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JANUARY 2006, VOL. 64, NO. 1
The formation of advanced glycation endproducts (AGEs) has been recognised as an important pathophysiological mechanism in the development of vascular complications in diabetic patients. Nonenzymatic glycation involves the condensation reaction of the carbonyl group of sugar aldehydes with free amino groups of proteins, resulting in the rapid formation of a Schiff base. Subsequently, this labile adduct undergoes rearrangements to a more stable Amadori product. Only a small number of these intermediate Amadori products are oxidised and can give rise to irreversibly formed AGEs, such as the cross-link pentosidine and N\(\varepsilon\)-(carboxymethyl)lysine (CML). Because of the slow formation, it was long believed that AGEs accumulate only on long-lived extracellular proteins. However, a rapid intracellular and extracellular AGE formation on short-lived proteins has attracted attention. Of importance for the intracellular Maillard reaction are glycolytic intermediates such as the dicarbonyl compounds glyoxal, methylglyoxal and 3-deoxyglucosone. In the context of intracellular glycation, it is important to emphasise that of all sugars, glucose has the slowest rate in the glycation reaction.

Several mechanisms have been proposed by which AGEs lead to diabetic complications: 1. the accumulation of AGEs in the extracellular matrix causing aberrant crosslinking, resulting in a decrease in the elasticity of vessels, 2. the binding of circulating AGEs to the receptor of AGEs (RAGE) on different cell types and activation of key cell signalling pathways such as NF-\(\kappa\)B activation with subsequent modulation of gene expression and 3. intracellular AGE formation leading to quenching of nitric oxide and impaired function of growth factors.

Because of the many deleterious effects of AGEs on vascular structure and function, prevention or reversal of AGE accumulation is an attractive therapeutic target. The data presented by Mentink et al. in this issue suggest that six months of optimised metabolic control by insulin therapy is not accompanied by a decrease in circulating AGEs. The fact that optimised metabolic control decreased markers of endothelial function but did not affect circulating AGEs suggests either the involvement of other circulating AGEs than the ones detected in this study or that other AGE-induced mechanisms such as crosslinking and intracellular glycation may be responsible for endothelial dysfunction. On the other hand, other hyperglycaemia-induced biochemical pathways, such as the sorbitol pathway or protein kinase C activation, may be involved in endothelial dysfunction.

These results encourage us to make some comments on the detection of different AGEs. Mentink et al. have measured different AGEs with immunoassays and high performance liquid chromatography (HPLC). Immunoassays are often used for the quantification of AGEs, but for several reasons the use of antisera for quantitative immunoassays of protein-bound AGEs is questionable. One reason is that the specificity of the antibodies is often difficult to define with certainty and thus far no monospecific antibodies are commercially available. Another reason is that proteins used to block nonspecific binding in immunoassays may also contain AGE epitopes and thus interact with the antibody. In addition, because of steric constraints, not all AGE epitopes on the protein may be available for interaction with the antibody. Finally, there is evidence for the presence in plasma of factors competing for the reaction between the anti-AGE antibody and its antigen. These factors include anti-AGE autoantibodies and, possibly, complement. As a consequence, AGE immunoassays may only yield semiquantitative results and these should be interpreted with care. The possibility that the results of the study by Mentink et al. are due to imperfections of the immunoassays can not be excluded. A better approach for the quantitative determination of specific AGE epitopes in proteins is to use a specific analytical technique for the analysis of these AGEs in protein hydrolysates. A major restriction of this approach is that not all AGE epitopes are stable during the harsh
conditions used for protein hydrolysis. Fortunately, CML is stable under the conditions used for acid protein hydrolysis and can be measured accurately by analytical techniques as performed by Mentink et al. Application of this kind of analytical technique in the laboratory could lead to a more comprehensive understanding of the role of nonenzymatic chemistry in disease.

The fact that Mentink et al. found that CML, which is a major AGE and a ligand for RAGE, did not decrease by improvement of metabolic control suggests that not hyperglycaemia, but accumulation of reactive dicarbonyl intermediates and/or increased oxidative stress and lipid peroxidation play a more important role in the generation of this compound. This is corroborated by recent findings showing that oxidants generated by NADPH oxidase play a crucial role in CML formation. In addition to the formation, renal dysfunction is the most important determinant in CML levels in diabetic patients.

Despite these considerations, we can conclude that additional intervention therapies may be used to reduce AGE accumulation or to reduce AGE-induced effects, also in view of the difficulties in achieving optimal metabolic control in subsets of patients with diabetes. A short overview of the current status of these therapies is presented in Figure 1. For further detailed information, we refer you to an excellent review by Monnier.

**Inhibition of AGE formation**

The first approach is to reduce the formation of AGEs by intervention at one of the many steps involved in the formation of AGEs, such as by aminoguanidine. Aminoguanidine was the first compound designed to inhibit AGE formation and has undergone clinical trials. Despite promising early results, aminoguanidine is unlikely to be used for therapeutic purposes due to safety concerns and lack of efficacy. Metformin, which is routinely used in the treatment of type 2 diabetic patients, has some structural similarities to aminoguanidine. It reduces methylglyoxal, an important precursor of AGE formation in type 2 diabetes. One may speculate whether the beneficial effects of metformin in type 2 diabetic patients, as reported in the UKPDS study, are related to these specific effects on AGE accumulation. Pyridoxamine is a natural intermediate of vitamin B6 metabolism and is a potent inhibitor of the formation of AGEs. Marked effects of pyridoxamine, such as delayed development of nephropathy and retinopathy, have been

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**Figure 1. Potential sites of intervention in the formation of AGEs (by aminoguanidine, pyridoxamine, metformin and antioxidants), AGE cross-link breaking (by ALT-711) and AGE-mediated damage (by sRAGE and antioxidants)**

![Diagram of potential sites of intervention in the formation of AGEs, including antioxidant therapy, aminoguanidine, pyridoxamine, metformin, and antioxidants](image)

Huijberts, et al. AGE accumulation.
demonsrated in diabetic rats. Pyridoxamine is currently being investigated in phase 3 of clinical trials for the treatment of diabetic nephropathy. All doses are well tolerated, without serious adverse effects. The initial results suggest that albuminuric is markedly reduced.

REDUCTION OF AGE CROSS-LINKS

The second approach to reduce AGE-induced effects is to diminish AGE cross-links in cardiovascular tissue by ‘AGE breakers’. ALT-711 is the first drug from a new class of therapeutic agents that break established AGE cross-links. In a randomised, placebo-controlled trial eight weeks of ALT-711 treatment reduced pulse pressure and arterial compliance in elderly patients. In an open-label, observational study in stable patients with diastolic heart failure, 16 weeks of ALT-711 diminished left ventricular hypertrophy and improved indices of diastolic function. Other clinical trials demonstrated the antihypertensive effect ALT-711, while the prevalence of adverse events is low.

INTERVENTION IN THE AGE-RAGE PATHWAY

The third approach to reduce the deleterious effects of AGES is by intervention in the AGE-RAGE interaction or their induced signalling pathway. The soluble form of RAGE (sRAGE) counteracts deleterious effects of AGES, which suggests RAGE may be a new target for therapeutic intervention in diabetic disorders. In addition to these approaches, numerous existing drugs against diabetic complications, both natural and pharmacological, are being investigated for their possible therapeutic potential and most of them have anti-AGEing effects. Thiamine and benfotiamine, drugs with antioxidant or metal-chelation properties, such as aspirin, ibuprofen, indomethacin, and flavonoids as well as angiotensin II receptor blockers and angiotensin-converting enzyme inhibitors, were reported to be inhibitors in the formation of AGEs. The question is whether the biological activities of these drugs are (partly) due to AGE lowering. More specific studies are needed to address that question.

Although it is now well recognised that the accumulation of AGEs in tissues has an important role in the pathogenesis of diabetic complications, the major question remains which AGE(s) is or are the real bad guy(s) and what are the AGE pathways leading to their deleterious effects. Interfering in the glycation pathway may offer new treatments for glucose-derived vascular complications of diabetes.

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Regulatory T cells: back to the future

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ABSTRACT

Regulatory T cells seem to represent the resurrection of the old suppressor T cells. Although of a different phenotype, regulatory T cells are able to suppress many T cell-mediated immune responses. While most basic knowledge about these cells is derived from animal studies, the recent identification of these cells in humans has further attributed to their characterisation by in vitro analysis. Results obtained have led to broad speculations about therapeutic potential by interference with these regulatory T cells. This review is an introduction to the world of regulatory T cells and contains an historical overview with respect to the identification and characterisation of these T cells. A distinction is made between naturally occurring regulatory T cells (nT_{reg}), which require cell-cell contact for suppression, and inducible regulatory T cells (iT_{reg}), which predominantly mediate suppression via cytokine-dependent pathways. Although only limited studies on regulatory T cells in human disease are available today, the possible clinical applications are discussed in light of the other side of the coin, i.e. the danger of interfering with homeostatic mechanisms in the immune system.

KEYWORDS

Immune regulation, homeostasis, therapy, regulatory T cells

INTRODUCTION

Regulatory T cells (T_{reg}) are currently in the spotlight of immunological research. In the last decennium of the previous century the immunopathogenesis of immune-mediated diseases was explained by the T helper (T_{h})1/T_{h}2 balance. Nowadays, aberrant numbers and/or functions of T_{reg} are incorporated in our view of the disturbance in the immune system of these diseases. Consequently, manipulation of T_{reg} is considered very promising as a therapeutic option. What are these T_{reg} exactly? As a matter in fact, regulation by T_{reg} is limited to suppression, and not activation, of immune responses. However, since suppressor T cells were banned during the 1980s, this term could not easily be revived. At that time, research on suppressor T cells focussed on finding an antigen-specific soluble factor. When it appeared that this factor could not exist at all, suppressor T cells left the stage. Nevertheless, a couple of tenacious scientists demonstrated unequivocally that in animal models T cells are able to suppress several experimental autoimmune diseases. These suppressor T cells, which are different from the originally defined suppressor T cells, are now referred to as T_{reg}. The identification of these cells in the human has resulted in a publication boom on T_{reg} during the last four to five years. The most attention is being paid to the natural occurring CD25^{+}CD4^{+} T_{reg} (nT_{reg}) and the inducible T_{reg} (iT_{reg}), including the T_{h}1 and T_{h}3 cells. This introduction to the exciting world of T_{reg} will first highlight the relevant animal models that enabled the discovery of T_{reg}. Next, the main characteristics of human T_{reg} will be described in relation to health and disease. Finally, the therapeutic potential of these cells will be discussed in light of possible consequences of T_{reg} based therapies.

ANIMAL MODELS

Basically, there are three animal models that have significantly contributed to the current interest in T_{reg}. First, the Penhale model for autoimmune thyroiditis is induced in rats by thymectomy and subsequent repeated low-dose total body irradiation (4x 250 rad). Autoimmune disease can be prevented by the adoptive transfer of T cells derived from healthy, syngeneic rats. In particular T_{h} cells with a low-level expression of CD45RC (CD45RC^{lo}) appear responsible for this effect, which is mediated by the cytokines interleukin (IL)-4 and transforming growth factor (TGF)-β. Typically, depending on the
major histocompatibility complex (MHC) haplotype, rats develop thyroiditis or diabetes. Second, the colitis model, as explored by Powrie, also originated in the rat. The adoptive transfer of Th cells with highly expressed CD45RC (CD45RC<sub>high</sub>) to T cell deficient rats results in severe wasting disease. Just as in the Penhale model, the CD45RC<sub>low</sub> Th cells of healthy rats are able to counteract the pathogenicity of the CD45RC<sub>high</sub> Th cells. In mice the adoptive transfer of CD45RB<sub>high</sub> Th cells (CD45RB is the mouse equivalent of rat CD45RC) to T cell deficient recipients results in colitis, an experimental model for inflammatory bowel disease (IBD). The CD45RB<sub>low</sub> Th cells protect against colitis via the cytokines IL-10 and TGF-β, but not IL-4. Third, neonatal thymectomy of mice on day 3 (d3Tx), but not day 7, will result in autoimmune gastritis (AIG), thyroiditis and/or diabetes. This effect is attributed to insufficient thymic output of T<sub>reg</sub> during the first week of neonatal life. Indeed, reconstitution of d3Tx mice with T cells of healthy mice prevents the development of autoimmune disease. The responsible T<sub>reg</sub> have been characterised as being CD25<sup>+</sup>CD4<sup>+</sup> T cells (nT<sub>reg</sub>) by adoptive transfer studies. The cytokines IL-4, IL-10, and TGF-β could not be attributed any significant role in this model.

### Phenotypic Markers

As can be concluded from information obtained in the animal models, CD45RC and CD25 (IL-2Ra) are two cell-surface receptors that are associated with T<sub>reg</sub>. CD45RC is a splice variant of the cell membrane common antigen CD45. In the rat CD45RC expression divides Th cells in two subsets: CD45RC<sub>high</sub> and CD45RC<sub>low</sub> Th cells. The suppressor activity is confined to the CD45RC<sub>low</sub> subset as demonstrated in several in vivo situations. Upon in vitro stimulation these cells, but not the CD45RC<sub>high</sub> Th cells, produce the anti-inflammatory cytokines IL-4, IL-10 and IL-13. Also in humans, CD45RC expression enables the distinction of two CD4 T cell subsets with opposite cytokine profiles (Saoudi and Damoiseaux, to be published). While the suppressor activity of CD45RC<sub>low</sub> Th cells has never been demonstrated in vitro, this is definitely the case for the CD25<sup>+</sup>CD4<sup>+</sup> nT<sub>reg</sub>. These nT<sub>reg</sub>, which are typically confined within the CD45RC<sub>low</sub> Th cell subset, are pre-eminently able to inhibit the proliferation of CD25<sup>+</sup> T cells in vivo settings. In mice and men, cell-cell contact is indispensable for the suppressor effect observed in vivo, while soluble factors, such as cytokines, play no crucial role in their mode of action. It should be stressed that this mode of action holds only for nT<sub>reg</sub>, but not for iT<sub>reg</sub> (vide infra). The identification of a surface marker, in particular CD25, and the establishment of in vitro methods for functional analysis have greatly attributed to the identification of T<sub>reg</sub> in humans. In humans, the level of CD25 expression may even discriminate between true nT<sub>reg</sub> and activated T cells that also express CD25. Indeed, the nT<sub>reg</sub> appear to be confined within the cells with high CD25 expression.

Recently two more specific markers for nT<sub>reg</sub> have been identified: Neuropilin-1 (Nrp1) and Foxp3. Nrp1 is a cell-surface receptor that is involved in axon conductance, angiogenesis, and cellular activation. In mice this receptor is constitutively expressed by nT<sub>reg</sub> and clearly discriminates between nT<sub>reg</sub> and activated T cells since the latter do not express Nrp1. The expression of this marker has not yet been examined on human nT<sub>reg</sub>. Foxp3 is the second new marker associated with nT<sub>reg</sub> and receives a lot of attention these days. The name Foxp3 actually refers to the gene (FOXP3) encoding a transcription factor of the forkhead/winged-helix family (scurfyn). The relation between this gene and nT<sub>reg</sub> originates from the human disease IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome) as well as the Scurfy mouse. Mutations in FOXP3 result in the complete absence of nT<sub>reg</sub> and the spontaneous development of autoimmune diseases of the endocrine organs, but also IBD, atopic dermatitis, and fatal infections. Foxp3 not only controls development, but also function of nT<sub>reg</sub>. The expression of Foxp3 is almost specific for nT<sub>reg</sub> in mice; however, expression in the human population is less restricted. Expression is not observed on activation of conventional T cells or differentiation in Th<sub>1</sub>, Th<sub>2</sub>, or NK-T cells. Initially, expression of Foxp3 was only detected by reverse transcription polymerase chain reaction (RT-PCR) and this largely precluded single cell analysis. However, recently monoclonal antibodies have become available that enable detection of the transcription factor by intracellular staining in mice and men. The discovery of multiple markers for nT<sub>reg</sub> has raised the question whether nT<sub>reg</sub> are a homogeneous population. Selective depletion studies in mice have revealed that this is not the case. Reconstitution of nonobese diabetic (NOD) T cell deficient mice with CD25<sup>+</sup> T cells results predominantly in AIG, comparable with the d3Tx model. Besides, these animals develop late-onset diabetes, but no colitis. Reconstitution with CD62L<sup>+</sup> T cells, on the other hand, induces full-blown diabetes in the same type of recipients, but neither colitis nor gastritis. Finally, reconstitution with CD45RB<sub>high</sub> T cells results only in severe colitis. These results underline the diversity and organ specificity of nT<sub>reg</sub> in the control of distinct autoimmune diseases.

### Innate nT<sub>reg</sub> vs Adaptive iT<sub>reg</sub>

The CD25<sup>+</sup>CD4<sup>+</sup> nT<sub>reg</sub> is a naturally occurring T cell subset that constitutes about 1 to 5% of the overall...
lymphocyte population in peripheral blood. As concluded from murine studies, the nT\textsubscript{reg} are generated as a separate T cell subset in the thymus.\textsuperscript{1,2} The important role of the thymus was already apparent in the Penhale thyroiditis model as well as the 3dTx model. Intrathymic development of nT\textsubscript{reg} requires a relatively high avidity interaction between the T cell receptor of the nT\textsubscript{reg} and the MHC expressed by thymic stroma, in particular the cortical epithelium. Additionally, also IL-2 and coligation by B7-CD28 and CD40-CD40L interactions have appeared to be important in the development of nT\textsubscript{reg}. Since selected thymocyte subsets are able to suppress the pathogenicity of autoreactive T cells, it can be concluded that nT\textsubscript{reg} already acquire their function in the thymus.\textsuperscript{3,4} The latter observation has been confirmed with human CD4\textsuperscript{+}CD25\textsuperscript{+} thymocytes.\textsuperscript{5} Finally, as observed in the Penhale thyroiditis model, the generation of nT\textsubscript{reg} is dependent on the presence of the autoantigen. Indeed, peripheral T cells of rats that have undergone thyroid destruction in utero are unable to prevent thyroiditis, but retain the ability to prevent diabetes.\textsuperscript{6}

In contrast to nT\textsubscript{reg}, which are considered innate, T\textsubscript{reg} may also develop from conventional, naïve T cells during an immune response.\textsuperscript{7} The best characterised, inducible T\textsubscript{reg} (iT\textsubscript{reg}) are known as the T\textsubscript{H}1 cells. These T\textsubscript{H}1 cells were initially described in mice upon long-term in vitro stimulation in the presence of IL-10.\textsuperscript{8} Next, T\textsubscript{H}1 cells were also identified in humans and it appeared that besides IL-10, also IFN-α is important for the development of these cells. Although it is evident that not all iT\textsubscript{reg} are identical to the originally described T\textsubscript{H}1 cells, in this review we will further use iT\textsubscript{reg} as the general term. The mode of action of iT\textsubscript{reg} is definitely different from the one described for nT\textsubscript{reg} (vide supra). Suppression by iT\textsubscript{reg} is contact independent and mediated by cytokines, in particular IL-10 and to a lesser extent TGFB-β. The coexistence of nT\textsubscript{reg} and iT\textsubscript{reg} with each a different mode of action, may explain the observed discrepancy between in vivo (cytokine dependent) and in vitro (cytokine independent) data, since in vivo the respective mechanisms will be intermingled. Besides the cytokine profile there are no characteristic markers for iT\textsubscript{reg}. The iT\textsubscript{reg} can be induced in several different ways. First of all, human nT\textsubscript{reg} are able to transfer suppressor activity to conventional T cells.\textsuperscript{9} In transplantation biology this process is referred to as infectious tolerance. While the induction is cell-contact dependent, the newly developed iT\textsubscript{reg} mediate their suppression via cytokines.\textsuperscript{10} Second, also dendritic cells are able to generate iT\textsubscript{reg}. Based on murine studies, it has been speculated that in particular semimature dendritic cells induce tolerance instead of effector responses due to a reduced expression of costimulatory molecules and high production of IL-10.\textsuperscript{11} Finally, as observed in both human and animal research, also infections are able to stimulate development of iT\textsubscript{reg}. This may seem a contradiction because the host should benefit from an effective immune response to destroy the invading pathogen. However, keeping the immune response in control is relevant to limit infection-induced immunopathology.\textsuperscript{12}

**REGULATORY T CELLS IN HUMAN DISEASES**

While most studies on human T\textsubscript{reg} were performed with cells from healthy individuals, several studies in human diseases have been published during the last two years.

These studies have mainly concentrated on autoimmune diseases and infections and deal with frequency and/or function of T\textsubscript{reg}.

In multiple sclerosis (MS), a T cell-mediated autoimmune disease, it was hypothesised that the control of peripheral autoreactive T cells, which are known to have similar frequencies in patients and healthy individuals, is hampered by a defect in the T\textsubscript{reg} compartment. While no differences in the frequencies of CD4\textsuperscript{+}CD25\textsuperscript{+} nT\textsubscript{reg} were observed, there is a marked decrease in the effector function of nT\textsubscript{reg} in MS patients as compared with healthy controls. This includes a defective inhibition of both conventional T cell proliferation as well as T\textsubscript{H}1 cytokine production.\textsuperscript{13} Human autoimmune polyglanudal syndrome (APGS) is characterised by involvement of multiple endocrine organs and thereby somewhat resembles the d3Tx model in mice. While APGS type I is caused by loss of central tolerance, APGS type II might be the result of defective peripheral tolerance, i.e. nT\textsubscript{reg} CD4\textsuperscript{+}CD25\textsuperscript{+} nT\textsubscript{reg} are found at a normal frequency in patients with APGS type II, but the suppressor function, as demonstrated in proliferation experiments, is significantly reduced.\textsuperscript{14} In myasthenia gravis, a prototypical antibody-mediated autoimmune disease, possible disturbances in the regulatory CD4\textsuperscript{+}CD25\textsuperscript{+} thymocytes were examined. Results indicate that also frequencies of regulatory thymocytes are normal, while the suppressive function is clearly impaired.\textsuperscript{15} Finally, in rheumatoid arthritis, characterised by uncontrolled production of inflammatory cytokines, a compromised function of nT\textsubscript{reg} has been described.\textsuperscript{16} However, functional deficits involve the inhibition of cytokine production (TNF-α) in conventional T cells as well as the capacity of nT\textsubscript{reg} to convey a suppressive phenotype (iT\textsubscript{reg}) to conventional T cells, but not the suppression of T cell proliferation. Both deficits are typically restored to normal by anti-TNF-α therapy. Altogether, these data are indicative of a central role of functional nT\textsubscript{reg} defects in the aetiology of the wide spectrum of autoimmune diseases. While autoimmune diseases are associated with a defective T\textsubscript{reg} compartment, it can be anticipated that chronic
infections may be due to increased numbers and/or function of T\textsubscript{reg}. In particular CMV and HIV are proposed to induce T\textsubscript{reg} that inhibit the virus-specific immune response.\textsuperscript{19} However, in the case of HIV infection, this seems to be a double-edged sword. While in the majority of healthy HIV-infected individuals CD\textsubscript{4}+CD\textsubscript{25}+ T cells suppress HIV-specific T cell responses \textit{in vitro}, and thereby may be responsible for \textit{in vivo} tolerance induction to HIV, these CD\textsubscript{4}+CD\textsubscript{25}+ T cells also prevent CD\textsubscript{4}+ T cell activation and thereby reduce the availability of target cells for HIV replication.\textsuperscript{34}

**THERAPEUTIC POTENTIAL**

Nearly all scientific publications about nT\textsubscript{reg} and iT\textsubscript{reg} speculate about the possible clinical applications. If there is excessive immunity, as in autoimmune diseases, asthma and allergy, transplant rejection, and certain cases of early pregnancy loss, increasing the amount and/or function of T\textsubscript{reg} is supposed to be beneficial. Since iT\textsubscript{reg} are inducible these cells are the best candidate for manipulation in the diseases mentioned above, but also expansion of nT\textsubscript{reg} may be achieved by retroviral transduction of T cells with FOXP\textsubscript{3}.\textsuperscript{16} Shortage of immunity, as in malignancy or chronic infections, may be due to an excess of T\textsubscript{reg}. These situations, and also vaccination against infectious diseases, are considered to benefit from a reduction in T\textsubscript{reg}. Elimination of T\textsubscript{reg} can be achieved by depleting antibodies reactive with surface receptors of these cells. Since the phenotype of nT\textsubscript{reg} is better characterised, this subset is the best candidate for depletion. Noteworthy is the fact that anti-CD\textsubscript{25} therapy is clinically successful. However, this therapy is aimed at the elimination of activated T cells and to suppress the immune response, in particular in case of transplant rejection.\textsuperscript{19} This therapy does not result in the generation of autoimmune disease. Also in mice, anti-CD\textsubscript{25} therapy does not result in autoimmune disease.\textsuperscript{16} Apparently, autoreactive T cells present in the periphery need prior activation, for instance by immunisation with the autoantigen or in a lymphopenic situation, before they are able to induce autoimmune pathology in the absence of iT\textsubscript{reg}.\textsuperscript{16} Alternatively, the T\textsubscript{reg} system may be redundant due to the presence of several other T\textsubscript{reg} subpopulations, such as NK-T cells and CD\textsubscript{8}+ T cell subsets. For details about these T\textsubscript{reg} subpopulations see references 37 and 38, and the tables therein.\textsuperscript{37,38}

Interference with the homeostatic mechanisms of the immune system, however, will remain a risky business. The rediscovery of the suppressor T cell has changed the concept of this homeostasis. While the pathology of most immune-mediated diseases can still be explained by the T\textsubscript{h}1/T\textsubscript{h}2 paradigm, the reciprocal regulation of both subsets is apparently outdated.\textsuperscript{37} It appears that the combination of nT\textsubscript{reg} and iT\textsubscript{reg} keeps both the T\textsubscript{h}1 as well as the T\textsubscript{h}2 responses in control (figure 1A). Shortage of T\textsubscript{reg} may result in an excessive T\textsubscript{h}1 or T\textsubscript{h}2 response with the associated immunopathology (figure 1B), while excess of T\textsubscript{reg} may suppress the respective immune responses (figure 1C). The latter will eventually prevent the generation of effector mechanisms that are required to inhibit tumour outgrowth and to clear the body of infectious agents. Moreover, the induction of iT\textsubscript{reg} by infections gives further support to broadening the hygiene hypothesis: not only the prevalence of T\textsubscript{h}2-mediated diseases, such as asthma and allergy, is increasing due to

![Figure 1](image-url)
a reduced challenge of the immune system by infections, but also the \( T_{reg} \)-mediated autoimmune diseases.19-20

**CONCLUSION**

Altogether it is evident that since the beginning of this century T cells with suppressor functions are again being recognised as new players in the field. Data originally obtained in animal research have recently been confirmed in humans. These include that there are at least two distinct types of \( T_{reg} \): the naturally occurring \( nT_{reg} \) which require cell-cell contact for suppression, and the inducible \( iT_{reg} \), which predominantly mediate suppression via cytokine-dependent pathways. The \( nT_{reg} \) are recognisable by the simultaneous expression of CD25 and Foxp3, while \( iT_{reg} \) are characterised by their cytokine profile. The analysis of \( nT_{reg} \) frequencies and function in several distinct types of autoimmune diseases has revealed that in particular the suppressive function of \( nT_{reg} \) is affected in patients with autoimmune disease. The role of \( T_{reg} \) in infectious disease seems to behold a paradox since \( T_{reg} \) may control severe immunopathology, but at the same time facilitate transition to chronic infections. Although there is extensive speculations about the therapeutic options involving \( T_{reg} \), caution with this therapy is warranted because \( T_{reg} \) take part in the homeostatic regulation of the immune system by enabling protective Th1 and/or Th2 responses but preventing excessive Th1 and/or Th2 responses.

**NOTE**

This review is based on a presentation at the Symposium on Clinical Immunology on 31 March 2005, in Amsterdam, the Netherlands.

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Time course of specific AGEs during optimised glycaemic control in type 2 diabetes

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ABSTRACT

Background: Several advanced glycation endproducts (AGEs) are formed in the hyperglycaemic state. Although serum AGEs correlate with average glycaemic control in patients with type 2 diabetes and predict the development of complications, it is not known how serum AGEs change during optimisation of diabetes therapy.

Methods: We evaluated the change in serum levels of total AGE and the AGEs CML (N-ε-carboxymethyllysine) and MGHI (methylglyoxal-derived hydroimidazolone), as well as markers of endothelial function in 28 subjects with type 2 diabetes, who were poorly controlled on oral agents, before and after the institution of insulin therapy.

Results: Mean subject age (± SEM) was 58 ± 2 years, body mass index 27.7 ± 0.8 kg/m², and known duration of diabetes was 8.1 ± 0.9 years. With insulin treatment fasting blood glucose levels dropped from 12.1 ± 0.9 mmol/l to 6.9 ± 0.3 and 8.1 ± 0.4 mmol/l after three and six months, respectively (both p<0.001), while HbA₁c decreased from 10.0 ± 0.3 to 7.8 ± 0.2% (p<0.001). Endothelial function improved as indicated by a small but significant decrease in soluble intercellular cell adhesion molecule (sICAM-1) (152 ± 10 to 143 ± 8 ng/ml, p<0.02) and sE-selectin (111 ± 16 to 102 ± 12 ng/ml, p<0.02) levels. In contrast, we observed only a tendency towards a decrease in CML levels (110 ± 22 to 86 ± 13 µg/mg protein, p=ns), but a small increase of MGHI (from 0.23 ± 0.02 to 0.29 ± 0.04 U/mg protein, p<0.02). At baseline, 16 patients were on metformin, which is known to reduce methylglyoxal levels and reduce generation of reactive oxygen species. They had similar levels of CML and MGHI to the non-metformin users, although their HbA₁c was lower (9.4 ± 0.3 vs 10.7 ± 0.6 %). During insulin, patients receiving concomitant metformin therapy showed a similar course of CML and MGHI to those not taking metformin.

Conclusion: Although insulin therapy improved HbA₁c and markers of endothelial function, the levels of serum AGEs did not follow the same time course. This suggests that these specific AGEs are influenced by other factors in addition to overall glycaemia, such as oxidative stress.

KEYWORDS

Adhesion molecules, AGE, endothelial dysfunction, insulin therapy, methylglyoxal-derived hydroimidazolone, N-ε-carboxymethyllysine, type 2 diabetes

INTRODUCTION

Hyperglycaemia is a major factor responsible for the development of diabetic complications. In recent years, several studies have reported on the effects of intensive glucose-lowering therapy in preventing both microvascular and macrovascular complications. Two of these major trials were the United Kingdom Prospective Diabetes Study (UKPDS) and the Diabetes Control and Complications Trial (DCCT). It was clearly shown that optimised diabetes treatment resulted in a reduction in the development and progression of microvascular complications in both type 2 and type 1 diabetic patients, and to a lesser extent of macrovascular disease. The biochemical changes induced by hyperglycaemia are complex and several mechanisms are involved, including the polyol pathway, activation of protein kinase C,
increased oxidative stress and the formation of advanced glycation endproducts (AGEs). AGE formation results from the reaction of a carbonyl group of a reducing sugar with the free amino groups of a protein. Known as Schiff’s base, this will react further to an Amadori product which rearranges to the AGEs.\textsuperscript{1,6} This glycation process leads to the formation of a group of heterogeneous components which are associated with several pathological processes in patients with diabetes.\textsuperscript{7} Next to this glycation process, lipid peroxidation or oxidative stress in general can lead to the formation of reactive carbonyl compounds (methylglyoxal, glyoxal or 3-deoxyglucosone), which can react with proteins thus forming advanced lipid peroxidation endproducts or advanced lipoxidation endproducts (ALEs), respectively.\textsuperscript{8} Circulating AGEs can react with their receptors and induce several cellular responses in the vessel wall, such as formation and activation of cytokines and increased expression of adhesion molecules including E-selectin, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) by the endothelium.\textsuperscript{9-11} Endothelial dysfunction plays an important role in the development of long-term diabetic complications,\textsuperscript{12} and it has been shown in vivo as well as in vitro that these adhesion molecules correlate well with endothelial dysfunction.\textsuperscript{13-18}

It is expected that intensive glucose-lowering therapy will lead to a gradual reduction in the formation of AGEs, as was shown for haemoglobin AGE several years ago.\textsuperscript{19} Although serum AGEs correlate with average glycaemic control and predict the development of complications, it is not known how other specific serum AGEs change during optimisation of diabetes therapy.\textsuperscript{20}

In this study we assessed the metabolic effects of improved glucose control by institution of insulin therapy in subjects with type 2 diabetes, who failed to respond adequately to oral blood glucose-lowering therapy. We followed the changes of specific AGEs over time, their interaction and studied whether improvement in glycaemic control resulted in an improved endothelial function indicated by the levels of circulating adhesion molecules.

**METHODS AND MATERIALS**

**Subjects and study design**

Twenty-eight subjects (14 males, 14 females) with type 2 diabetes, who were insufficiently controlled on oral glucose-lowering medication (sulphonylurea with or without metformin) and therefore started insulin therapy, participated in this study. Their mean age (± SEM) was 58 ± 2 years, body mass index 27.7 ± 0.8 kg/m\(^2\), and known duration of diabetes was 8.1 ± 0.9 years. Subjects were attending the outpatient departments of four Dutch hospitals, Maastricht University Hospital, Radboud University Nijmegen Medical Centre, and the hospitals of Boxmeer and Leidschendam. They were all participating in a larger international study assessing the effects of therapy with a new recombinant human insulin preparation (Sanofi-Aventis). Men and women between the age of 30 and 80 years were included in this study if they had documented type 2 diabetes, had normal kidney function, and failed to achieve adequate glycaemic control on oral blood glucose lowering agents. Written informed consent was obtained from all participants.

At the start of the study subjects were screened and demographic data as well as fasting blood samples were taken. Data on concomitant medication were obtained throughout the study. Nine patients were taking an ACE inhibitor, six patients a β-blocker, while eight patients were treated with a statin and two patients were on acetylsalicylic acid. These treatments were not altered during the study.

The choice for a specific insulin regimen was made by the treating physician for the individual patient, and based on the results of home blood glucose monitoring. Patients were seen at regular intervals in the outpatient clinic to monitor insulin therapy, and the insulin dose was adjusted by the treating physician on the basis of home blood glucose monitoring. For the purpose of the study, fasting blood samples were taken at baseline, i.e. before insulin therapy, and three and six months after initiation of insulin therapy. On these occasions, blood samples were directly centrifuged and serum was stored in small aliquots at -80°C until further analysis.

**Methods**

HbA\(_{\text{lc}}\) levels were measured with HPLC (Bio-Rad Variant II, Hercules CA). The nondiabetic reference range was 4.2 to 6.5%, and the assay was linear up to 17.9%. Fasting blood glucose was measured by a hexokinase method (Olympus, Southall, UK).

Measurements of the adhesion molecules (sE-selectin and sICAM-1) were performed with an ELISA.\textsuperscript{21} sICAM-1 standard was obtained from Bender MedSystems (Vienna, Austria); sE-selectin standard was prepared as described elsewhere.\textsuperscript{21}

Serum total AGE levels were determined with a polyclonal antibody raised against AGE RNase by the DELFIA method.\textsuperscript{22} One AGE unit was defined according to Makita et al. as the competitive activity of 1 μg AGE-BSA standard.\textsuperscript{23} The final serum concentration of AGEs was corrected for total protein concentration in each serum sample in the following equation [AGE, U/ml] x [sample protein/mean protein concentration of all sera measured]. All analyses were performed in the same run. Methylglyoxal-derived hydroimidazolone (MGHI) levels were determined using a method similar to the total AGE
measurement. One hydroimidazolone unit was defined as the competitive activity of 1 µg of MG-modified BSA standard. The serum concentration of hydroimidazolone was adjusted for the total protein concentration in each sample, and is expressed as U/mg protein. In this way, possible systematic errors due to the differences in protein content between groups were avoided. Nε-carboxymethyllysine (CML) was measured using a newly developed HPLC method. CML data were then normalised against plasma protein concentration resulting in the final concentration of ng CML/mg plasma protein.

Statistics
At baseline several patients were on metformin. Since this was an open study, some subjects continued this medication concomitantly with their insulin treatment, while others stopped all oral agents and switched to insulin alone. As it is known that metformin may scavenge intermediate glycation products such as methylglyoxal, the results of insulin therapy were analysed in the whole group, and post-hoc for metformin users and non-users separately. Also the observed changes were compared between patients who showed a good improvement in metabolic control (decrease in HbA1c of >1.5%; good responders), and those with only moderate changes (decrease in HbA1c of ≤1.5%; poor responders).

All results were expressed as means ± SEM. Data were analysed using one-way ANOVA, paired t-tests and Pearson correlations. Statistical analysis was performed using SPSS 10.0, SPSS, Chicago, Il, USA. P values <0.05 were considered to indicate statistical significance.

RESULTS
At baseline metabolic control was insufficient, indicated by mean HbA1c levels of 10.0 ± 0.3 % and fasting blood glucose (FBG) levels of 12.1 ± 0.9 mmol/l. Insulin therapy resulted in a significant improvement in glycaemic control: HbA1c in the total group decreased to 7.8 ± 0.2 % at three months with no additional change after six months (table 1). Mean daily insulin dose was 39 ± 5 U at six months, and at that time four subjects were using one daily insulin injection, 16 used a mixture of fast-acting and neutral protamine Hagedorn (NPH) insulin twice daily, and eight were on a four-injection regimen comprising fast-acting insulin before meals and NPH insulin at bedtime. The serum levels of the adhesion molecules E-selectin and ICAM-1 also decreased significantly, indicating an improvement in endothelial function. It appeared that total AGE serum levels did not change significantly after six months. We observed a small decrease in CML levels, which was not statistically significant, while levels of MGHI increased significantly (table 1). The course of HbA1c and CML in the individual patients is depicted in figure 1.

Overall, HbA1c levels were not correlated with serum AGE levels (total AGE, CML and MGHI) nor with serum adhesion molecule levels (E-selectin and ICAM-1). Total AGE and CML did correlate with sE-selectin (R_{Pearson}=0.36, p=0.013 and R_{Pearson}=0.29, respectively, p=0.044), but not with sICAM-1.

Influence of metformin use
The relevant laboratory parameters at baseline were compared between subjects who were only on sulphonylurea (SU) and those taking SU with metformin. As shown in table 2, FBG and HbA1c were significantly lower in metformin users, although their diabetes duration was longer. No differences were observed in the levels of adhesion molecules or the different AGE measurements. Of the 16 metformin users, six continued this drug during insulin therapy, and ten switched to therapy with insulin alone. The decrease in HbA1c in subjects who continued metformin in addition to insulin

Table 1. Changes in the main variables during the course of the study, after initiation of insulin therapy at baseline

<table>
<thead>
<tr>
<th>Variable</th>
<th>Baseline</th>
<th>Three months</th>
<th>Six months</th>
</tr>
</thead>
<tbody>
<tr>
<td>FBG (mmol/l)</td>
<td>12.1 ± 0.9</td>
<td>6.9 ± 0.3*</td>
<td>8.1 ± 0.4*</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>10.0 ± 0.3</td>
<td>7.8 ± 0.2*</td>
<td>7.8 ± 0.2*</td>
</tr>
<tr>
<td>Insulin dose (U/day)</td>
<td>-</td>
<td>38 ± 4</td>
<td>39 ± 5</td>
</tr>
<tr>
<td>sICAM-1 (ng/ml)</td>
<td>152 ± 10</td>
<td>143 ± 8†</td>
<td>147 ± 8†</td>
</tr>
<tr>
<td>sE-selectin (ng/ml)</td>
<td>111 ± 16</td>
<td>96 ± 12‡</td>
<td>102 ± 14‡</td>
</tr>
<tr>
<td>Total AGE (U/ml)</td>
<td>6.7 ± 0.7</td>
<td>6.7 ± 0.5</td>
<td>7.4 ± 1.0</td>
</tr>
<tr>
<td>MGHI (U/mg protein)</td>
<td>0.23 ± 0.03</td>
<td>0.23 ± 0.02</td>
<td>0.29 ± 0.04‡</td>
</tr>
<tr>
<td>CML (ng/mg protein)</td>
<td>1.14 ± 2.3</td>
<td>1.26 ± 1.8</td>
<td>0.86 ± 1.3†</td>
</tr>
</tbody>
</table>

* p<0.001 vs baseline; † p<0.02 vs baseline; ‡ p=0.086.

FBG = fasting blood glucose; MGHI = methylglyoxal-modified hydroimidazolone; CML = Nε-carboxymethyllysine.
treatment was 1.7% (from 9.3 ± 0.4 to 7.6 ± 0.4%), which was comparable with subjects who switched to insulin alone 1.3% (from 9.4 ± 0.4 to 8.1 ± 0.4%). There were, however, considerable differences in insulin dose: the metformin users injected 18 ± 3 U of insulin per day at six months, while subjects on insulin alone used 45 ± 5 U daily (p=0.009). We were unable to demonstrate significant differences in the changes of the various AGE levels between metformin users and nonusers, but the group sizes may be too small.

### Influence of insulin efficiency

As insulin therapy has a variable effect on glycaemic control in patients, we assessed whether the observed changes were related to the efficiency of insulin therapy. Poor responders were at better metabolic control at baseline (HbA\(_1c\) 8.9 ± 0.2 vs 10.7 ± 0.5%), but had a longer duration of diabetes (table 3). Baseline levels of total AGE, MGHI and CML were not different between the groups. At six months no significant difference could be seen between the two groups, their insulin dose was identical, and the change in total AGE, MGHI, CML and adhesion molecule levels over time was not significantly different between the two groups.

### Discussion

It has been suggested that the formation of AGEs plays an important role in the development of microvascular and macrovascular complications in patients with diabetes mellitus, and the level of glycated haemoglobin (HbA\(_1c\)) is a major predictor. However, it is not known how serum AGEs change during optimisation of diabetes therapy. In the present study we observed that insulin therapy improved glycaemic control after three and six months
as indicated by a considerable decrease in both FBG and HbA<sub>1c</sub>. However, this improvement of glycaemic control did not result in a significant decrease in total AGE levels. A slight but not significant decrease in CML levels was observed, while MGHI even increased significantly.

In addition to the insulin therapy, several patients received concomitant medication such as metformin. The UKPDS has shown that metformin treatment in obese type 2 diabetic patients reduced cardiovascular complications to a greater extent than could be expected from its glucose-lowering potential. This resulted in the hypothesis that metformin also interacts with another molecular mechanism resulting from hyperglycaemia. Several studies have shown that metformin plays an important role in inhibiting dicarbonyl-mediated AGE formation and accumulation, which should be reflected in decreased MGHI and CML levels. At baseline, metformin users had a lower HbA<sub>1c</sub> and fasting blood glucose levels than nonusers. However, in this small group of patients we observed no differences in total AGE, MGHI or CML levels. Of the 16 metformin users, six subjects continued this medication in addition to insulin. No different changes in the levels of HbA<sub>1c</sub>, fasting blood glucose or the various AGE measurements were seen.

Thus it is apparent that HbA<sub>1c</sub> and the levels of AGEs did not follow the same time course. This suggests that these specific AGEs, such as CML and MGHI, are influenced by other factors in addition to overall glycaemia, and that these factors are of greater importance in determining AGE levels. It has been suggested that CML and MGHI in serum may also be derived from lipid peroxidation or be formed as a consequence of the generation of reactive oxygen species. Again this may relate to changes in metformin treatment. It has been shown that metformin may also decrease production of reactive oxygen species. In addition, elevated methylglyoxal levels, increasing after withdrawal of metformin, may directly increase oxidative stress, as was demonstrated in vitro in vascular smooth muscle cells.

Furthermore, increased oxidative stress can be generated by postprandial blood glucose excursions, occurring in diabetic patients even when on good metabolic control. Several studies have shown the potential use of AGEs as marker for the progression and severity of diabetic complication independent from markers for hyperglycaemia such as HbA<sub>1c</sub>. The discrepancy in the changes in HbA<sub>1c</sub> and AGEs after insulin treatment supports the notion that they are independent determinants of prognosis.

In addition to the time course of AGEs, we measured serum levels of ICAM-1 and E-selectin as an estimate of endothelial function. Insulin therapy resulted in a slight, but consistent and statistically significant decrease of both adhesion molecules, which indicates an improvement in endothelial function. Baseline levels of total AGEs correlated with E-selectin, but not with ICAM-1 levels. It has previously been suggested that AGEs induce the upregulation of adhesion molecules. Since we observed that the decrease in adhesion molecules was not paralleled by a decrease in AGE levels, this suggests that other (glycaemic or nonglycaemic) factors add to the effect of AGEs in upregulation of adhesion molecule expression. As receptor for AGE (RAGE) is a multiligand member of the immunoglobulin super family of cell surface molecules, other ligands can interact with RAGE resulting in the same effect as activation of intracellular signalling pathways such as MAP-kinases and NF-κB and the resulting upregulation of adhesion molecules.

Table 3. Characteristics of subjects responding or not responding to insulin therapy

<table>
<thead>
<tr>
<th></th>
<th>Poor responders (n=12)</th>
<th>Good responders (n=16)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Six months</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>5/7</td>
<td>9/7</td>
</tr>
<tr>
<td>Diabetes duration (years)</td>
<td>11.1 ± 1.2</td>
<td>8.1 ± 0.7</td>
</tr>
<tr>
<td>FBG (mmol/l)</td>
<td>9.5 ± 0.7</td>
<td>8.3 ± 0.3</td>
</tr>
<tr>
<td>HbA&lt;sub&gt;1c&lt;/sub&gt; (%)</td>
<td>8.9 ± 0.2</td>
<td>40 ± 7</td>
</tr>
<tr>
<td>Insulin dose (U/day)</td>
<td>146 ± 18</td>
<td>158 ± 16</td>
</tr>
<tr>
<td>sICAM-1 (ng/ml)</td>
<td>146 ± 18</td>
<td>158 ± 16</td>
</tr>
<tr>
<td>sE-selectin (ng/ml)</td>
<td>98 ± 23</td>
<td>109 ± 29</td>
</tr>
<tr>
<td>CML (ng/mg protein)</td>
<td>90 ± 21</td>
<td>73 ± 22</td>
</tr>
<tr>
<td>MGHI (U/mg protein)</td>
<td>0.26 ± 0.05</td>
<td>0.33 ± 0.08</td>
</tr>
<tr>
<td>Total AGE (U/ml)</td>
<td>6.1 ± 0.9</td>
<td>9.5 ± 1.8</td>
</tr>
</tbody>
</table>

†p<0.05 vs poor responders; †p<0.05 vs baseline.

Poor responders were considered those subjects in whom HbA<sub>1c</sub> decreased by ≤1.5% (at six months compared with start of the study) and good responders were those individuals in whom a decrease in HbA<sub>1c</sub> of >1.5% was observed.

Mentink, et al. Specific AGES during optimised glycaemic control in type 2 diabetes.
To assess the efficiency of insulin therapy on both endothelial function and AGE levels, patients were divided in two groups. Poor responders were considered those subjects in whose HbA1c decreased by ≤1.5% (at seven months compared with start of the study) and good responders were those individuals in whom a decrease in HbA1c of >1.5% was found. Good responders did achieve an average HbA1c of 7.3%, whereas in poor responders HbA1c after six months was 8.3%. Endothelial function, total AGE, CML and MGG-derived hydroimidazolone showed no significant differences at the different time intervals, nor a significant different increase or decrease over time. Insulin therapy has a varying efficiency in different patients resulting in different levels of glycemic control. However, this variance in glycemic control did not result in a difference in the underlying molecular mechanisms as AGE production and accumulation and the resulting change in adhesion molecule levels.

We conclude that improvement in glycemic control by glucose-lowering therapy ameliorates endothelial function as assessed by sICAM-1 and sE-selectin levels. Although insulin therapy improved HbA1c, the levels of serum AGEs, CML and MGHI did not follow the same time course. This indicates that these AGEs are formed in different pathways and improving glycemic control in diabetic subjects will not automatically lead to a reduction in AGE levels. This suggests that their presence in serum is influenced by other factors in addition to overall glycemia, such as lipid peroxidation or in general oxidative stress. The influence of specific treatment, as metformin, may be of significance as well, since this drug can scavenge methylglyoxal and reduce generation of reactive oxygen species.

ACNOWLEDGEMENT

We thank Professor J. Lutterman (Radboud University Nijmegen Medical Centre), Dr A.J. van Bork (Maas Hospital, Boxmeer) and Dr J. van Hooghenhuize (Antoniushove Hospital, Leidschendam) for their participation.

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Fascioliasis: a report of five cases presenting with common bile duct obstruction

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ABSTRACT

Fascioliasis is a zoonotic infection caused by Fasciola hepatica. It is rarely seen with icterus caused by obstruction of the common bile duct. We report five patients with obstructive jaundice due to Fasciola hepatica, who were diagnosed and managed with endoscopic retrograde cholangiopancreatography (ERCP). All cases were admitted to hospital with complaints of icterus and pain in the right upper quadrant of the abdomen; their biochemical values were interpreted as obstructive jaundice. Ultrasound and computer tomography (CT) revealed biliary dilatation in the common bile duct, but did not help to clarify the differential diagnosis. ERCP showed the presence of Fasciola hepatica in the common bile duct. After removing the flukes, the symptoms disappeared and the biochemical values returned to normal. Biliary fascioliasis should be considered in the differential diagnosis of obstructive jaundice. This report confirms the diagnostic and therapeutic role of ERCP in patients with obstructive jaundice caused by biliary fascioliasis.

KEYWORDS

Extrahepatic cholestasis, Fasciola hepatica, fascioliasis, parasitosis

INTRODUCTION

There has been an increase in Fasciola hepatica infections worldwide in the last decade and it is reported that 2.5 million people have been infected in 61 countries and more than 180 million people are at risk.1 As seen in the related literature, this disease is not only seen in developing countries but also in developed ones. For this reason it can be considered a worldwide problem. In nonendemic areas, it can be difficult for physicians to diagnose this disease as it is not often encountered. This may also lead to a delay in making the diagnosis. We present five cases of Fasciola hepatica in the common bile duct associated with jaundice. The patients could not be diagnosed by conventional methods, but were diagnosed and treated by ERCP.

CASE REPORTS

Between 2000 and 2003, five patients were referred to our hospital with suspicion of choledocolithiasis. The clinical presentations and general conditions of the cases were fairly similar. The patients’ main complaints were severe jaundice and pain in the right upper quadrant of the abdomen. All cases were females over 60 years (range 62-70 years, except for one male, aged 32). All cases showed a typical obstructive jaundice clinically. Abdominal ultrasound revealed minimal intrahepatic and mild extrahepatic biliary dilatation while other organs were normal, and no stones were observed. Since no definite diagnosis could be reached by ultrasound, CT was used but it did not provide any additional information. Two patients confirmed that they had previously undergone similar episodes of right upper quadrant pain without jaundice within the last four to five years. Blood tests revealed mild eosinophilia in only two cases, while haemoglobin and white blood cell count were normal in all patients. The erythrocyte sedimentation rates were normal, but asparate aminotransferase and alamine aminotransferase values were two to three times the upper limit, while γ-glutamyltransferase was four to six times greater. Total bilirubin and direct bilirubin levels were three to seven times and ten to 15 times the upper limit, respectively. ERCP demonstrated extrahepatic biliary dilatation, the common bile ducts were about 12 to 15 mm in diameter, with a small linear filling defect and crescent-
like shadows and a jagged appearance in the distal dilated parts. Sphincterotomy was performed, and the living mobile parasites were removed from the common bile duct by a balloon (figures 1-3). After ERCP, triclabendazole was administered at a dose of 10 to 12 mg/kg for one or two days, after which the symptoms disappeared and biochemical values soon returned to normal. A history of ingestion of watercress and other freshwater plants was confirmed in all the patients. Later at their first control visit, ultrasound revealed an almost normal CBD in all patients.

**DISCUSSION**

Humans, usually an accidental host, most commonly and classically get infected by eating watercress grown in sheep-raising rural areas. When eating infected material, infective metacercariae excyst in the duodenum and larvae emerge. The larvae penetrate the wall of the small intestine into the peritoneal cavity, then penetrate the liver capsule and pass through the liver tissue into the biliary tract. The disease is not only acquired by eating watercress but also by raw or undercooked liver of infected animals, or other plants such as lettuce and spinach, or drinking infected water. For this reason, if the infected materials stated above are eaten, the disease will not be limited to rural areas, and can be seen in the centres of developed cities.\(^1\)

While fascioliasis used to be seen mainly in developing countries, in the last decade the number of cases in developed countries has increased, reaching 61 countries worldwide because of the increase in worldwide travelling and immigration.\(^4\) The total estimated number of infected people is 2.4 million and the number at risk is more than 180 million throughout the world.\(^7\) For this reason, physicians who are not aware of this increase or who have not encountered many cases related to fascioliasis may waste time by performing multiple diagnostic procedures.\(^5\)

There are three phases of the disease: the acute or liver phase, the chronic or biliary phase, and ectopic or pharyngeal fascioliasis.\(^6\) Although the biliary phase is usually asymptomatic, it is rarely reported in the medical literature that it can lead to extrahepatic cholestasis, as was the case with our patients. In a report published in 2000, only 19 cases were reported to have had common bile duct obstruction by *Fasciola hepatica* during the last ten years.\(^7\) Besides, the parasite itself can obstruct the duct mechanically, and it can lead to hyperplasia and hypertrophy in the duct epithelium by increasing the concentration of proline.\(^8\) As a result periductal fibrosis and thickening of the duct walls may occur, causing obstruction.

The image seen on ultrasound and CT is sometimes confused with malignancy or stones.\(^8\) In the diagnosis of this disease, ultrasound may not provide certain information and CT is not superior. The most useful

![Figure 1. Fasciola hepatica being removed through the papilla](image1)

![Figure 2. Fasciola hepatica in the intestinal lumen](image2)

A colour version of this figure can be found on our website www.njmonline.nl.
A diagnostic test for viewing the bile ducts is cholangiography by ERCP, and more recently, by magnetic resonance cholangiopancreatography (MRCP). Some technical limitations make bile duct detail obtained by ultrasound, CT or MRCP imaging methods inferior to that obtained with ERCP. For this reason, ERCP is considered to be the gold standard for bile duct imaging. Likewise, ERCP should also be considered the first choice in patients in the chronic phase, even if the diagnosis is established by ultrasound or CT. However, the number of reported cases described by radiological features in chronic fascioliasis is small. The chronic phase is managed by endoscopic mechanical clearance of the bile ducts because of the risk of biliary obstruction caused by drug therapy, while the acute stage of the disease can be treated adequately by drugs only. In biliary obstruction due to fascioliasis, ERCP and sphincterotomy have been used successfully and safely to extract parasites from the biliary tree by balloon or basket. However, inadequate incision of papilla may result in cholangitis as well as stones in the common bile duct. Surgery is only indicated in complicated cases. We treated our patients with triclabendazole after effective sphincterotomy, and no relapse occurred in the two-year follow-up. In conclusion, because fascioliasis is increasingly encountered worldwide, physicians should be aware of this disease and they should take into consideration the travel and immigration history of patients during diagnosis of the disease. And also, ERCP still maintains its importance in the diagnosis and treatment of the disease, and can be used safely.

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Gulsen, et al. Fascioliasis
Mural aortic thrombus and peripheral embolisation in a patient with hyperhomocysteinaemia

M. Slabbekoorn*, O.D.F. Henneman†, P.H.L.M. Geelhoed-Duijvestijn, R.F. Veldkamp

A mobile thrombus of the descending thoracic aorta in young people is extremely uncommon. We describe a 38-year-old woman with a mural thrombus in the proximal aorta complicated by peripheral embolisation, due to hyperhomocysteinaemia.

KEYWORDS
Aortic thrombus, embolisation, hyperhomocysteinaemia

INTRODUCTION
Homocysteine has been identified as an independent risk factor for atherosclerosis. It can lead to both venous and arterial thrombotic disease. We report on a young woman with hyperhomocysteinaemia and a mural thrombus of the proximal descending aorta without identifiable atherosclerotic lesions.

CASE REPORT
Two weeks after her third delivery, a 38-year-old woman came to the emergency room because of abdominal pain radiating to the shoulders of sudden onset. Her medical history was remarkable for a 20 pack-year smoking habit and a history of pregnancy complications. Her first pregnancy at the age of 35 years was complicated by pulmonary embolism during the second trimester. At 33 weeks of pregnancy the foetus was lost due to a partial lissencephalia in combination with intracranial bleeding, as a result of high levels of anticoagulation. The second pregnancy was uncomplicated; she was then on low-molecular-weight heparin (LMWH). In her third pregnancy, she stopped the LMWH therapy herself directly after delivery. She continued smoking during this pregnancy. One week after discontinuing the LMWH, the abdominal pain developed. Physical examination revealed normal blood pressure, pulse rate and temperature. Auscultation of lungs and heart was normal. Palpation of the left upper part of the abdomen was tender without palpable mass. All peripheral pulses were palpable. Striking laboratory results included a high sedimentation rate (102 mm/h) and a lactate dehydrogenase (LDH) of 900 U/l. Cholesterol was normal. A computed tomography (CT) scan did not reveal pulmonary embolism, but a low-density intraluminal lesion, 0.5 to 1.0 cm in size, was seen on the anterior wall of the proximal descending aorta (figure 1). Magnetic resonance imaging (MRI) confirmed these findings and revealed a large splenic infarction and a smaller renal infarction on the left side (figure 2). Transoesophageal echocardiography also showed a highly mobile mass, 0.5 to 1 cm in size, in the very proximal descending aorta. There were no signs of intracardiac thrombi, valvular disease, dilation of the ventricles or abnormal systolic function. In view of these findings the patient was admitted to hospital for intravenous anticoagulant therapy. Search for a hypercoagulable state revealed normal thrombin, prothrombin and partial thromboplastin times, as well as antithrombin III, protein C and S antigens, protein C pathway, and activated protein C resistance. Screening for anticardiolipin antibodies
and lupus anticoagulant was negative. Factor VIII was markedly elevated (405%) as frequently seen in the course of pregnancy. Postload plasma homocysteine level was elevated (68 umol/l, normal <45 umol/l). Vitamin B12 and folic acid levels were normal. A total body gallium SPECT scan was performed and showed no abnormalities. Media and intima thickness of the carotid arteries, measured by ultrasound, were normal.

In view of the hyperhomocysteinaemia, treatment was started with folic acid (0.5 mg once daily) and pyridoxine (20 mg once daily). The sedimentation rate and LDH normalised. She was discharged from hospital after two weeks. After two months transoesophageal echocardiography showed resolution of the thrombus and the nonfasting homocysteine blood level normalised (7 umol/l, normal <12 umol/l).

DISCUSSION

This young patient presented with acute onset of abdominal pain radiating to the shoulder. Taking into account her prior pulmonary embolism, the first differential diagnosis was a new pulmonary artery thrombus after she had stopped anticoagulant treatment. The CT scan in combination with the MRI of the aorta revealed unusual findings: ischaemic regions in the spleen and left kidney probably caused by emboli of a small mural thrombus in the proximal descending aorta.

A mobile intraluminal thrombus of the descending thoracic aorta is an unusual cause of peripheral embolisation. In this patient aortitis or vasculitis was excluded by gallium SPECT scan and atherosclerosis was unlikely in the light of physical examination, normal media and intima thickness and absence of calcifications in the large arteries on the CT scan. Primary aortic tumours may present with peripheral emboli but are extremely uncommon. The diagnosis is most often made after surgery or autopsy while growth is aggressive. CT, MRI and ultrasound did not show any solid masses except the thrombus. Although rare, mural aortic thrombi must be considered in a patient with otherwise unexplained peripheral embolisation. Most patients with this phenomenon have several risk factors for atherosclerosis. A high blood level of homocysteine has been identified as a risk factor for venous and arterial thrombotic disease. Patients with a mild hyperhomocysteinaemia are at risk of atherosclerotic vascular disease independent of diabetes, smoking, hypertension and hyperlipidaemia. The association with arterial thrombosis in the absence of atherosclerosis has rarely been described. It has been suggested that homocysteine can stimulate thrombosis by enhanced tissue factor expression and factor V activity in combination of suppression of thrombomodulin activity and decreased fibrinolysis. Furthermore, increased oxidative damage and proliferation of vascular smooth muscle cells have been noticed. The mobile aortic thrombus in our young patient without atherosclerosis...
strongly supports the diagnosis of a hypercoagulable state. A high homocysteine blood level was the only prothrombotic abnormality besides her pregnancy. Nevertheless our patient is a smoker. Graham described a two- to four-fold increased risk of cardiovascular events for smokers in the presence of hyperhomocysteinaemia. In this case, smoking could certainly have promoted the hypercoagulable state.

Anticoagulation is an effective treatment for aortic mural thrombi. In this case the thrombus was completely resolved within two months. To the best of our knowledge, only one other case report has mentioned a patient with hyperhomocysteinaemia, aortic thrombus and peripheral embolisation. That patient died before institution of treatment. With this case we provide clinical evidence to support the relationship of hyperhomocysteinaemia and mural thrombus in the proximal aorta even without signs of atherosclerosis. Hyperhomocysteinaemia should be considered when evaluating peripheral arterial thrombosis in a young person especially when atherosclerosis is absent.

ACKNOWLEDGEMENT

The authors thank Dr A.P.C. van de Maas for his critical comments on the manuscript.

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Cyclical Cushing’s syndrome due to an atypical thymic carcinoid

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ABSTRACT

A 43-year-old man presented with fluctuating symptoms of weight gain, shortness of breath, pretibial oedema, associated with anxiety and memory disturbances. Laboratory investigation revealed an adrenocorticotropin (ACTH)-dependent cyclical Cushing’s syndrome characterised by remarkable variations in urinary cortisol excretions ranging from 27 to 28,050 nmol/24 h. Magnetic resonance imaging (MRI) of the pituitary was normal and ectopic ACTH production was suspected. A tumour in the right anterior mediastinum was revealed on octreotide receptor scintigraphy, which had initially been overlooked on computed tomography (CT) scanning. A thymic carcinoid tumour was suspected, which was supported by increased levels of urinary serotonin, while platelet serotonin and urinary 5-hydroxyindoleacetic acid levels were normal. The tumour was removed surgically and histological examination revealed an atypical thymic carcinoid tumour. Postoperatively, the patient’s symptoms disappeared rapidly. He underwent external radiotherapy and is still free of symptoms after almost two years of follow-up. Clinicians should be aware of the existence of this entity, as it easily leads to confusion, misdiagnosis and consequently to treatment delay.

We present here a patient with cyclical Cushing’s syndrome due to ectopic ACTH production by an atypical thymic carcinoid.

KEYWORDS

Cushing’s syndrome, hypercortisolism, cyclical, thymic carcinoid

INTRODUCTION

Cushing’s syndrome is a rare disorder, characterised by an inappropriately high synthesis of cortisol by the adrenal cortex. It usually results from overproduction of ACTH by a pituitary corticotroph tumour, representing the classic Cushing’s disease. This accounts for 70% of patients with Cushing’s syndrome. In about 20% of cases, hypercortisolism occurs without ACTH stimulation, as is found in adrenal adenoma, adrenal carcinoma or adrenal nodular hyperplasia. In the remaining cases, Cushing’s syndrome is due to ectopic, i.e. nonpituitary, secretion of ACTH or (very rarely) corticotropin-releasing hormone (CRH). Ectopic ACTH production is mainly associated with small-cell carcinoma of the lung but can also be found in various neuroendocrine tumours, such as bronchial, thymic, or pancreatic carcinoid, medullary carcinoma of the thyroid and pheochromocytoma.

In some cases, overproduction of cortisol occurs intermittently, resulting in high peaks of serum cortisol followed by periods with completely normal cortisol levels. This phenomenon is known as cyclical Cushing’s syndrome, as first described by Bailey in 1971. Clinicians should be aware of the existence of this entity, as it easily leads to confusion, misdiagnosis and consequently to treatment delay.

We present here a patient with cyclical Cushing’s syndrome due to ectopic ACTH production by an atypical thymic carcinoid.
medical history was unremarkable. Spironolactone had been used to treat his fluid retention without effect. Other medications were not given. He smoked 20 cigarettes daily and seldom used alcohol. His family history was negative with respect to endocrine diseases. On physical examination his height was 1.83 m and his weight 90 kg with truncal obesity. Blood pressure was 140/90 mmHg. Most prominent features were a red face and bilateral oedema of the lower limbs. No typical Cushingoid stigmata, as buffalo hump, striae, acne, ecchymoses, or cutaneous hyperpigmentation, were present. Muscle strength appeared normal.

Laboratory investigation, carried out in the referring hospital, suggested ACTH-dependent Cushing’s syndrome. Plasma ACTH varied between 43 and 75 pmol/l and serum cortisol between 581 and 2205 nmol/l. Urinary free cortisol excretion was sometimes extremely high (26,000–300,000 nmol/24 h; normal range 55 to 300 nmol/24 h), interspersed by periods of normal urinary cortisol excretion. Both low- and high-dose dexamethasone suppression tests had been performed showing no suppression of serum cortisol. Extensive radiological studies had been carried out, including MRI of the pituitary as well as chest X-ray, and CT of chest and abdomen, which were all judged as normal. At the time of referral cyclical Cushing’s syndrome, surreptitious use of corticosteroids (factitious Cushing’s syndrome) or cortisol resistance were all considered. However, the last two diagnoses seemed unlikely as elevated ACTH levels essentially ruled out use of corticosteroids, whereas intermittent hypercortisoluria is very unusual in cortisol resistance.

In our hospital, laboratory evaluation showed mild hypokalaemic alkalosis (potassium 3.1 (normal range 3.6 to 4.8 mmol/l), bicarbonate 31 (21 to 25 mmol/l)) with normal blood cell counts and chemistry. Excretion of free cortisol in 24-hour urine was initially normal, but strongly elevated thereafter with a maximal cortisol excretion of 28,050 nmol/24 h (normal <270 nmol/24 h) (Figure 1). Analysis of urinary steroid metabolites was performed by gas chromatography. The sum of the 24-hour urinary excretion of tetrahydrocortisol, allo-tetrahydrocortisol, tetrahydrocortisone, cortols and cortolones (reference range 11.6 to 45.9 µmol/24 h) was used as an estimate of cortisol production. The excretion of these glucocorticoid metabolites was strongly increased (821.4 µmol/24 h) during a period of hypercortisoluria (cortisol excretion 28,050 nmol/24 h) and was normal (29.8 µmol/24 h) during a period of normocortisoluria. Furthermore, androgen metabolites were also elevated in parallel with increased excretion of glucocorticoid metabolites, but no abnormal glucocorticoid metabolites were detected by additional mass spectrometry. These findings essentially ruled out factitious Cushing’s syndrome.

Random serum cortisol levels fluctuated from 130 to 2098 nmol/l (normal range 200 to 800 nmol/l) and plasma ACTH levels from 50 to 435 ng/l (normal range 10 to 100 ng/l). The results so far demonstrated ACTH-dependent hypercortisolism, characterised by a cyclic pattern of cortisol secretion. To elucidate the source of ACTH production (pituitary or ectopic), we repeated the dexamethasone suppression tests (DST) and performed a corticotrophin-releasing hormone (CRH) stimulation test. A low-dose DST (0.5 mg dexamethasone 6 hourly for 48 h) showed inadequate suppression of serum cortisol (290 nmol/l), in line with previous findings. Similarly, a high-dose DST (7 mg intravenously for 7 h) showed no suppression of serum cortisol levels, suggestive of ectopic ACTH secretion. The CRH test (100 µg human CRH intravenously) revealed a rise in serum cortisol of 68% (from 220 to 370 nmol/l) and a rise in ACTH of 700% (from 15 to 106 ng/l). Once Cushing’s syndrome is confirmed, a ≥20% increase in cortisol and/or ≥25% increase in ACTH is regarded as consistent with Cushing’s disease. Additional endocrinology evaluation showed normal thyroid function tests, normal plasma testosterone as well as a normal insulin-like growth factor-1. Plasma prolactin was modestly elevated (603, normal range <200 mU/l). Considering the normal pituitary images and the absence of a medication-related cause, its slight increase was possibly attributable to stress at the time of blood sampling. Plasma calcitonin level was normal as was the urinary excretion of catecholamine metabolites. Indium111 labelled pentreotide scintigraphy showed pathological uptake in the right part of the upper chest. Hence, the CT scans of chest and abdomen of the referring hospital were revised. On CT, a small tumour of 2 x 3 cm appeared to be present in the upper right anterior mediastinum, which had been overlooked initially. The anatomic localisation

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**Figure 1. Excretion of urinary free cortisol (nmol/24 h) before surgical removal of the thymic carcinoid**

![Figure 1](image-url)
corresponded with the results of pentreotide scintigraphy. Taken together, a thymic carcinoid was suspected. To evaluate possible growth or metastases, an MRI of the neck, chest and abdomen was carried out, identifying the described tumour without evidence of location elsewhere.

To biochemically demonstrate carcinoid tumour, platelet serotonin and urinary excretion of both serotonin and 5-hydroxyindoleacetic acid (5-HIAA) were measured. Furthermore, serum chromogranin A was measured as a neuroendocrine tumour marker. Of these, urinary serotonin (90 µmol serotonin/mmol creatinine, normal range <66 µmol serotonin/mmol creatinine) and serum chromogranin A (282 µg/l, normal range <100 µg/l) were found to be elevated, indeed consistent with a carcinoid tumour.

Selective venous catheterisation for ACTH sampling was carried out to demonstrate a gradient in ACTH concentration along the veins in the tumour area. ACTH levels in the femoral vein were simultaneously measured. The highest ACTH levels (422 ng/l) were found in the superior caval vein, just above the entrance of the azygos vein. Although this sampling approach evaluated the tumour region, the mediastinal to femoral vein ACTH ratio was only 1.25. On thoracotomy, a weak tumour of 4 x 3 x 3 cm, adjacent to the brachiocephalic vein, was removed. Histological examination showed an atypical thymic carcinoid tumour, angioinvasive and infiltrating into the surrounding fat tissue as far as the resection margin. Mitotic activity was high (5 per 10 high-power fields). Tumour cells were positive for immunohistological staining with ACTH, CAM 5.2, keratin, AE1/3, chromogranin, CD56 and synaptophysin.

In tumour tissue, profiling of indoles (tryptophan, 5-hydroxytryptophan, serotonin and 5-HIAA) was carried out by HPLC with fluorescence detection, as described previously. This revealed low concentrations of tryptophan (0.8 µmol/l) and 5-HIAA (23 nmol/l), whereas serotonin and its precursor 5-hydroxytryptophan (5-HTP) could not be detected. Furthermore, catecholamines were isolated from carcinoid tissue by paired ion extraction followed by HPLC with electrochemical detection, showing remarkably high concentrations of adrenalin (0.17 nmol/gram tissue) and noradrenaline (1.24 nmol/gram tissue), as compared with previous findings in foregut carcinoids.

After surgery, the patient showed a spectacular clinical improvement. Particularly, his ‘attacks’ of confusion and anxiety as well as weight fluctuations and full moon face disappeared. As expected, he temporarily developed partial cortisol deficiency, treated with cortisone suppletion. ACTH levels declined to 15 ng/l. Plasma chromogranin A and urinary serotonin decreased to normal values. A repeated whole body indium pentreotide scintigraphy showed no abnormalities. Two and eight months after surgery, a new CT of chest and abdomen was performed to evaluate possible tumour recurrence and/or metastases. All proved normal. To prevent recurrence, external radiotherapy was given to the mediastinal region (30 times 2 Gy). Since surgery, he has now been free of symptoms for 20 months.

**DISCUSSION**

Cyclical Cushing’s syndrome due to ectopic ACTH secretion is an intriguing but very rare clinical disorder. Its relation with a thymic carcinoid has only been described in five patients so far.6,7 Besides its rarity, our patient had extreme fluctuations in his urinary cortisol excretion, ranging from 27 to as high as 28,050 nmol per 24 h. Such striking cortisol fluctuations have rarely been reported before. The clinical and laboratory features of a cyclical Cushing’s syndrome are often misleading so that it may take years before the correct diagnosis is made. Disease-free periods may last from days to even years.8 Therefore, once Cushing’s syndrome is highly suspected, urinary free cortisol excretion should be repeatedly measured, even when initial values are normal.

ACTH-dependent cyclical Cushing’s syndrome may result either from a pituitary corticotroph adenoma or, less often, from ectopically secreted ACTH. The distinction between these entities is usually difficult to make. Clinical features of patients with ectopic ACTH production may mimic those found in patients with the classic Cushing’s disease, particularly when the underlying tumour is slow growing, as with carcinoid tumours. Although ACTH plasma levels are usually higher in the ectopic ACTH syndrome, considerable overlap exists with Cushing’s disease. Additional dynamic endocrine testing may be helpful but has a limited accuracy.9 Furthermore, the need to carry out these tests during an episode of hypercortisolism often introduces logistic problems.

The search for the source of the ectopic ACTH production may be extremely difficult since as many as 50% of these patients harbour an occult underlying tumour.1 The recommended diagnostic approach is a CT or an MRI scan of neck, chest and abdomen. MRI is preferable as it may detect bronchial carcinoid tumours overlooked on CT. It should be noted that on CT scans of the anterior mediastinum, thymic remnant tissue and small thymic carcinoid tumours might have a similar appearance, potentially leading to an incorrect diagnosis and unnecessary thoracotomy.14,15 Since thymic tissue regresses with age, this confusion will not often arise in patients aged older than 40 year.15 As most carcinoid tumours and other neuroendocrine tumours express somatostatin receptors, octreotide

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receptor scintigraphy has been evaluated for localisation of ACTH-producing tumours. Although its sensitivity has proved to be high, it generally does not disclose tumours that are not seen on conventional imaging, although exceptions have been reported. Hence, octreotide receptor scintigraphy is especially indicated when an ACTH-producing tumour is suspected but CT or MRI results are negative. If the tumour remains undetected, positron emission tomography (PET) may be useful, but its exact diagnostic position has to be established. For detection of carcinoid tumours, PET using the serotonin precursor \( \text{C}5\text{-hydroxytryptophan} \) is valuable. Finally, selective venous sampling for ACTH may be a last tool for localising an occult underlying tumour.

Carcinoid tumours produce biogenic amines, such as serotonin, which allows specific biochemical detection. Carcinoid tumours are classified into foregut, midgut, and hindgut tumours, according to their supposed origin from the primitive gut. Thymic carcinoids belong to the foregut tumours. For screening, urinary excretion of the serotonin metabolite 5-HIAA is widely used but has a low specificity due to interaction with ingestion of serotonin-containing foods. Platelet serotonin does not show such an interaction and is the most discriminative marker as it detects smaller increases in serotonin production. Foregut carcinoids have a lower serotonin metabolism than midgut carcinoids, as reflected by their low frequency of serotonin-related symptoms. These tumours usually do not secrete serotonin but its precursor 5-HTP, which is supposed to result from deficiency of the enzyme aromatic-L-aminoacid decarboxylase (AADC) required to convert 5-HTP to serotonin. Consequently, platelet serotonin may be normal in up to 55% of patients with foregut carcinoids, as in our patient. Noteworthy, elevated circulating 5-HTP in foregut carcinoids can be converted to serotonin by renal AADC activity, leading to increased urinary serotonin levels. Indeed, elevated urinary serotonin levels have been found in 40% of patients with foregut carcinoids. Our patient illustrates that urinary serotonin may be elevated despite normal platelet serotonin levels, underlining its value in carcinoid screening for foregut carcinoids. Unexpectedly, the tumour tissue from our patient contained neither serotonin nor its precursor 5-HTP, which is not in line with a postulated AADC deficiency. Moreover, the high concentration of tumour catecholamines rules out AADC deficiency, as this enzyme is also required for formation of catecholamines. The precise function of AADC in foregut carcinoids, therefore, remains to be established.

Thymic carcinoid tumours are very rare. As the annual incidence of carcinoids is about 3 per 10,000,000 persons a year, and only 2% of the carcinoids originate from the thymus, the estimated incidence of thymic carcinoid tumours is three per 10,000,000 persons a year. To date, about 150 patients with a thymic carcinoid tumour have been reported. Thymic carcinoid tumours tend to metastasise either lymphogenously, or haematogenously to lungs, bone, adrenals, liver, or spleen. Invasion of local thoracic structures is found in 50% of patients and extrathoracic metastases in 20 to 30%. Cushing’s syndrome is found in about 20% of patients with thymic carcinoid tumours. Sometimes, thymic carcinoid forms part of the multiple endocrine neoplasia type I syndrome. Despite aggressive treatment, thymic carcinoid tumours have a poor prognosis with a ten-year survival rate less than 50%, with lowest survival rate (35%) in patients associated with Cushing’s syndrome. In a large series of 342 cases with mediastinal or thymic carcinoid tumours, an overall survival of 38% after ten years was observed, similar to atypical and typical carcinoid tumours. Surgical treatment, as radical as possible, is the treatment of choice. The role of adjuvant radiotherapy or chemotherapy has not been well assessed as the number of studied patients is low. Nevertheless, several reports suggest a response to postoperative irradiation, particularly when the thymic carcinoid tumour is invasive.

Chemotherapy has been tried in some patients without a significant effect on recurrence rate or survival. The precise mechanism of periodic hypercortisolism is largely unknown. Changes in dopaminergic tone as well as influences of ghrelin have been described as possible underlying mechanisms. Tumour infarction would be an alternative explanation for periodic ACTH and cortisol release. Furthermore, Cushing’s syndrome may be food dependent and hence lead to periodic hypercortisolism. In our patient, the cause of intermittent ACTH secretion remained unclear, although potentially relevant observations were made. Most remarkable was the high concentrations of catecholamines in the carcinoid tissue, of which the adrenalin concentration was clearly higher than previously found in other foregut carcinoid tumours. It is therefore tempting to speculate that local overproduction of catecholamines may play a role in the cause of cyclic ACTH release. Interestingly, studies in rats have demonstrated that serotonin release in carcinoid tumour cells is controlled by adrenergic stimulation.

Assuming ACTH and serotonin are co-secreted, ACTH release might similarly be influenced by local (fluctuating) production of catecholamines, as could have been the case in our patient. This case report illustrates several clinically relevant aspects. Firstly, cyclical Cushing’s syndrome should be suspected in a patient with typical clinical findings of Cushing’s syndrome but normal biochemistry. Repeated measurement of urinary cortisol excretion is then required to establish or rule out the diagnosis. Secondly, octreotide receptor scintigraphy may be helpful to detect an ectopic ACTH-producing tumour, not disclosed (or overlooked).
on CT. Finally, measurement of urinary serotonin in addition to platelet serotonin or urinary 5-HIAA might be valuable to detect foregut carcinoids.

REFERENCES


Melaena in a liver transplant recipient

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A 25-year-old female presented with melaena. At birth, she had been diagnosed with biliary atresia, initially treated with Kasai hepatic portoenterostomy. At the age of 5, she required liver transplantation with Roux-en-Y biliary anastomosis. For 20 years following transplantation, her medical history had been uneventful. On admission, the patient was haemodynamically unstable and was admitted to the intensive care unit. The haemoglobin level was 6.2 mmol/l. Subsequent evaluation including upper and lower gastrointestinal endoscopy, angiography and bleeding scans did not reveal the source of the bleeding. An abdominal CT was performed with intravenous contrast and selected images are shown (figures 1 and 2).

WHAT IS YOUR DIAGNOSIS? WHAT ARE THE TREATMENT OPTIONS?

See page 30 for the answer to this photo quiz.

Figure 1.

Figure 2.
Online submission to the Netherlands Journal of Medicine: embracing the electronic future

J.P.H. Drenth, for the Editorial Board

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Some six months after the introduction of open access for the Netherlands Journal of Medicine, the Editorial Board wishes to implement another change. Until now, we have mostly put our trust in surface mail for the delivery of manuscripts and for all our contacts with reviewers and authors. Over the last year, however, we have noticed an increased use of electronic mail in communication with authors and reviewers. Some authors already use electronic mail for submission of manuscripts. These facts argue for the internet as favoured medium for communication and as a consequence we have decided to step in.

From 1 February onwards we will be implementing an online submission system for both authors and editors of the Netherlands Journal of Medicine. We have decided to use the software package ‘Manuscript Central’, a web-based submission and peer-review system. This system has the advantage that over 1000 biomedical journals use it and that it is widely known among authors and reviewers. It is easy to use and offers maximum convenience to authors and editors. The system supports the complete process of submitting and reviewing manuscripts, as well as the correspondence between authors, editorial office and the reviewers. Manuscript Central requires no additional preparation beyond having your manuscript files in an electronic format: text can be submitted as Microsoft Word. In addition graphics can either be embedded in the word-processed document or uploaded separately, for example as a joint photographic experts group (JPEG) file or as a tagged image file format (TIFF) file. The text and graphic files are automatically converted into an adobe portable document format (PDF) document for distribution and review. After verification by the corresponding author, the system generates immediate confirmation by electronic mail. In addition, the status of the manuscript can be tracked as it moves through the peer-review process. Once the Editors have reached a decision regarding the manuscript the authors will be updated immediately. Subsequent revisions, if required, will be handled online in a similar fashion. Likewise, peer review will also be an online process. We will invite a reviewer from our panel of referees by electronic mail to review the manuscript which will be made available online as a PDF file from the same website. The system has clear rewards; its use will enhance the administrative efficiency and shorten the handling time of manuscripts. In the end, this will further decrease the lag time between submission and final publication in the Journal.

The consequence of this move is that the Editorial Office will not handle conventional mail submissions from 1 February 2006 onwards. Full instructions for manuscript submission are available on the website at http://mc.manuscriptcentral.com/nethjmed. We have thoroughly tested the system and we do not anticipate any problems, but authors who experience technical difficulties may contact the Editorial Office via electronic mail.

During the last year the Journal has witnessed many changes. As recently reiterated, the number of people accessing and downloading papers published in the Netherlands Journal of Medicine has progressively increased since the introduction of the full text online version of the Journal. We feel that by embracing the new technology the Journal has obtained the competitive edge. We invite you as reader to be part of our Journal by submitting your clinical observations and/or fruits of your research to us. The benefits for authors publishing in the Journal are clear: because of open access the widest possible dissemination of your work is possible. Therefore, we encourage you to submit your manuscripts online at http://mc.manuscriptcentral.com/nethjmed.

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Following the CT, percutaneous transhepatic venography was performed (figure 3) and portal venous pressure measurements were obtained. The CT scan in the portal venous phase showed a dilated portal vein and contrast extravasation in the Roux-Y limb (figure 1, arrow) and downstream in the gut lumen (figure 2, arrow). A bleeding focus was suspected at the Roux-en-Y site, probably associated with portal hypertension. Percutaneous transhepatic venography showed the presence of a portal vein stenosis (figure 3, white arrow) with portal hypertension illustrated by marked dilatation of the coronary vein (figure 3, black arrow). The portal venous pressure gradient across the stenosis was 10 mmHg. The stenosis was treated by transhepatic balloon venoplasty (figure 4), after which the coronary vein dilation disappeared (figure 5). The gradient completely resolved following the intervention. After the procedure, the bleeding stopped and further recovery was uneventful.

Upper gastrointestinal bleeding is a common complication following liver transplantation. In a series of 92 gastrointestinal bleeding episodes in liver transplant recipients with endoscopic diagnoses the most common causes were ulcers (n=25), enteritis (n=24), oesophageal or gastric varices (n=15) and Roux-en-Y bleeds (n=6). In the patients with Roux-en-Y bleeds, endoscopic investigations were nondiagnostic and the diagnosis was established by laparotomy. In the setting of gastrointestinal bleeding and portal hypertension, one should be aware of bleeding sources not only from the oesophagus or stomach, but also uncommon sites with possible varices, such as duodenum, Roux-en-Y limb, gallbladder or colorectum. In liver transplant recipients with an anastomotic portal vein stenosis, treatment with percutaneous transhepatic balloon venoplasty has been shown to be effective, eliminating the need for surgical revision, portacaval shunting, or retransplantation.

**REFERENCES**

This month’s cover is a print by Andrea Anatas. This print shows a snake; as the snake and the staff of Aesculapius are both symbols of medicine, Anatas thought this print would be perfect for the cover of the Netherlands Journal of Medicine. The technique used is a gouache print on cardboard.

Andrea Anatas was born in Kleve, Germany and brought up in an artistic family, so her choice to become a graphic artist was a logical one. Because of her Dutch roots, she decided to attend the Academy of Arts in Arnhem, the Netherlands. There her talent did not remain unnoticed; after competing her studies, the Dutch Ministry of Welfare, Health and Culture granted her a two-year starter’s scholarship. Since 1985 she has been living and working in Cologne. She has exhibited her work in many group and solo expositions in Germany and the Netherlands.

An original of this print (size 50 x 70) is available at a price of €300 and can be ordered from Galerie Unita, Rijksstraatweg 109, 6573 CK Beek-Ubbergen, the Netherlands or by e-mail: galerie-unita@planet.nl of www.galerie-unita.com.
Aims and scope
The Netherlands Journal of Medicine publishes papers in all relevant fields of internal medicine. In addition to reports of original clinical and experimental studies, reviews on topics of interest or importance, case reports, book reviews and letters to the editor are welcomed.

Manuscripts
Manuscripts submitted to the Journal should report original research not previously published or being considered for publication elsewhere. Submission of a manuscript to this Journal gives the publisher the right to publish the paper if it is accepted. Manuscripts may be edited to improve clarity and expression.

Language
The language of the Journal is English. English idiom and spelling is used in accordance with the Oxford dictionary. Thus: Centre and not Center, Tumour and not Tumor, Haematology and not Hematology.

Preparation of manuscripts
Type all pages with double spacing and wide margins on one side of the paper. To facilitate the reviewing process, number the lines in the margin and the pages. Subheadings should not exceed 55 characters, including spaces.
Abbreviations: Measurements should be abbreviated according to SI units. All other abbreviations or acronyms should be defined on the first appearance in the text. Use a capital letter for generic names of substances and materials.
A Covering letter should accompany the manuscript, identifying the person (with the address, telephone number, fax number and e-mail address) responsible for negotiations concerning the manuscript. The letter should make it clear that the final manuscript has been seen and approved by all authors. Conflicts of interest, commercial affiliations, consultations, stock or equity interests should be specified. In the letter one to three sentences should be dedicated to what this study adds. All authors should sign the letter.

Divide the manuscript into the following sections: Title page, Abstract, Keywords, Introduction, Materials and methods, Results, Discussion, Acknowledgements, References, Tables and Figures with Legends.

The Title page should include authors' names, degrees, academic addresses, correspondence address, including telephone number, fax number, e-mail address and grant support. Also the contribution of each author should be specified.
The title should be informative and not exceed 90 characters, including spaces. Avoid use of extraneous words such as ‘study’, ‘investigation’ as well as priority claims (new, novel, first). Give a running title of less than 50 characters. If data from the manuscript have been presented at a meeting, list the name, date and location of the meeting and reference and previously published abstracts in the bibliography. Give a word count (including references, excluding tables and legends) at the bottom of this page.
The Abstract, not exceeding 250 words, should be written in a structured manner and with particular care. In original articles, the Abstract should consist of the following paragraphs; Background, Methods, Results and Conclusion. They should briefly describe the problem being addressed in the study, how the study was performed and which measurements were carried out, the most relevant results, and what the authors conclude from the results.

Keywords: Include three to five keywords.
The Introduction should be brief and set out the purposes for which the study has been performed.
The Materials and methods should be sufficiently detailed so that readers and reviewers can understand precisely what has been done without studying the references directly. The description may be abbreviated when well-accepted techniques are used.
The Results should be presented precisely, without discussion.
The Discussion should directly relate to the study being reported. Do not include a general review of the topic, but discuss the pertinent literature.

Acknowledgement: All funding sources should be credited here. Also a statement of conflicts of interest should be mentioned.
References should be numbered consecutively as they appear in the text (after the punctuation and in square brackets). Type the reference list with double spacing on a separate sheet. References should be in the language they are published in, conform the 'Vancouver' style for biomedical journals (N Engl J Med 1991;324:424-8).
Journal abbreviations should conform to the style used in the Cumulated Index Medicus. Examples:


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Case reports containing concise reports on original work will be considered for publication. Case reports which are relevant for understanding the pathophysiology or clinical presentation of disease may also be accepted under this heading. Articles published in this section should be no longer than 1000 words, and supplied with a summary of about 60 words, preferably no more than two figures and/or tables, and no more than 15 references.

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