ASCA

ASCA: genetic marker, predictor of disease, or marker of a response to an environmental antigen?

F Seibold

Anti-*Saccharomyces cerevisiae* antibodies (ASCA) may be a marker of an immune response to an environmental antigen that occurs in the context of early stage Crohn's disease

The presence of antibodies against the yeast *Saccharomyces cerevisiae* (ASCA) and against neutrophils (pANCA) has been used as diagnostic serological markers for inflammatory bowel disease (IBD) for many years. The combination of a positive ASCA test with a negative pANCA test has a positive predictive value of 96% and a specificity of 97% for Crohn's disease (CD).¹ However, both antibodies have been found in other diseases, such as autoimmune liver disease, primary sclerosing cholangitis (pANCA), and in gluten sensitive enteropathy (ASCA). Therefore, their role as diagnostic serological markers for IBD seems to be limited.

Antibody determination is of interest in patients with indeterminate colitis. However, almost 50% of these patients do not develop ASCA or pANCA antibodies whereas in antibody positive patients, ASCA+/pANCA- predicts CD in 80% of patients with indeterminate colitis and ASCA-/pANCA+ predicts ulcerative colitis (UC) in 64%.²

Generation of both antibodies is poorly understood. Several studies have shown that titres of both antibodies do not correlate with disease activity, as known from classical autoimmune disease. Antibody titres seem to be stable over long periods of time. Surprisingly, pANCA in UC persist after colectomy,³ and we have observed patients who have had their last flare up of CD more than 20 years ago and currently display normal findings in gastroscopy, colonoscopy, and histology, but still have high titres of ASCA. Thus these antibodies seem to represent stable serological markers. The only clinical parameter confirmed by several groups is the correlation between ASCA positivity and ileal involvement of disease and penetration as well as structuring disease behaviour.⁴

The question has been raised whether pANCA and ASCA represent genetic

markers for susceptibility to IBD. Several studies tried to elucidate this question. Family studies showed that 16–30% of healthy first degree relatives of patients with UC were pANCA positive.^{5–6} Although these studies could not be confirmed by others, probably due to methodological problems, they indicate that pANCA may be a genetic marker.⁷ Comparable with pANCA research, several studies showed that ASCA were detectable in 20–25% of first degree relatives of patients with CD.^{8–9} Healthy spouses however were generally antibody negative, indicating that genetic and not environmental factors play a decisive role. The prevalence of ASCA in families with more than two affected members suffering from CD was significantly higher than in families with only two affected members, which points towards the role of ASCA as a genetic marker.¹⁰ In the same study however, the prevalence of these serological markers did not differ in pure Crohn's families overall from sporadic cases. Therefore, the question needs to be raised whether these antibodies develop as an epiphenomenon during the onset of disease. It is known that luminal antigens such as bacteria and yeast seem to play an essential role for the perpetuation of inflammatory processes. In patients with CD, loss of immune tolerance towards the resident bacterial flora is one of the major pathogenetic concepts for this disease. Possibly, pANCA are due to cross reactivity to bacterial antigens.¹¹ Bacterial and yeast antigens are ubiquitous, permanently present in the gastrointestinal tract. Therefore, it would be of great interest to evaluate when these antibodies are generated.

The study of Israeli and colleagues¹² in this issue of *Gut* is the first to provide an answer to this question (see page 1232). In this study, ASCA were detected in 31% of patients before the clinical diagnosis of CD. Furthermore, an

increase in ASCA frequency was observed over time, with the highest frequency documented in the 36 months before the diagnosis of CD. These results indicate that ASCA development occurs before or during the early stages of disease. This thesis was confirmed by one patient who was ASCA negative 80 months before diagnosis but was ASCA positive 48 months before diagnosis. Hence ASCA do not seem to be generated as genetic markers in early childhood but in the context of early disease. ASCA may therefore be a marker of an immune response to an environmental antigen that occurs in the context of an early stage of disease. In some patients with other autoimmune diseases, such as lupus erythematosus and rheumatoid arthritis, antibodies were detected up to nine years before diagnosis.^{13–14} However, whether inflammatory bowel disease (IBD) autoantibodies are markers of future disease, as has been suggested for classical autoimmune disease, has yet to be determined. If this hypothesis is true, the high frequency of ASCA in family studies would indicate that the frequency of diseased family members is substantially higher than actually known.

In Israeli's study,¹² four of eight patients had an increase in ASCA titres whereas in two patients the titres decreased. The increase in titres was interpreted as crescendo autoimmunity by Israeli *et al*, although this finding must be interpreted carefully considering the small number of patients available.¹² Furthermore, antibody titres in IBD are generally stable, in contrast with various other autoimmune diseases where there is a correlation between clinical activity and titre levels. Therefore, it is questionable whether IBD and other classical autoimmune diseases can be compared.

The initial event leading to IBD is still unclear but of major interest. ASCA positivity has been found to be associated with a deficiency in mannan binding lectin, a component of the innate immune system.¹⁵ The theory that the initial incident leading to IBD is an infection in patients with a defect in their innate immune system is still speculation. The data of Israeli *et al* can be interpreted in two ways: either the autoimmune reactions precede the disease or a latent subclinical disease is followed by generation of antibodies as an epiphenomenon. Which came first, the chicken or the egg?

Approximately seven decades after the first description of CD, our knowledge about this disease is still limited. Above all we do not know the number of undiagnosed cases, if there are

asymptomatic patients, or whether a subclinical form of CD exists, as is in the case of individuals who are hepatitis C virus RNA positive but have normal transaminases. Ten to 20 year since the description of ASCA and pANCA, the paper of Israeli and colleagues¹² shows that ASCA and pANCA precede the clinical diagnosis of IBD. This study should encourage the scientific world to perform large studies where antibody positive and negative healthy family members are followed over a long period of time. This will help determine whether CD develops more frequently in antibody positive persons compared with those who are antibody negative. Furthermore, regular precise clinical observation may detect subclinical disease in antibody positive and negative patients.

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Osteopontin

Osteopontin: a new addition to the constellation of cytokines which drive T helper cell type 1 responses in Crohn's disease

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Osteopontin, a cytokine which promotes Th1 immune responses, is overexpressed in the gut of patients with Crohn's disease or ulcerative colitis. The main cellular source of this cytokine appears to be gut plasma cells.

Crohn's disease appears to be caused by an excessive CD4⁺ T helper cell type 1 response directed against undefined antigens of the commensal bacterial flora.¹ T cells from affected areas of Crohn's disease mucosa produce enhanced amounts of interferon γ (IFN- γ) and tumour necrosis factor α . Other markers of Th1 cells, such as expression of the transcription factor T-bet, the high affinity β 2 chain of the interleukin 12 (IL-12) receptor, and activated STAT4, all indicate that the mucosal environment in Crohn's disease favours Th1 polarisation.¹

It is important to emphasise that CD4 T cells in normal bowel are also Th1 skewed and express T-bet, so that the differences seen in Crohn's disease are quantitative rather than qualitative.² Normal mucosal T cells are however susceptible to apoptosis, whereas this is not the case in Crohn's disease,³ suggesting that it is the persistence and accumulation of Th1 cells which drives tissue injury.

Factors which commit virgin T cells to the Th1 or Th2 pathway are still under investigation. T-bet appears to be of primary importance.⁴ It is induced by

IFN- γ itself and is capable of promoting IFN- γ production, not only in Th1 cells, but also in Th2 cells. T-bet also increases expression of the IL-12R β 2 chain. Macrophage and dendritic cell derived IL-12 is crucial in Th1 immune responses.⁵ The IL-12 receptor is made of two chains, β 1 and β 2, but only the β 2 chain can signal and phosphorylate STAT4 which then migrates to the nucleus and further boosts IFN- γ production. Other factors also augment Th1 polarisation. IL-18 activates the transcription factors AP-1 and nuclear factor κ B in T cells, and acts synergistically with IL-12 to boost IFN production. Type 1 interferons can also activate STAT4. IL-7, IL-15, and IL-21 also act in synergy with IL-12 to boost IFN- γ production and all, including IL-18, are overexpressed in Crohn's disease.⁶ The IL-12 p40 chain can form a heterodimer with p19 protein to form a recently described cytokine, IL-23, which appears to be important in the activation of memory Th1 cells.⁵ There are no publications on IL-23 in Crohn's disease.

In this issue of *Gut*, Sato and colleagues⁷ identify osteopontin (also known as early T lymphocyte activation Eta-1) as yet another cytokine involved in the Th1 response of Crohn's disease (see page 1254). Osteopontin is a 60 kDa phosphoprotein constitutively secreted by epithelial cells and bone.⁸ It contains the characteristic RGD sequence seen in extracellular matrix proteins and shares receptor binding on cells with

Figure 1 Diagrammatic indication of the cytokines, transcription factors, and signalling molecules which polarise virgin CD4 cells into T helper cell type 1 cells. Interferon γ (IFN- γ) induces the transcription factor T-bet which induces the high affinity interleukin (IL)-12 receptor on T cells. IL-12 can then bind to its receptor, and activate STAT4, which goes to the nucleus and activates IFN- γ transcription. A plethora of other cytokines can synergise with IL-12 to boost IFN- γ production. IL-23, an IL-12-like cytokine, seems to be more important in activating memory cells. A crucial component of this pathway is the feedback loop by which IFN- γ continues to drive T cells along a Th1 pathway.

extracellular matrix proteins, including α v and β 1 integrins. It also binds to CD44. Osteopontin is expressed in many distinct forms depending on differential splicing and the glycosylation/phosphorylation status of the core protein. Proteolytic cleavage by thrombin and matrix metalloproteinases also changes its function. Osteopontin is important in many different tissues. In the bone it regulates calcium deposition and it is also a chemoattractant for cancer cells. However, it is also important in the immune system as it is made by activated T cells, macrophages, and dendritic cells. These cells also have osteopontin receptors.⁹

Osteopontin increases the adhesion of activated T cells and is a T cell chemoattractant, but importantly it supports Th1 responses and inhibits Th2 responses⁹; thus its identification in Crohn's disease adds another member to the increasing number of cytokines which drive Th1 immune responses (fig 1). Osteopontin deficient mice show impaired Th1 immune responses and fail to make granulomas.⁹ There was therefore a strong rationale for looking at osteopontin in Crohn's disease. Sato and colleagues⁷ now show that osteopontin transcripts and protein are elevated in Crohn's disease and ulcerative colitis. Osteopontin also increases IL-12 production specifically in Crohn's disease lamina propria mononuclear cells but has little effect on IL-10 production. Arguably, the most important finding of the paper is the source of the osteopontin. There was a predictable increase in immunoreactivity in epithelial cells, macrophages, and around granulomas in Crohn's disease. However, the most

striking feature in inflammatory bowel disease (IBD) was coexpression of osteopontin with mucosal IgM, IgG, and IgA plasma cells. Especially in Crohn's disease, osteopontin was particularly associated with IgG plasma cells. This result has some similarities with a previous study on osteopontin in Crohn's disease and normal ileum where production in plasma cells was also noted, although overall no differences were seen between controls and Crohn's samples.¹⁰ However, gene array analysis has shown markedly increased osteopontin transcripts in ulcerative colitis colon compared with normal colon.¹¹

The role of plasma cells in IBD has been somewhat overlooked in recent years, with most emphasis on cellular immunity. However, quantitatively, plasma cells are as abundant as T cells in normal and inflamed gut. By far the largest population of plasma cells in normal and inflamed gut secrete IgA, generally considered to be a beneficial non-phlogistic antibody. In IBD, the largest quantitative increase is in IgA plasma cells, but the biggest proportional increase is in IgG plasma cells as these are uncommon in the normal gut. In Crohn's disease, IgG plasma cells tend to be found around ulcers whereas in ulcerative colitis they are present along the length of the diseased mucosa.¹² It has been suggested that some of this IgG has specificity for gut autoantigens, such as epithelial tropomyosin, indicating that at least part of the pathogenesis of ulcerative colitis may involve antibody mediated autoimmunity.¹² The results of Sato *et al* suggest that IgA and IgG plasma cells

may be far more important than previously considered, with a role greater than simply being antibody secreting factories.

The work of Sato and colleagues⁷ also poses some interesting questions. Is osteopontin always made by plasma cells and is its presence in the gut merely a feature of the fact that the gut contains more plasma cells than the rest of the body combined? Do plasma cells secrete bioactive osteopontin? If osteopontin is present in plasma cells in healthy gut, why does it not deliver a survival signal to CD4 cells? Is osteopontin involved in the migration of T cells into normal and inflamed gut? It would also be of interest to examine trinitrobenzene sulphonic acid colitis in osteopontin deficient mice.

The importance of the therapeutic benefit of neutralising Th1 inducing cytokines in Crohn's disease is well demonstrated by the clinical success of anti-IL-12 antibodies.¹³ However, the antibody used in this study is against the p40 subunit of IL-12 and theoretically can also neutralise IL-23. However, there may be considerable heterogeneity between patients in the relative importance of other Th1 inducing cytokines, such as IL-18 and osteopontin, which deserve further investigation.

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Pancreatic cancer

Silencing RNA: a novel treatment for pancreatic cancer?

N R Lemoine

The high sequence specificity of RNA interference may make it suitable to treat diseases that are linked to selective or elevated expression of particular identified genes, such as in pancreatic cancer

The antiapoptotic gene Bcl-2 has been a target for downregulation by nucleic acid based strategies for more than a decade but the recent failure of the synthetic antisense oligonucleotide agent Genasense in phase III clinical trial caused many to think again about the worth of this approach. However, a new optimism about this and other targets is spreading through the community following several encouraging applications of the recently discovered technology of RNA interference, which is essentially a new biological version of the antisense system.¹

RNA interference is considered to have begun as an evolutionarily ancient mechanism for protecting organisms from viruses. Many viruses have RNA, rather than DNA, as their genetic material and go through at least one stage in their life cycle in which they make double stranded RNA. Perhaps not surprisingly, all multicellular organisms have evolved a well conserved protein apparatus that destroys double stranded RNA but this has also been found to play a role in maintenance of the organism's own genome stability by suppressing the movement of mobile genetic elements, such as transposons and repetitive sequences.

The gene silencing process of RNA interference (RNAi) involves the manufacture of short double stranded RNA molecules by an enzyme called DICER, which cleaves RNA duplexes into 21–26 base pair oligomers. These small interfering RNAs (siRNA) cause sequence specific, post-transcriptional gene

silencing by guiding an endonuclease, the RNAi induced silencing complex (RISC), to mRNA. This process has been seen in a wide range of organisms such as *Neurospora fungus* (in which it is known as quelling), plants (post-transcriptional gene silencing), and mammalian cells (RNAi). Downregulation of target gene expression has been found to involve interactions at multiple levels. Where there is complete or near complete sequence complementarity between the small RNA and the target, the Argonaute 2 component of RISC mediates cleavage of the target transcript.^{2–3} In contrast, where there is sequence mismatch between the miRNA and the target transcript, the mechanism appears to involve repression of translation predominantly.⁴ More recently, it has been recognised that siRNA molecules can induce transcriptional silencing through promoter methylation.^{5–6}

In principle, the high sequence specificity of RNA interference might make it suitable to treat disease that is linked to selective or elevated expression of particular identified genes. This may make it particularly appropriate for combating cancers associated with mutated endogenous gene sequences. An early example of the potential power of this approach came in a study of pancreatic cancer. *RAS* genes are frequently mutated in human cancers, particularly in pancreatic and colon carcinomas. Mutant *RAS* oncogenes often contain point mutations that alter only a single amino acid, which locks the oncogenic *RAS* proteins in a persistently activated

GTP bound state. A complication in using *RAS* oncogenes as targets in anti-cancer therapy is that at present it is not possible specifically to inhibit the biochemical function of only the oncogenic *RAS* alleles. This may be essential as the wild-type *K-RAS* gene appears to be required for viability, as evidenced by the embryonic lethal phenotype of mice nullizygous for *K-ras*.⁷ Retroviral delivery of siRNAs can specifically inhibit the mutant *K-RASV12* allele in human pancreatic carcinoma cells, while leaving the wild-type *K-RAS* allele untouched.⁸ In spite of the fact that pancreatic carcinoma cells have many genetic alterations, loss of *K-RASV12* expression leads to loss of tumorigenicity in experimental animal models.

In this issue of *Gut*, Ocker and colleagues⁹ explore the use of siRNAs against another gene that is aberrantly expressed at high frequency in pancreatic cancer, the antiapoptotic gene bcl-2 (see page 1298). Their results suggest that the target can be selectively down-regulated in tumour cells in vitro and that intraperitoneal administration of the naked nucleic acid agent can produce variable antitumour effects against malignant deposits growing subcutaneously in vivo. In this study—as in most experiments using RNAi to target particular genes in mammalian cells—the results are interpreted as representing induction of sequence specific transcript cleavage. However, at this early stage in our understanding of RNAi, it is important not to rule out the possibility that interference mediated through protein translational repression or genomic modification (DNA methylation or histone modification) may also be playing a role in mediating gene specific silencing and any derived RNAi phenotype. The great attraction of therapeutic epigenetic gene specific silencing lies in its heritable nature, meaning that, unlike post-transcriptional gene silencing that requires the continued presence of an siRNA molecule targeting a coding sequence, long lasting suppression of gene expression could be achieved from a single exposure to a specific methylation inducing RNAi agent targeting a promoter sequence.

The major challenge in turning RNA interference into an effective therapeutic strategy is the delivery of RNA

interference agents, whether they are synthetic, short double stranded RNAs (as in the paper by Ocker and colleagues⁹) or viral vectors directing production of double stranded RNA, to the target cells within the body. While siRNA technology has proven extremely powerful and robust for cell culture work, translating this success reliably to animals or humans is proving very difficult, due to insufficient bioavailability of the compounds. However, an important step in the right direction is that Jürgen Soutschek and colleagues have recently been able to demonstrate siRNA mediated downregulation of apolipoprotein B in the liver (and jejunum) of mice using cholesterol conjugates delivered systemically.¹⁰ The effects were preferentially seen in the liver, which is a relatively easy organ to target, and relatively high dosages (three injections each of 50 mg/kg) were required for the effect. In view of the extremely high potency of the siRNA in in vitro cell cultures, one must conclude that only a very small fraction of the injected siRNA actually reaches its molecular mRNA target in liver cells. Thus it is unfortunately not likely that simple cholesterol conjugation will solve the general delivery problem of siRNA. Other cationic cell penetrating peptides such as penetratin, Tat, and more recently transportan and oligo arginine have been proposed as

general transmembrane carriers for a variety of cargoes, including oligonucleotides and PNA.^{11,12} However, it appears that the main uptake route for most, if not all, of these peptides is endosomal, and thus the reagents have to escape the endosomal compartment in order to enter the cellular compartments of action: the cytoplasm and/or nucleus. The problem of cellular delivery is yet more complex for clinical application where the real challenge for an adjuvant therapy agent is delivery and maintenance of the compound in cancer cells in multiple organs in humans. However, the biotechnology industry has readily recognised the potential silencing properties of RNA mediated interference, with at least 15 companies active in the field, and two companies (Ribopharma and Benitec) have patents for RNAi based clinical applications. Significant progress in delivery technology is required before the concept of RNAi can realistically benefit cancer patients, but exploitation of the decade of clinical experience of antisense and viral gene therapy agents gives investigators a head start.

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