker that reliably differentiates AIH from other immune-mediated, viral, metabolic or drug-induced liver diseases. Thus, clinicians have traditionally arrived at a diagnosis by looking for clues suggestive of the disease (epidemiology, biochemistry, immunoserology, histology), while trying to exclude alternative causes. This intuitive approach has been formalised by the International Autoimmune Study Group, resulting in a scoring system that recently has been updated,[1] and which is very helpful in clinical practice. Unfortunately, liver diseases have a tendency not to respect classic textbook boundaries. Overlap syndromes combining features of AIH and PBC (primary biliary cirrhosis) or PSC (primary sclerosing cholangitis) occur frequently.[2] In such cases, the various components all have to be recognised and properly treated. Autoantibodies, e.g. directed against F-actin (smooth muscle antibodies, SMA) or antineutrophil cytoplasmatic antibodies (ANCA) are frequently present in chronic hepatitis C infection, probably as an epiphenomenon. They should not lead to institution of immunosuppressive treatment, however, as this will promote viral replication and further increase liver damage.

Therapeutic problems

Corticosteroids with or without azathioprine induce remission of clinical and biochemical abnormalities in over 85% of patients. Treatment needs to be continued for at least two years, and toxicity may be troublesome. Steroid-related side effects can be limited by careful dose titration using transaminases and total IgG as indicators of disease activity. Alternatively, increasing the dose of azathioprine to 2 mg/kg may allow complete discontinuation of steroids.
Firm guidelines are lacking on how to treat patients who do not, or only partially respond. The side effects of prolonged treatment with high doses of steroids may appear worse than the disease itself. It has been advised to aim for doses that reduce but not eliminate inflammation and that have manageable toxicity. Uncontrolled studies have suggested that mycophenolate mofetil[3] and cyclosporin A[4] may be effective in such cases. These drugs may also be helpful in patients experiencing troublesome side effects. It may be difficult in patients presenting with impending liver failure to decide whether immunosuppressive treatment still is worthwhile, or whether the patient should be offered a

transplant right away. Survival rates of patients transplanted because of AIH are generally excellent, but patients who fail on conservative treatment are extremely susceptible to opportunistic infections, and may have to be transplanted in a far worse condition than on initial presentation (if still transplantable at all). No firm guidelines can be given here, but early referral to a transplant centre is recommended.

New therapeutic options

Budesonide is a potent corticosteroid that has an extremely high first-pass effect in the liver after oral administration. Theoretically, this should provide effective anti-inflammatory and immunosuppressive activity in the liver, with minimal extrahepatic side effects. An uncontrolled study[5] showed evidence supporting this hypothesis, and a randomised trial comparing budesonide *versus* prednisolone, both combined with azathioprine, is presently being conducted. This study may not only improve patient care by ameliorating side effects, but – more interestingly – will also tell us whether 'local immunosuppression' as a concept is valid in AIH.

AIH after liver transplantation

A minority of patients with AIH develops liver failure and needs to be transplanted. It has become clear in recent years that AIH may recur after liver transplantation, and that it may even lead to transplant failure.[6] This may occur despite the fact that no HLA matching is performed in liver transplantation, generally resulting in an (almost) complete HLA mismatch. It is the impression that the recurrence of AIH is related to the type of immunosuppression, since recurrences are observed predominantly in centres striving to maintain patients on tacrolimus or cyclosporine monotherapy, but not in those keeping their patients on steroids. We have not seen a single case of recurrent AIH in over 30 patients maintained on prednisolone/azathioprine therapy.

Immunopathogenesis

AIH may be considered to be initiated by a specific event, such as an infection, occurring in a genetically predisposed individual. In white northern Europeans the disease is related to HLA alleles encoding the six amino acid sequence LLEQKR at positions 67-72 of the DRB1 polypeptide, suggesting a role in optimal T-cell recognition of autoantigen(s). Moreover, there is a distict relation with CTLA-4 gene polymorphisms.[7] There is observational evidence that AIH may be triggered by viral infections such as hepatitis A or EBV. This suggests that a failure to switch off immune responses lies at the heart of this disease.
Autoantibodies directed against a variety of self-proteins (such as histone, F-actin, et cetera) can be demonstrated in most AIH patients, but their role in the immunopathogenesis of the disease (target, innocent bystander or epiphenomenon) remains uncertain.
These observations indicate that AIH provides immunologists with an excellent opportunity to study the pathogenesis of autoimmune diseases. This might hopefully lead to radically new treatment approaches, no longer based on prolonged, non-specific immunosuppression, but directed towards restoring the normal immunological homeostasis and tolerance of 'self'.

References

1. Alvarez F, Berg PA, Bianchi FB, et al. International Autoimmune Hepatitis Group Report: review of criteria for diagnosis of autoimmune hepatitis. J Hepatol 1999;31:929-38.
2. Czaja AJ. Frequency and nature of the variant syndromes of autoimmune liver disease. Hepatology 1998;28:360-5.
3. Richardson PD, James PD, Ryder SD. Mycophenolate mofetil for maintenance of remission in autoimmune hepatitis in patients resistant to or intolerant of azathioprine. J Hepatol 2000;33:371-5.
4. Malekzadeh R, Nasseri-Moghaddam S, Kaviani MJ, Taheri H, Kamalian N, Sotoudeh M. Cyclosporin A is a promising alternative to corticosteroids in autoimmune hepatitis. Dig Dis Sci 2001;46:1321-7.
5. Danielsson A, Prytz H. Oral budesonide for treatment of autoimmune chronic active hepatitis. Aliment Pharmacol Ther 1994;8:585-90.
6. Hubscher SG. Recurrent autoimmune hepatitis after liver transplantation: diagnostic criteria, risk factors, and outcome. Liver Transpl 2001;7:285-91.
7. Czaja AJ, Donaldson PT. Genetic susceptibilities for immune expression and liver cell injury in autoimmune hepatitis. Immunol Rev 2000;174:250-9.

INTESTINAL TRANSPLANTATION

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The results of the first human intestinal transplants in the early 60s where overshadowed by early rejection and sepsis resulting in a high mortality rate. At the same time total parenteral nutrition became an acceptable option with a good quality of life for those suffering from intestinal failure. An important turning point for intestinal transplantation in humans was the introduction of FK 506/ tacroliminus as an immunosuppressive agent. In the pretacrolimus period, the three-year patient survival rate ranged from 0 to 28% with graft survival rates of 0 to 11%. Long-term survival now exceeds 50% in large series.[1,2] Also combined small bowel/liver and multivisceral transplantations have been successful. The Intestinal Transplant Registry reported 55 active intestinal transplant centres between April 1985 and May 2001. In total 651 patients have been transplanted and there are currently 335 survivors. Of note is that 83% of these survivors have a modified

Karnofsky index between 90 and 100 points, indicating a good quality of life.[3]

Intestinal failure itself is not an indication for small bowel transplantation, but should only be considered as a life-saving option for patients with severe complications from (home) parenteral nutrition (HPN) or if HPN is not an option. Therefore only a small number of patients are candidate for an isolated or combined small bowel/liver transplantation. Identifying the right candidates in cooperation with referring HPN centres is a prerequisite for a small bowel transplant programme.

A characteristic problem encountered in intestinal transplantation is that rejection immediately results in loss of integrity of the barrier between the outer world and the human body, offering a condition for massive bacterial translocation and sepsis.
Histological diagnosis of intestinal allograft rejection is therefore crucial in monitoring postoperative patients. Increase in apototic bodies in the crypts can be an early signal of rejection without any clinical symptoms. Balancing between immunosuppressive therapy, which should protect the integrity of the bowel mucosa, and preventing CMV and EBV proliferation and/or opportunistic infections is the clinical challenge in small bowel transplantation programmes.

Intestinal transplantation has evolved from an experimental therapy to a standard therapeutic option for selected patients with intestinal failure. Identifying good candidates for intestinal transplantation, development of novel immunosuppressive strategies and improving postoperative monitoring techniques will be the base for improving clinical outcome of intestinal transplantation in the near future.

References

1. Asfar S, Atkinson P, Ghent C, et al. Small bowel transplantation: a life saving option for selected patients with intestinal failure. Dig Dis Sci 1996;41:875-83.
2. Gerber DA. Intestinal transplantation. Available at http://www.medscape.com.
3. The intestinal transplant registry. Current results. 6th International Small Bowel Transplant Symposium. University of Nebraska Medical Centre, 1999 Oct 6-8. Available at: http://www.lhsc.on.ca/itr/.

CELIAC DISEASE: THE ROLE OF (AUTO)ANTIBODY DETECTION IN DIAGNOSIS AND FOLLOW-UP

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Celiac disease is characterised by hypersensitivity to cereal grain storage proteins (gluten) of the taxonomic closely related species wheat, barley, and rye (Triticeae), but not oats, rice or maize. The prevalence of clinically diagnosed celiac disease is estimated to be 1:1000, however, screening trials suggest a prevalence of up to 1:100.[1] The gluten-sensitive enteropathy results in weight loss, diarrhoea, symptoms due to nutritional deficiencies, such as anaemia and fatigue, and growth failure. These symptoms are the result of the mucosal lesions that develop in a sequence of progression.[2] The initial event in the mucosal lesions is a lymphocytic infiltration of the lamina propria (stage 1) and is followed by crypt hyperplasia (stage 2) and villous atrophy (stage 3).[3] Celiac disease may also present as extra-intestinal manifestation, such as dermatitis herpetiformis, or remain clinically silent. Because of the wide spectrum of symptoms the European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) has defined diagnostic criteria of celiac disease.[4] First, the diagnosis is based on the appearance of flat small intestinal mucosa with the histological features of hyperplastic villous atrophy while the patient is still eating adequate amounts of gluten. Second, there should be unequivocal and full clinical remission after withdrawal of gluten from the diet. The clinical response to a gluten challenge is not essential for diagnosis.

Active celiac disease is accompanied by the presence of circulating (auto)antibodies.[5] Since celiac disease is a mucosa-associated disease, these antibodies are predominately of the IgA isotype. The discovery of the presence of agglutinating antibodies against wheat gluten eventually resulted in the development of sensitive methods for measuring IgA antibodies to gliadin (AGA), the ethanol-soluble fraction of gluten, as a screening tool for celiac disease (sensitivity 30 to 100%; specificity 81 to 100%). Methods used for AGA determination include enzyme-linked immunosorbent assays (ELISA) and fluorescent-enzyme immunoassays (FEIA). Besides AGA, several types of autoantibodies to extracellular matrix constituents have been associated with celiac disease. The first-described autoantibodies are the antireticulin autoantibodies (ARA) as detected by indirect immunofluorescence (IFT) on rat tissues including liver, kidney, and stomach. The ARA includes five subtypes, but only the IgA R1 subtype is

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associated with celiac disease (sensitivity 53 to 92%; specificity nearly 100%). The most distinctive feature of R1 type ARA is the staining of the peritubular and periglomerular connective tissue fibres in rat kidney sections. Next, IgA antibodies to the lining of smooth muscle bundles, i.e. endomysium, of the distal part of monkey oesophagus were described (sensitivity nearly 100%; specificity nearly 100%). These antiendomysium antibodies (EMA) also react with similar antigenic structures in human umbilical cord vein. Furthermore, adsorption studies have demonstrated that there is no difference between EMA and ARA of type 1. The endomysial antigen has been identified as tissue transglutaminase (tTG), and recently recombinant human (rh)-tTG has become available for testing of IgA anti-rh-tTG antibodies (rh-tTGA) in solid-phase assays (sensitivity 95 to 100%; specificity 90 to 97%).[6,7]

Although the presence of circulating (auto)antibodies is not essential for diagnosis according to the ESPGHAN criteria, the high sensitivity and specificity of these antibodies for celiac disease enables the selection of suspected patients for the requirement of an intestinal biopsy for verification of the diagnosis. The high sensitivity, however, has been disputed in a couple of situations. First, in clinical practice autoantibodies are primarily tested for the IgA isotype and given the relatively high incidence of IgA deficiency (~10%) in patients with celiac disease, it is surprising that several studies claim sensitivities of nearly 100% when only testing for the IgA isotype.[5] To enable serological screening for possible celiac disease in patients with IgA deficiency, additional testing for the IgG isotype is recommended. However, due to the reduced specificity of IgG isotype (auto)antibodies, interpretation of positive test-results should be done in relation to defined IgA deficiency. In a small cohort of untreated patients with biopsy confirmed celiac disease, we recently confirmed that indeed ~10% was IgA deficient, and that all these IgA deficient patients were positive when tested for AGA, EMA, and/or rh-tTGA of the IgG isotype.[8] Second, tests for (auto)antibodies may be negative in celiac patients less than two years of age, as well as in patients with only partial villous atrophy.[9]

It is well established that AGA, ARA, and EMA disappear when a gluten-free diet is installed. Furthermore assays for these (auto)antibodies are helpful in therapy control of celiac disease. Detection of AGA in these situations is superior to ARA and EMA since AGA are measured in a quantitative assay and may respond within a period of three to six months. Whether detection of rh-tTGA, combining the highest sensitivity in a quantitative assay, performs even better than AGA, remains to be determined. Another application of the serological tests for celiac disease is epidemiological screening of the general population in order to detect patients with silent disease. The rationale for this approach is the relatively high incidence of small bowel lymphoma as well as decreased bone mineral density in patients with untreated celiac disease.[1,2] Besides population-wide serological screening for silent celiac disease, it was recently suggested that a two-step strategy should be performed for screening, based on selection of the individuals with potential celiac disease by HLA-DQ typing and on longitudinal serological screening in this selected group.[10] This approach is based on the strong linkage of celiac disease with HLA-DQ2 (90 to 99%), whereas the remaining patients express HLA-DQ8.[2] However, since also 20 to 30% of the general population expresses HLA-DQ2, the advantage of this preselection may not be cost effective.

Altogether, serological assays for celiac disease are useful for three reasons. First, these assays are valuable in screening suspected patients for the necessity of an intestinal biopsy; second, they are helpful in therapy control; and third, population-wide screening may reveal silent celiac disease and thereby enable the prevention of associated side effects like small bowel lymphoma. Until recently qualitative detection of EMA was the golden standard, but this assay may be replaced by the quantitative detection of rh-tTGA in the near future.

References

1. Maki M, Collin P. Coeliac disease. Lancet 1997;349:1755-9.
2. Schuppan D. Current concepts of celiac disease pathogenesis. Gastroenterology 2000;119:234-42.
3. Marsh MN. Gluten, major histocompatibility complex, and the small intestine. A molecular and immunobiologic approach to the spectrum of gluten sensitivity ('celiac sprue'). Gastroenterology 1992;102:330-54.
4. Walker-Smith JA, Guandalini S, Schmitz J, et al. Revised criteria for diagnosis of coeliac disease. Report of Working Group of European Society of Paediatric Gastroenterology and Nutrition. Arch Dis Child 1990;65:909-11.
5. Dieterich W, Storch WB, Schuppan D. Serum antibodies in celiac disease. Clin Lab 2000;46:361-4.
6. Dieterich W, Ehnis T, Bauer M, et al. Identification of tissue transglutaminase as the autoantigen of celiac disease see comments. Nat Med 1997;3:797-801.
7. Sblattero D, Berti I, Trevisiol C, et al. Human recombinant tissue transglutaminase ELISA: an innovative diagnostic assay for celiac disease. Am J Gastroenterol 2000;95:1253-7.
8. Damoiseaux JGMC, Linders A, Austen J, et al. Antibodies to endomysium (EMA), tissue transglutaminase (tTGA), neutrophil cytoplasmic antigens (ANCA), and Saccharomyces cerevisiae (ASCA) in patients with celiac disease abstract. Clin Immunol 2001;99:146.
9. Rostami K, Kerckhaert J, Tiemessen R, et al. Sensitivity of antiendomysium and antigliadin antibodies in untreated celiac disease: disappointing in clinical practice. Am J Gastroenterol 1999;94:888-94.
10. Csizmadia CG, Mearin ML, Oren A, et al. Accuracy and cost-effectiveness of a new strategy to screen for celiac disease in children with Down syndrome. J Pediatr 2000;137:756-61.

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IMMUNOTHERAPY OF CELIAC DISEASE: FACT OR FALLACY?

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Introduction

Celiac disease is a disease of the small intestine. Approximately one out of 200 individuals suffer from the disease, corresponding to roughly one million patients in Western Europe alone. Since the 50s it is known that the gluten proteins in wheat and related grains cause celiac disease. Recent studies have revealed a complex interaction between gluten, the enzyme tissue transglutaminase and components of the immune system. These observations provide an explanation for the disease inducing capacity of gluten.

Background

Celiac disease (CD) is a permanent intolerance to gluten. Typical disease symptoms include chronic diarrhoea, fatigue, and failure to thrive. These are the result of a lesion in the small intestine. The only known cure is a lifelong gluten free diet. Gluten is a complex mixture of storage proteins found in wheat. The major components are the gliadins and glutenins of which multiple variants are found in any wheat variety.

CD is almost exclusively found in individuals with a particular genetic background: they express the HLA-DQ2 and/or -DQ8 molecules. The function of HLA molecules is to bind protein fragments, peptides, and to 'present' these to cells of the immune system. The immune system should only respond when these peptides are derived from a pathogen. In the case of celiac disease the immune system makes a mistake and responds to gluten peptides. Inflammation and tissue damage is the result.

Which gluten peptides initiate this response?

The sequences of five gluten peptides that stimulate the immune system have now been published[1-6] and seven more are known Koning, *et al*. unpublished. These peptides are derived from α-gliadins, γ-gliadins and glutenins. Moreover, some of these peptides represent repetitive sequences and homologue sequences are also present in gluten. One of the glutenin sequences, for example, has 32 homologues[2] and the majority of these can stimulate the immune system.[2] Thus, multiple gluten peptides that can stimulate the immune system are present in a variety of gluten proteins.

Why do HLA-DQ2 and/or HLA-DQ8 molecules predispose to disease?

HLA molecules bind peptides but they do not bind just any peptide. Such peptides have to fulfil particular requirements. In the case of HLA-DQ2 and HLA-DQ8, the molecules found in CD patients, the peptides must contain one or two amino acids with a negative charge.[7] When this was first observed it did not seem to make any sense because gluten molecules contain very few negative charges. This puzzle was solved when it was found that gluten could be modified by an enzyme, tissue tranglutaminase (tTG).[3,4] Due to the modification, negative charges are introduced in gluten peptides and this facilitates the binding to HLA-DQ2 or -DQ8 molecules.[3,4,7] While some unmodified gluten peptides can stimulate the immune system, the majority of gluten peptides require the tTG modification for optimal stimulation of the immune system.[1-6] In conclusion, the expression of HLA-DQ2 and/or- DQ8 molecules allows disease development because tTG generates gluten peptides that bind with high affinity to these HLA molecules. Individuals that do not express HLA-DQ2 and/or -DQ8, therefore, will not develop disease.

Unresolved issues

There are two major unresolved issues. First, the majority of individuals that express HLA-DQ2 and/or -DQ8 *do not* develop CD: while 25% of the population is HLA-DQ2 positive, only 0.5% develops CD. We do not know why that is. Second, and related to the first issue: we do not know what triggers the disease development. While in some individuals CD develops after the first contact with gluten, in others it develops much later in life. Also, the disease heavily affects some patients while others have only mild or even absent clinical symptoms. Two possibilities warrant further investigation: the role of intestinal infections that could lead to uncontrolled immune responses in the intestine, including those to gluten, and the role of additional genetic factors that predispose to disease development. These are topics for future investigations.

How can we use this knowledge?

There are several options:

- Since we now know which peptides in gluten appear responsible for disease development we can develop a test to screen food products for the presence of such peptides. This would certainly be an improvement on the current tests that either screen for the presence of a single gliadin type or nitrogen content, and thus only give a rough estimate of gluten content.
- We could use such a test system to screen for wheat varieties that lack one or more of the toxic gluten peptides. Such varieties could form the basis for a dedicated breeding programme to develop wheat varieties that contain gluten molecules with considerably less toxicity for CD patients.[8]

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- Given the importance of gluten modification by tTG a seemingly logical step would be to block the enzyme activity in the intestine of CD patients in order to prevent the immune response to gluten. Unfortunately, tTG has important biological functions other than gluten modification, in particular the cross-linking of extracellular matrix proteins upon tissue damage as part of the wound-healing process. Prolonged blocking of tTG activity may thus have adverse side effects.[9]
- Finally, the dream of an immunologist: manipulate the immune system so that it will no longer respond to gluten. This goal is more realistic now we know which gluten peptides stimulate the immune system but complicated by the fact that so many peptides are involved.[10]

Conclusions

In the last four years we have gained detailed insight in the events that play a key role in the development of celiac disease. Peptides derived from gluten bind to the HLA-DQ2 and/or HLA-DQ8. Subsequently these HLA-DQ-peptide complexes stimulate the immune system. The interaction between gluten peptides and HLA-DQ-molecules is facilitated by the activity of the enzyme tTG. Multiple gluten peptides derived both from the gliadin and glutenin molecules can stimulate the immune system. This knowledge opens novel approaches for the development of safer food products and novel therapies for CD patients.

Acknowledgements

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References

1. Wal Y van de, Kooy YM, Veelen PA van, et al. Small intestinal cells of celiac disease patients recognise a natural pepsin fragment of gliadin. Proc Natl Acad Sci USA 1998;95:10050-4.
2. Wal Y van de, Kooy YM, Veelen PA van, et al. Glutenin is involved in the gluten-driven mucosal T cell response. Eur J Immunol 1999;29:3133-9.
3. Wal Y van de, Kooy YM, Veelen PA van, et al. Selective deamidation by tissue transglutaminase strongly enhances giadin-specific T cell reactivity. J Immunol 1998;161:1585-8.
4. Molberg O, McAdam SN, Korner R, et al. Tissue transglutaminase selectively modifies gliadin peptides that are recognised by gut-derived T cells in celiac disease. Nature Medicine 1998;4:713-7.
5. Arentz-Hansen H, Korner R, Molberg O, et al. The intestinal T cell response to α-gliadin in adult celiac disease is focused on a single deamidated glutamine targeted by tissue transglutaminase. J Exp Med 2000;191:603-12.
6. Anderson RP, Degano P, Godkin AJ, Jewell DP, Hill AV. In vivo antigen challenge in celiac disease identifies a single transglutaminase-modified peptide as the dominant A-gliadin T-cell epitope. Nature Medicine 2000;6:337-42.
7. Wal Y van de, Kooy YMC, Drijfhout JW, Amons R, Koning F. Peptide-binding characteristics of the Coeliac disease associated DQ (a1*0501, β1*0201) molecule. Immunogenetics 1996;44:246-53.
8. Asfar S, Atkinson P, Ghent C, et al. Small bowel transplantation: a life saving option for selected patients with intestinal failure. Dig Dis Sci 1996;41:875-83.
9. Gerber DA. Intestinal transplantation. Available at http://www.medscape.com.
10. The intestinal transplant registry. Current results. 6th International Small Bowel Transplant Symposium. University of Nebraska Medical Centre, 1999 Oct 6-8. Available at: http://www.lhsc.on.ca/itr/.

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[2.] Kaplan NM. Clinical Hypertension. 7th Edition. Baltimore: Williams & Wilkins; 1998.

[3.] Powell LW, Isselbacher KJ. Hemochromatosis. In: Harrison's Principles of Internal Medicine, 15th Edition, Braunwald E, Fauci AS, Kasper DL, et al. (eds). New York: McGraw-Hill; 2001. p. 2257-61.

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