Chapter 1

Hot Topics in Autoimmunity

Intravenous immunoglobulins: the best biologic treatment available for autoimmune diseases

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Keywords

IVIG, intravenous immunoglobulins, biotherapy, autoimmune diseases, autoantibodies, anti-idiotypic antibodies

Abbreviations

Tumor Necrosis Factor α (TNFα), Interleukin (IL), Intravenous Immunoglobulin (IVIG), Specific IVIG (sIVIG), Anti-citrullinated protein anti-idiotypic-antibodies (ACPA), Collagen-induced arthritis (CIA), Systemic Lupus Erythematosus (SLE), Recurrent Pregnancy Loss (RPL), Acetylcholine receptor (AChR), Rheumatoid arthritis (RA), B cell antigen receptor (BCR), Subcutaneous immunoglobulins (SCIG), Tuberculosis (TB)

Abstract

Novel biologic treatments are introduced recently for patients with autoimmune diseases**.** Previous studies demonstrate the effectiveness of intravenous immunoglobulins (IVIG) in the treatment of systemic lupus erythematosus, rheumatoid arthritis, pemphigus vulgaris and other immune-mediated diseases. Here, we suggest the notion that currently, IVIG remains the leading biologic treatment available for autoimmune diseases.

Introduction

In recent years, novel biologic treatments are introduced to patients with autoimmune and inflammatory diseases. Drugs such as tumor necrosis factor (TNF) α inhibitors, anti-interleukin (IL)-1 and others, offer new therapeutics modalities (1-3).

Intravenous immunoglobulin (IVIG) was found to reduce morbidity in autoimmune, inflammatory and immune-mediated diseases (4, 5). Such diseases include graulomatosis and polyangiitis (6), systemic lupus erythematosus (SLE)(7-14), anti-Ro/anti-La associated congenital atrioventricular block(15, 16), antiphospholipid syndrome(17), heparin-induced thrombocytopenia(18), immune-mediated

thrombocytopenia (19), Churg-Strauss vasculitis(20) and many others. IVIG was demonstrated to improve survival in patients with polymyositis and dermatomyositis (21). It was even suggested to help in chronic ocular inflammatory diseases such as refractory uveitis, birdshot disease and ocular cicatricial pemphigoid (22).

Studies comparing IVIG and other modes of biotherapy are scarce. However, evidences for the effectiveness of IVIG in treatment with autoimmune diseases are increasing. Here, we suggest the notion that currently, IVIG remains the leading biologic treatment available for autoimmune diseases.

Efficacy of IVIG as treatment for autoimmune diseases

a) The presence of anti-idiotypic antibodies in IVIG

Recently, the presence of anti-idiotypic antibodies (specific IVIG (sIVIG)), which are found in IVIG and bind to autoantibodies, was found to be effective in ameliorating symptoms and improving outcome in different autoimmune diseases models(23).

In one study, anti-anti-citrullinated protein anti-idiotypic-antibodies (anti-ACPA) were fractionated from IVIG and were tested as a treatment for collagen-induced arthritis (CIA) in mice (24). ACPA-sIVIG was significantly more effective than IVIG in reducing the development of CIA. Treatment of splenocytes from CIA mice with ACPA-sIVIG reduced the ACPA and anti-collagen-antibody titers. Moreover, higher levels of anti-inflammatory cytokines and lower pro-inflammatory cytokines were detected in the treated cells (24).

Anti-anti-β2GPI antibodies were demonstrated to inhibit human trophoblast cell invasion and to improve significantly the pregnancy outcome in mice with anti-phospholipid syndrome in comparison to mice treated with IVIG(25).

Comparison between mice with systemic lupus erythematosus (SLE) treated with anti-dsDNA anti-idiotypic antibodies to untreated mice demonstrated a significant reduction in the titer of anti-dsDNA antibodies, the level of proteinuria and capillary IgG deposits in the kidneys, as well as an increased survival time in the sIVIG treated mice (26). Interestingly, IVIG was also found to contain anti-idiotypes to oxidized LDL antibodies (27). As one of the complications of SLE is accelerated atherosclerosis, it may be helpful in immunomodulation of atherosclerosis in these patients.

IVIG was previously demonstrated to be useful in patients with recurrent pregnancy loss (RPL), which is characterized by high titers of anti-elastin IgG autoantibodies (17, 28, 29). In one study IVIG was shown to contain anti-elastin and anti-anti-elastin idiotypes, which may account for its beneficial effect in RPL(30).

In an experimental model of myasthenia gravis in rats successful IVIG treatment was dependent on the presence of anti-anti-acetylcholine receptor (AChR) IgG specific IVIG (31).

In mice with pemphigus vulgaris, anti-desmoglein/3-idiotype-IVIG was also found to decrease cutaneous lesions and prevent the formation of acanthylosis and the deposition of IgG in the intercellular spaces (32).

In rheumatoid arthritis (RA), autoantibodies play a role in the pathogenesis of synovial inflammation and joint destruction through the activation of the complement and Fc-γ receptor pathways. Thus, IVIG treatment may be beneficial in patients with vasculitis, overlap Rhupus syndrome, severe infections with active disease, and pregnancy, where anti-cytokine blockers or rituximab may be unwarranted (33).

b) Anti- inflammatory activity of IVIG

IVIG also possesses anti-inflammatory activity, which is mainly mediated by antibodies containing terminal α2,6-sialic acid linkages at the Asn297-linked glycan of Fc region. This sialic acid-rich IVIG fraction possesses an anti-inflammatory effect by the induction of the expression of the inhibitory IgG Fc-IIB receptor, which is exclusively expressed on B cells and serves as a negative regulator for inhibiting B cell antigen receptor (BCR)-elicited activation (5, 32).

c) The additive effect of IVIG in autoimmune- associated primary immune deficiencies

IVIG therapy appears to be beneficial in patients with primary immune deficiency and immune dysregulation. It appears to have a double effect both as supplementation of immunoglobulins in these patients and as immune-modulation therapy. It was successfully used in X-linked agammaglobulinemia, common variable immunodeficiency, X-linked hyper-IgM, severe combined immunodeficiency, Wiskott-Aldrich syndrome, and selective IgG class deficiency(34). The additive effect of IVIG without immune-suppression is an important advantage.

Safety and financial issues of IVIG in treatment for autoimmune diseases

IVIG's immediate adverse reactions include headache, flushing, malaise, chest tightness, fever, chills, myalgia, fatigue, dyspnea, back pain, nausea, vomiting, diarrhea, blood pressure changes, tachycardia, and anaphylactic reactions, especially in IgA-deficient patients (35). Late adverse events are rare and include acute renal failure and thromboembolic events (36) .

Other important disadvantages of IVIG are the need of an intravenous route of administration and high costs. The recently introduced subcutaneous Ig (SCIG) may offer an alternative. Its use is easier, has fewer adverse reactions with longer IgG serum levels and it is cost effective, as compared to IVIG (37). Like IVIG, SCIG was found to be useful in immune-mediated diseases, such as inflammatory myositis and demyelinating neuropathies (37).

Unlike TNFα modulating drugs (infliximab, etanercept and others), IVIG does not possess a risk of the development of opportunistic infections such as tuberculosis (TB). It can be used safely in patients with latent TB and the pre-emptive tuberculin testing is not needed. IVIG lack immunosuppressive properties and it has a wide therapeutic range (1).

Biologic drugs are often expensive and their cost effectiveness in public health systems remains to be evaluated. In patients with rheumatoid arthritis, infliximab was found unlikely to be cost effective in the Medicare program compared to other TNFα inhibitors, as in the case of anakinra compared to infliximab, etanercept and adalimumab (38). Thus, IVIG may offer an alternative and decrease the financial burden of biologic drugs.

Conclusions

With the increasing use of SCIG and sIVIG for specific autoimmune diseases, it seems that immunoglobulins will remain the leading biotherapy for autoimmune diseases. Comparison between IVIG, sIVIG and other biological drugs in specific autoimmune, inflammatory and immune-mediated diseases remains to be evaluated.

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Can we cure autoimmune diseases?

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Abstract

The treatment of autoimmune diseases mainly comprises glucocorticoids, immunosuppressive drugs and biologics, which more selectively affect the disturbances in the immune system. So far, the available therapies can reduce disease activity or induce remission. However, a permanent treatment is needed to maintain this clinical improvement, which has substantially prolonged the survival of severe autoimmune disease. But then chronic administration of these drugs is related to adverse events such as accelerated atherosclerosis or infections. Nevertheless, the autoimmune process may continue on a subclinical level and even a subset of patients is refractory to the available treatments. There are proof-of-concept studies providing evidence that the ablation of the adaptive immune system in patients with severe autoimmune diseases who were refractory to the available therapies including immunosuppressants and biologics can lead to long-term treatment-free remissions. The data explicitly show that the autoreactive immunological memory, which is resistant to immunosuppression can be eliminated by an immunoablative regimen including antithymocyte globulin within the setting of autologous stem cell transplantation. The immunoablation provides the basis for the regeneration of an adaptive immune system, which is free of autoimmunity and tolerant against self. Consequently, therapeutic approaches that target the autoreactive memory have a curative potential.

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Scleroderma-like skin fibrosis caused by genetic defect of ribonucleotide excision repair

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Abstract

Systemic sclerosis (SSc) is a severe fibrotic disease associated with systemic autoimmunity. A subset of SSc patients develop autoantibodies against topoisomerase I (Top1). SSc etiology and reasons for anti-Top1 (Scl70) antibody formation are enigmatic until today. We propose that genome damage may contribute to the pathogenesis of anti-Top1 positive SSc. This is based on our observations in RNase H2-deficient mice, which reproduce important features of SSc. RNase H2 is essential for the removal of ribonucleotides misincorporated into genomic DNA during replication. Increased ribonucleotide loads resulting from RNase H2 deficiency cause DNA damage. Mice with epidermis-specific loss of RNase H2 and consecutive p53-mediated DNA damage response in keratinocytes develop dermal fibrosis and spontaneous type I IFN responses similar to SSc patients. We propose that accumulating DNA damage may functionally alter epithelia in SSc to induce fibrosis of subepithelial tissues. Importantly, unrepaired ribonucleotides in the genome of RNase H2-deficient cells represent a substrate for Top1, which introduces single strand breaks at these sites. More stable or higher numbers of Top1cc in RNase H2-deficient cells with high genomic ribonucleotide load could explain the increased immunogenicity of Top1 in SSc.

Breaching intestinal tight junction permeability associated with industrial food additives might explain the surge in autoimmune disease incidence.

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Keywords

autoimmune disease, food additive, tight junction, permeability, intestine, food industry

Abbreviations

tight junction (TJ), autoimmune disease (AD), transglutaminase (TG)

Abstract

Autoimmune disease (AD) incidence increases. Simultaneously, the food industry introduces ingredients that revolutionize food qualities by constantly introducing food processing technologies. The intestinal intercellular tight junction, controls the equilibrium between tolerance and immunity to non-self antigens and its dysfunction is important in autoimmunity progression. Tight junction leakage is enhanced by many luminal components, including food additives. Neo-linked and post-translational modified molecules, represent mucosal load with altered immunogenic properties. It is hypothesized that commonly used industrial food additives abrogate human epithelial barrier function, thus, increasing intestinal permeability, resulting in activation of the autoimmune cascade.

Glucose, salt, organic solvents, emulsifiers, gluten, microbial transglutaminase, and nanoparticles are being exponentially used by food industries to improve the qualities of the food. All these food additives increase intestinal permeability by opening tight junction, paracellular transfer, by the following described mechanisms: change of the distribution and functions of key proteins, condense or rearrange the cytoskeleton, induce post-translational modifications, dissociate complexes, disband and separate tight junction (TJ) molecules, impact enterocyte intracellular pathways, change luminal microbial load and imitate known TJ breachers.

In fact, in multiple AD, a breach in TJ integrity and function is observed. Future research on food additive exposures on intestinal permeability and autoimmunity interplay will enhance our knowledge of the common environmental mechanisms associated with AD.

Introduction

Epidemiological data provide strong evidence of a steady rise in autoimmune disease (AD) throughout westernized societies over the last decades (1). Multiple sclerosis, type 1 diabetes, inflammatory bowel diseases (mainly Crohn's disease), systemic lupus erythematosus, primary biliary cirrhosis, myasthenia gravis, autoimmune thyroiditis, hepatitis, rheumatic diseases, bullous pemphigoid, and celiac disease are several examples. Parallel, the role of the environment in AD development is gradually becoming clear [2].

The geoepidemiological distribution of the ADs , the world-wide North-South gradient and the West-East gradient in Europe related to the socioeconomic status, the rapid increase in developed countries and population migration observations are indicative of an environmental impact, rather genetic factors, driving these rapid and recent evolutionary processes (3). Among others, two major environmental factors, strongly related to socioeconomical status are suspected to drive these phenomena: infections and nutrition (4, 5). The present review will expand on the nutritional aspects, concentrating on the major food additives, used frequently in the industrial food processing, that affects TJ integrity and potentially enhancing autoimmunity progression (6).

Before embarking on the nutritional aspect and autoimmunity, TJ function and regulation, will be expanded.

Intestinal tight junction function and regulation

Only a single layer of epithelial cells separates the luminal contents from effector immune cells in the lamina propria and the internal milieu of the body. Epithelial TJs are the key structures regulating paracellular trafficking of macromolecules. It is a multi-protein complex that forms a selective permeable seal between adjacent epithelial cells and demarcates the boundary between apical and basolateral membrane domains. Disruption of the intestinal TJ barrier, followed by permeation of luminal noxious molecules, induces a perturbation of the mucosal immune system and inflammation, which can act as a trigger for the development of autoimmune, allergic or cancerous systemic diseases (7).

TJs are composed of a complex network of proteins, the interaction of which dictates their competency. Zonulins, occludins, claudins and junctional adhesion molecules are a few examples that modulate movement of fluid, micro and macromolecules and immune cells from intestinal lumen to the blood stream and vice versa. The intestinal epithelial barrier, with its intercellular TJs, controls the equilibrium between tolerance and immunity to non-self-antigens.

Dysfunction seems to be a primary defect in AD [8]. Intestinal permeability is decreased in many AD: Ulcerative colitis, Crohn''s disease, celiac disease, inflammatory joint disease, ankylosing spondylitis, juvenile onset arthritis, psoriatic arthritis, type 1 diabetes mellitus , Behcet's syndrome, multiple sclerosis and primary biliary cirrhosis. In fact, in addition to genetic predisposition and exposure to triggering non-

self-antigens, the loss of protective function of mucosal barriers that interact with the environment is necessary for autoimmunity to develop.

Therefore, therapeutic targets that restore TJ barrier integrity may provide effective therapeutic and preventive approaches against ADs. (7)

Industrial food processing and food additive consumption is expanding. The recent increased knowledge on the functions, mechanisms and abnormalities of intestinal permeability and the specific relationship between some common food additives and their deleterious effects on the tight-junction, prompted us to put forward the hypothesis that increased intestinal permeability induced by these industrial food additives explains the observed surge in autoimmune disease (6).

Increased consumption of processed food additives.

The world's capacity to provide food through increased productivity and diversity, decreased seasonal dependency and seasonal pricing is a currently ongoing phenomenon. These changes in food consumption at a global and regional level result in considerable health consequences. Among many other factors including improved industrial food technologies, processing and marketing, the policies of trade liberalization over the past decades have implications for health outcomes associated with rising rates of obesity and chronic diseases such as cardiovascular disease and cancer, as well as autoimmune diseases (6).

Living in western countries has a strong impact on nutritional habits collectively termed the “western diet” including high fat, trans fatty acids, cholesterol, protein, sugar and salt intake, as well as frequent consumption of processed and “fast food” [9]. Figure 1 shows the net fold increases %/year of the 7 industrial food additives, actual and forecasted sales and consumption over the last 4–6 decades (adapted from 6). As can be noticed, in an increasing order, emulsifiers, salt, industrial food enzymes and nanoparticles are sold and consumed the most. In fact all of them increased except organic solvents usage that decreased in the last years.

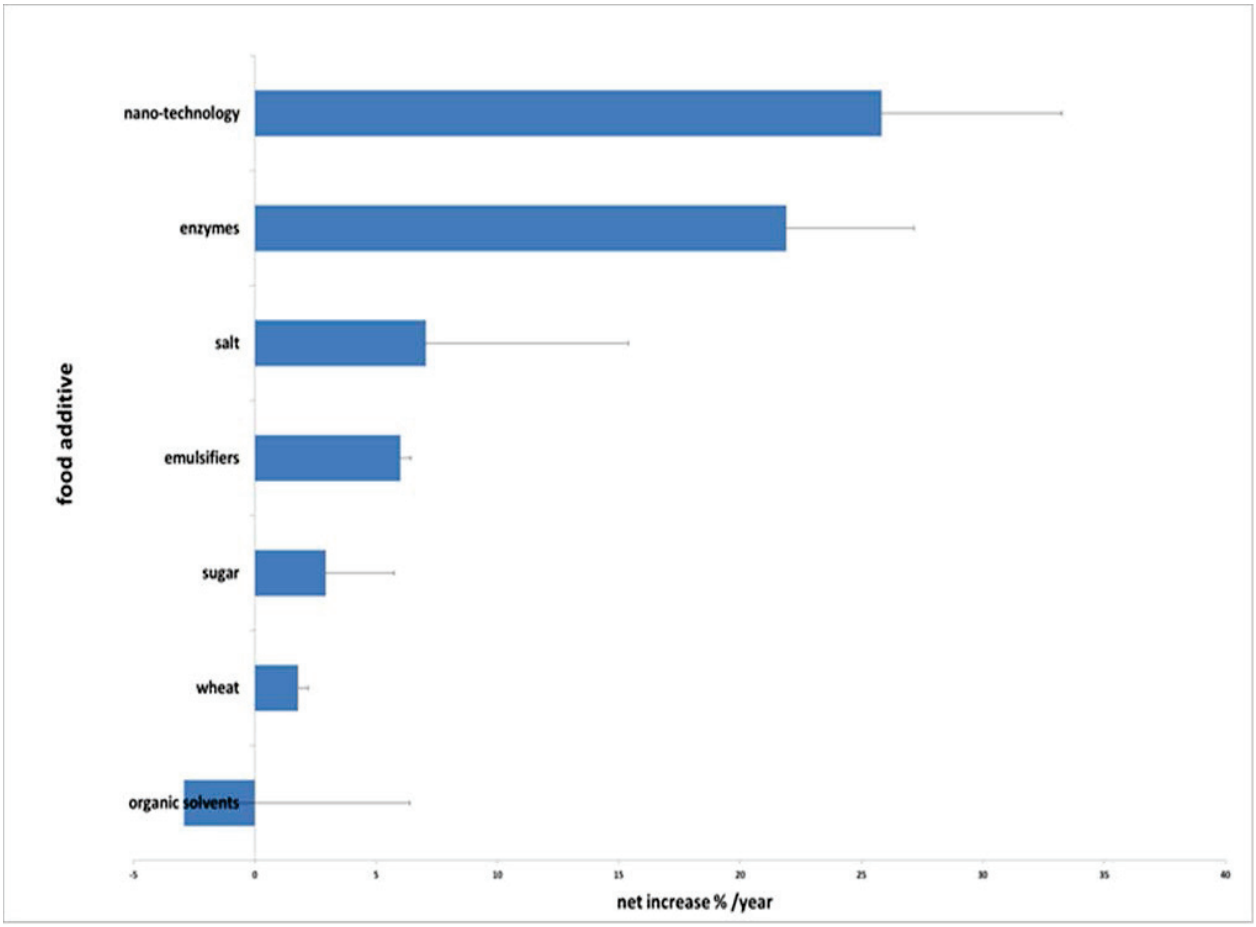

Figure 1. Following are the effects of those food constituents on the intestinal TJ functions
The net fold increases %/year of 7 industrial food additives, actual and forecasted sales and consumption over the last 4–6 decades (adapted from 6).

Commonly ingested food ingredients increase intestinal permeability.

Table 1 describes the various mechanisms by which the 7 industrial food additives, commonly used during food processing, disrupt the intestinal TJ integrity and result in increased permeability (6). Most of the investigations were performed ex vivo, on cell lines or on animals, very few were performed in humans. It can be summarized that these nutrients change the distribution and functions of key proteins, condense or rearrange the cytoskeleton, induce post-translational modifications, dissociate complexes, disband and separate TJ molecules, impact enterocyte intracellular pathways, change luminal microbial load and imitate known TJ breachers.

Table 1. The various net % surges/year and the mechanisms by which industrial food additives disrupt the intestinal TJ integrity and result in increased permeability (adapted from ref. 6).

Food additives	Net % increase/year	Mechanism of TJ integrity breaching
Glucose	2.9±2.8	Change in distribution of ZO-1, claudin-1, E cadherin. Perijunctional cytoskeleton condensation.
Salt	7.1±5.3	Increased phosphorylation of myosin light chain, contraction of the perijunctional actomyosin ring, loss of function of claudin 2 and 15.
Organic solvents	-2.9±9.3	Alterations in TJ proteins, dissociates the PTP1B-E-cadherin-beta-catenin complex
Emulsifiers	6±8.4	P-glycoprotein inhibition, decrease the hydrophobicity of the mucus layer, actine disbandment and structural separation of TJ, change the distribution of ZO-1 and actine.
Gluten	1.8±0.4	Rearrangement of the cytoskeleton through the zonulin pathway, reduces F-actin content, interaction between occludin and Zo-1 is compromised, zonulin release is leading to PKC-mediated cytoskeleton reorganization, zonulin release by binding to the CXCR3 receptor in intestinal cells, in a MYD88-dependent pathway and subsequent transactivation of EGFR by PAR2.
Microbial transglutaminase	21.9±7.4	Increases luminal microbial load, cross-linking TJ proteins, imitating emulsifiers and nanoparticles functions.
Nanoparticles	25.8±21.5	Redistribution of ZO-1 TJ proteins, clustering of integrin α(V)β(3) along the cell border, F-actin reorganization and claudin 4 down regulation, open epithelial TJ via C-Jun Nh2-terminal kinase-dependent pathway, TJ electrostatic interactions

The Hypothesis

The hypothesis that modern food additives (such as sugars, salt, organic solvents, emulsifiers, gluten, microbial TG, and nanoparticles) increasingly used in the food and beverage industries, are a major environmental factor for AD induction is forwarded (6, 10). All the food ingredients mentioned here abrogate human epithelial barrier function and increase intestinal permeability through the opened TJ, resulting in entry of foreign immunogenic antigens and activation of the autoimmune cascade. Figure 2 describes schematically the present hypothesis.

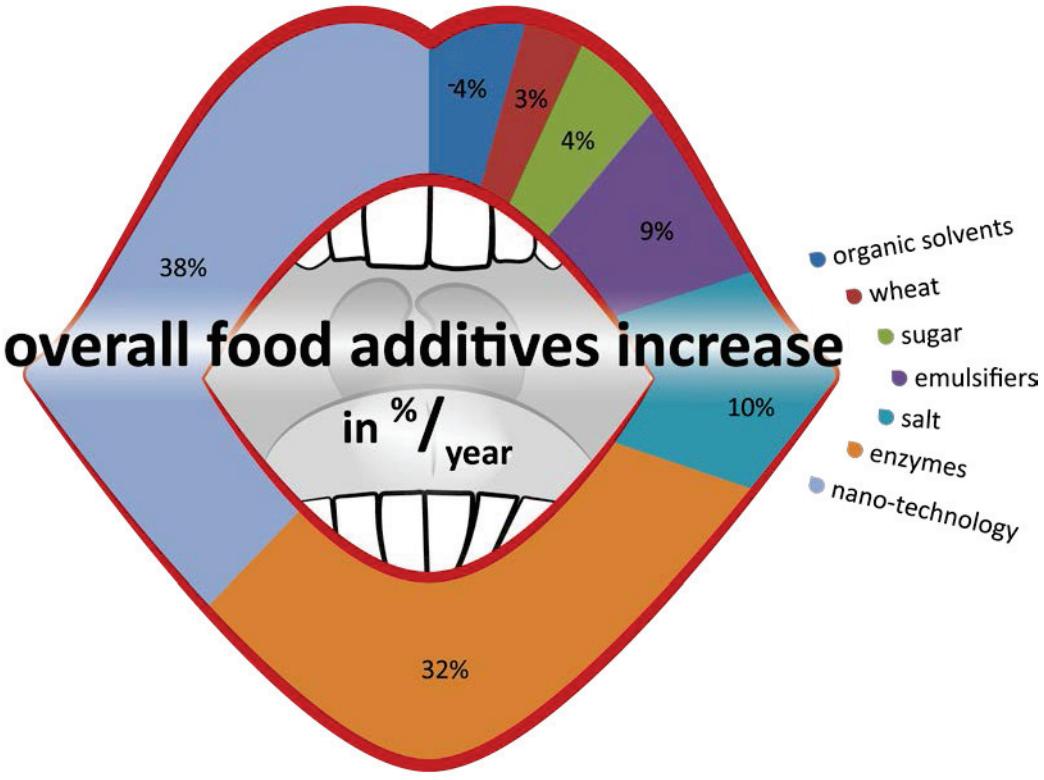

Figure 2. A schematic presentation showing the overall (%/year) food additives increase produced by the industries for human consumption (adapted from 6).

Cause and effect relationship was demonstrated for almost all the food additives mentioned above and the central role of TJ dysfunction in many autoimmune diseases is well described. Although, direct causality has not been proven, increases in the usage of the above mentioned food additives have paralleled increased incidences and prevalences of AD during the last decades.

Summary

It is hypothesized that commonly used industrial food additives (sugars, salt, organic solvents, emulsifiers, gluten, microbial TG, and nanoparticles) abrogate human epithelial barrier function, thus, increasing intestinal permeability through the opened tight junction, resulting in entry of foreign immunogenic antigens and activation of the autoimmune cascade. Future research on food additives exposure-intestinal permeability-–autoimmunity cross talks will enhance our knowledge of the common mechanisms associated with autoimmunogenesis.

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Autoinfectome: a tool to study the link between infection and autoimmunity

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Keywords

autoimmunity, disease, environment, infection, immunity, microbiome

Abbreviations

liquid chromatography-tandem mass spectrometry (LC-MS/MS), polymerase chain reaction (PCR)

Abstract

Autoimmune disease develops in genetically susceptible individuals following exposure to multiple environmental agents, including infectious and non-infectious triggers. Exposome serves to characterise all exogenous and endogenous environmental exposures and can be further broken down to infectome and autoinfectome, which consists of all infectious organisms that we are exposed to, and which contribute to the induction and/or progression of autoimmunity. Autoinfectome can be studied at high risk individuals (such as family members, professionals exposed to autoimmunity-linked environmental agents) over time. Multiplex technologies become less costly and may assist dissecting the complex nature of autoinfectome. It is expected that most of these infectious agents may be preventable and/or treatable, and could therefore stand for a set of risk factors which could be modified in their own right.

Introduction

Autoinfectome is the totality of infections throughout life which contribute to the development and progression of an autoimmune disease in genetically susceptible individuals (1-4) (Figure 1). Various studies are now using advanced toxicological, microbiological, biochemical, and immunological assays to identify the environmental causes of autoimmunity, which can include infections and xenobiotics. These

triggers do not act in a similar manner and their involvement may differ from one disease to another under the influence of genetic and epigenetic factors. Nevertheless, some generalized mechanisms may operate and could act as common pathogenic denominators of every single autoimmune disease or at least of a large group of them, with subsequent exposures differentiating one disease from the other (3, 5-7).

Referring to terminology, “exposome” is currently used as a means of assembling, and measuring the unwanted consequences of environmental (infectious and non-infectious) factors, both internal and external on the homeostasis of a susceptible host (Figure 1). Caution must be exercised as internal factors *per se* include the microbial flora (i.e the “microbiome”), as well as nutrition factors, antioxidants and immunomodulators. This chapter emphasizes the important role of the “infectome” as the infectious component of the microbiome (as part of exposome) which is pivotal for the induction of autoimmunity over the years. This part of the infectome is the one we have termed “autoinfectome”. It becomes apparent that “autoinfectome” is like a fingerprint which is unique for an affected individual and may differ even within a given autoimmune disease. Thus, the autoinfectome needs to be differentiated from the infectome not related to autoimmunity.

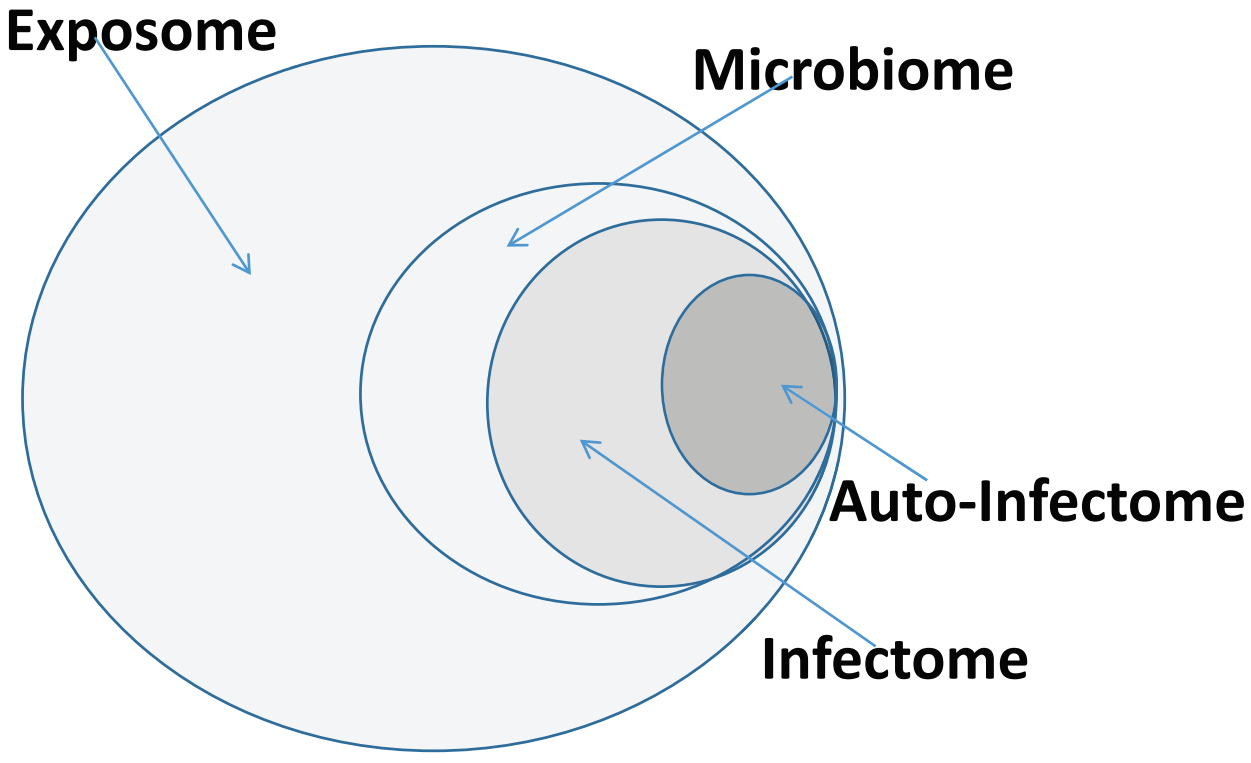

Figure 1. “Exposome” refers to all endogenous and exogenous environmental factors which we are exposed to in a lifetime. These include the infectious (microbiome) and non-infectious factors. The concept of “autoinfectome” that we introduce, describes the part of the exposome which refers to the collection of an individual’s exposures to infectious agents, which play a role in the inductions of autoimmunity and indeed autoimmune disease.

Understanding the exposome

The exposome corresponds to the totality of exogenous and endogenous environmental factors which begin preconception and continue over the years and throughout life (8-12). The endogenous factors are distinctive to the exposome (10). Thus, when studying the exposome we investigate the role of endogenous parameters such as oxidative stress, pro-infmamatory mediators and lipid peroxidationl (10-12). Amongst them, several serve as nucleophiles or electrophiles, being able to DNA and protein modification (13), especially during infection (14-16).

The quantification of exogenous agents is a critical step towards understanding the role of exposome in the induction of a disease. Equally important is to look at features of the causes, times and sequence of exposures and time-points of exposure events. Subsequent examination of isolated causes could assist attempts to identify true causes of autoimmune diseases. Measurable types of these agents in the form o biomarkers could be measured using technologies such as liquid chromatography-tandem mass spectrometry (LC-MS/MS) (17), and DNA adducts (18). Such measurements can be performed using the 'bottom-up' method which quantifies the external factors (10) and the 'top-down' method which measures internal factors. A combination of both provides an overall picture of a measurable exposome under specific conditions (10-12). For example breath analysis for specific biomarkers leads to a critical piece of information regarding the degree of exposure, the time-point of exposure, the dosage and the extent of elimination from the body of a toxicant (9). "Environmental-wide association study exposome, EWAS" in chronic diseases such as diabetes revealed significant associations with dozens of environmental factors including dioxins, polychlorinated biphenyls, organochlorine pesticides, as well as nutrients and vitamins (19). Immunological factors are also involved (20,21). Thus, despite the difficulty to trace down and follow up all these triggers, uniform platforms must be developed to incorporate and analyse the diverse analytes. Getting into infectious triggers, screening for individual infectious agents is currently used in a small/medium size scale (11). Multi-parametric testing of antibodies against infections or autoantigens using serum or other biological material are commercially available. Molecular testing of blood, saliva, tears, gastric juice, urine and stools using polymerase chain reaction (PCR) are also conducted by reference or large laboratories (11). Technological platforms that enable high-scale testing of antibodies against short peptidyl infectious sequences are under way but more are needed.

Approaches to study the autoinfectome

The study of autoinfectome involves a step-wise approach (1). We believe that it would be desirable to determine (if possible) the genetic make-up of the affected individuals and their siblings (at least HLA class I and II genes), ideally at birth. Subsequent stratification into groups with high or low risk to develop autoimmunity would allow further analysis. Second, urine, oro-nasal swabs, saliva, faecal material, and blood (for isolation of plasma, serum, and peripheral blood mononuclear cells) would be collected at regular intervals and/or during clinically relevant time points, such as those where affected individuals are treated with antibiotics for particular infections i.e recurrent urinary tract infections, cellulitis, pulmonary infections. These samples can be stored and analysed at a later time point as a whole using multiplex technology. Patterns of infection may differ from patient to patient and this may play a role for the progression of the disease. Some infections are incriminated for a fast pace while others appear to slow down disease progression.

What we can we learn from the microbiome and immunome projects

As we described earlier, microbiomes are region or tissue specific and are usually referred to the gut, oral cavity, etc where microbial genes are found to be most prevalent (22-31). Microbiomes provide a snapshot of the flora of that region and often reflect what would be termed normal flora, but may also be applied to a body site, such as gut during a gastrointestinal disease such as irritable bowel disease (IBS) (28). Most microorganisms of the human microbiome do not induce antigen specific immune responses which can provoke local or systemic inflammation and tissue injury. This is what differentiates the microbiome from the infectome/autoinfectome. The study of the autoinfectome necessitates monitoring of the extent of microbial/host immunity focusing mainly on those infectious agents potentially causing self-destruction. At times beneficial bacteria of the normal flora become pathogenic, as with *E. coli*, *Clostridia* and *Klebsiella* for example.

Metagenomic screens of bacterial populations have been performed to investigate the microbiome of the gut and of other regions (24-27, 30). It needs to be emphasized that what is normal in an individual may be abnormal in another. Race and regional differences have been noted and the percentage of prevalent bacteria is changing amongst generations. Investigation of multiple body-sites would likely prove to be useful (32-33).

Screening populations

Screening all populations to study their infectomes is not feasible from both a practical and financial standpoint. It is more logical to study individuals who have high risk to develop autoimmunity, as family members or individuals with a genetic make-up conferring susceptibility to a given disease. Longitidinal studies on large number of biological sample s from the affected individuals collected over the years are ideal for such testing. Also, asymptompatic individuals who are seropositive for autoantibodies can be studied. Special cohorts like professionals who are exposed over the years to heavy metals or other agents linked to autoimmunity can also be examined.

Screening applications

Samples to test preferentially will include blood, urine or saliva. The easiest testing will be that o IgM, IgA and IgG antibodies against infectious agents. Multiplex ELISA, line/dot assays and cell-based immunoflourescence assays are cost effective and informative (34-36). Large-scale multiplex technologies such as protein/peptide microarrays and peptidome libraries are costly but are able to detect antibody responses to dozens or hundreds of antigens (37-38).

Detecting genetic material in tissues by a multi-parametric approach can be performed by DNA sequencing technology using 'high-throughput DNA sequencers' (39-42). Massive, parallel sequencing permits the detection of several infectious agents at the same time (43). Multiplex PCR is a useful tool to detect infectious agents and the use of 16S/18S rRNA gene sequencing allows mass-analysis of samples (26, 28).

Sampling sites

Special attention must be given to the examination of the oral cavity as several infections affecting this site are linked to autoimmune diseases (44-46). Dysbiosis of the oral mucosa participates to the induction of inflammatory bowel disease (44). The high prevalence of dental problems in patients with these diseases may be due to alterations in oral flora and may participate in autoimmune rheumatic diseases

(46). The lung microbiome has emerged as another focus of interest. By far the best studied microbiome is that of gut and most studies are investigating its role in disease states.

In conclusion, the study of autoinfectome (3-4) helps to characterise which infections are linked to autoimmuinity and may assist clinicians to design early therapeutic interventions based on antibiotic and anti-viral treatments taking into account best patient care and safety.

Table 1. Multiparametric systems used to detect infectious agents at present including immunological and molecular tests (34-36)

Molecular detection	
Multiplex real-time PCR	Real time PCR and higly specific melting point analysis e.g LightCycler Septi*Fast* (25 pathogens)
Molecular hybridization	Commercially available platforms are already in use e.g. simultaneous detection of multiple viral types and subtypes from nasopharygeal swabs & simultaneous detection of viral, bacterial, and protozoan parasites causing gastrointestinal diseases
Nucleotide sequencing	Nucleotide (pyro) sequencing Next-generation sequencing (highly massive pyrosequencing technology, sequencing by synthesis (SBS), sequencing by oligonucleotide ligation and detection (SOLiD) system
Mass spectrometry	Post-culture microbial identification by MALDI-TOF Post-PCR microbial identification by PCR-ESI
Integrated fluidic systems	
Immunological Assays	Multiparametric ELISA, line blots/dots Multiparametric IFA chips Magnetic and non-magnetic bead multiplex immunoassays Lateral flow immunochromatographic Assays Triplex lateral flow immunoassay Optical Immunosensor Systems Electrochemical-based ELISA

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Chapter 2

From Research to Clinical Application

Anti-beta2 glycoprotein I epitope specificity: from experimental models to diagnostic tools

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Keywords

antiphospholipid syndrome, beta2 glycoprotein I, domain I, thrombosis, fetal loss

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Introduction

The diagnosis of the anti-phospholipid syndrome (APS) relies on the association between recurrent arterial/venous thrombosis and/or miscarriages and the persistent presence of antibodies against phospholipid (PL)-binding proteins (aPL) (1). The main antigenic target for aPL is beta2 glycoprotein I (β2GPI) although antibodies directed against other PL-binding proteins have been reported such as those against prothrombin, high molecular weight kininogen, protein C/S, thrombomodulin (2,3). However, anti-β2GPI antibodies are widely accepted to be responsible for the positivities in all the three formal diagnostic tests for APS. In particular, the Lupus Anticoagulant (LA) phenomenon mediated by anti-β2GPI antibodies displays a better diagnostic/prognostic power than LA mediated by anti-prothrombin antibodies (3). Similarly, only β2GPI-dependent antibodies testing positive in the anti-cardiolipin (aCL) assay are diagnostic for APS (1). Although the diagnostic specificity of the anti-β2GPI test is superior than that of the aCL assay, the results of the epidemiological studies on the association between APS clinical manifestations and anti-β2GPI antibodies is still weakend by the lack of standardization of the assay (4).

On the other hand, much stronger evidence supports the pathogenic activity of β2GPI-mediated aPL. In fact, several *in vitro* models have been described in which anti-β2GPI antibodies are responsible for mechanisms involved in clot triggering as well as in placental tissue damage (2,3,5). More importantly, APS animal models have been recently described in the literature that clearly show how anti-β2GPI antibodies may be responsible for both thrombosis and fetal loss (3,6-9). This is actually not the case for

antibodies against other PL-binding proteins such as anti-prothrombin. The only one exception is represented by a model of fetal loss mediated by anti-phospatidylamine reported by one group only (10).

Beta2GPI

a) Physiology of β2GPI and its role as antigenic target

Beta2GPI, also known as apolipoprotein H, is a 50-kDa protein that is present in normal plasma at a concentration of 50–500 μg mL. Its physiological funtion is still matter of research since individuals deficient in β2GPI seem to be healthy, and mice deficient in β2GPI do not express a clear phenotype, indicating that the presence of β2GPI is not essential for life (11). The finding of a reduction in the number of viable implantation sites as well as a reduced fetal weight and fetal: placental weight in β2GPI deficient in comparison with wild type mice suggested a role for the molecule in optimal placental development and fetal growth (12). These data are in agreement with the demonstration that β2GPI binding to endothelial cells can be found in placenta and uterus in resting animals, further supporting a physiological role of the molecule in reproduction (13).

On the other hand, even mild inflammatory stimuli can upregulate the binding of the molecule in other vascularized tissues through several cell membrane receptors including members of the Toll Like family (13, 14). This finding fits very well with the two hit theory for APS according which aPL are the first hit that requires an additional one such as an inflammatory stimulus to upregulate the presence of tissue β2GPI and to eventually allow aPL binding and complement activation (3, 13). Accordingly all the reported animal models for aPL-mediated clotting require a second hit such as a vessel traumatic injury or a lypopolysaccharide (LPS) stimulus (6-9).

We described for the first time that β2GPI-dependent aPL are formally the antibodies responsible for clotting since IgG fractions depleted of the anti-β2GPI activity were no more thrombogenic in the LPS-model (8). Moreover, we recently added a direct demonstration of such a pathogenic activity by showing that a human anti-β2GPI monoclonal IgG reproduces comparable results in models of clotting and fetal loss (15).

b) Epitope antigenic specificity

Beta2GPI is a relatively large molecule belonging to the complement control protein (CCP) superfamily with a good immunogenicity. It contains five CCP domains with the fifth one displaying a large positive charged patch responsible for the affinity for anionic PL. Electron microscopy studies showed that the molecule is present in a circular form in blood, with domain 1 (DI) linked to DV. After binding to anionic surfaces or to antibodies, the molecule opens up and adopts the hockey stick-like conformation exposing DI (11).

Studies with several linear peptides spanning different portions of the molecule showed that anti-β2GPI antibodies can be directed against all the β2GPI domains with no clear clinical associations among the different antibody subpopulations (16,17). More recently, evidence is emerging that β2GPI-DI may represent the immunodominat antigenic portion of the molecule and that a conformational short peptide located close to the junction between DI and DII may represent the immunodominant epitope (18-22). The DI of β2GP1 is usually hidden as a result of the linkage with DII in the circular form and is presented to the afferent limb of the immune system when the molecule is opened after binding to anionic PL monolayers or scavenging LPS (11). It has been suggested that while the other domains may induce

tolerance at high antigen concentrations, DI does not. Hence, even small amounts of DI presented to the immune system might break the tolerance (23).

c) Anti-β2GPI-DI antibodies are pathogenic in animal models of APS.

There is growing evidence that anti-β2GPI-DI antibodies are pathogenic. The first proof of concept on the pathogenicity of anti-β2GPI-DI antibodies was based on the demonstration that a peptide reproducing the antigenic epitope in DI was able to inhibit - at least in part - the in vivo thrombogenic activity of polyclonal human anti-β2GPI IgG passively infused in mice (24).

More recently we synthesized a human monoclonal IgG (MBB2) that recognizes β2GPI from different species. When tested against the different β2GPI domains, it displayed a selective reactivity against DI. The passive infusion of MBB2 in naive mice induced thrombosis and fetal loss through complement activation as demonstrated by complement deposition in the tissues. A parent monoclonal antibody, MBB2-CH2, displays the same antigen specificity of MBB2 but lacks the CH2 domain, being unable to activate complement and to display any thrombogenic or pro-abortive effect in animals. More importantly, we reported evidence that the CH2-variant prevented the pathogenic effects of anti-β2GPI IgG fractions from APS patients by competing with patient antibodies for β2GPI binding (15). Altogether these findings further support the pathogenic role of antibodies against β2GPI-DI.

There is evidence from preliminary experiments that whole sera and polyclonal IgG fractions from APS patients with reactivity against β2GPI-DI induce thrombus formation and fetal loss in our model (personal unpublished results).

d) Epidemiological evidence of the diagnostic/predictive value of anti-β2GPI-DI antibodies.

Although a reactivity against DI has been described since 1998 (25), the interest on anti-β2GPI-DI antibodies increased more recently when anti-β_2GPI antibodies with DI specificity were found in the majority of APS patients and significantly associated to LA and vascular thrombosis (mostly venous) (18). Such an association was also described for obstetric manifestations by the same group in a further multicentre study (19). Moreover, preliminary results on high levels of anti-DI antibodies detected by research ELISA kits and associated with an increased risk for thrombotic events have been reported by other groups in abstracts presented at the 14th International Congress on Antiphospholipid Antibodies (26, 27).

It is now widely accepted that patients with multiple positive test results (i.e. LA, aCL and anti-β_2GPI autoantibodies particularly of the IgG isotype) display a much higher risk for developing clinical complications. According to the hypothesis that anti-DI IgG may represent a more predictive aPL profile, a higher prevalence and higher titers of anti-β2GPI-DI antibodies have been recently reported in these patients (22, 28).

The presence of anti-β2GPI DI IgG not only in primary APS (PAPS) with thrombosis but also in PAPS with pure obstetric disease has been confirmed by a larger study (22). Comparable positivity rates were also detectable in patients with systemic lupus erythematosus (SLE) or undifferentiated connective tissue diseases (UCTD), while antibodies against DIV or DV were less frequent in the same populations. aPL positive asymptomatic carriers display a less polarized profile and anti-DIV-V antibodies have been detected in healthy children born from mothers with autoimmune diseases and in atopic dermatitis

patients (22). Moreover, almost all anti-β2GPI-DI IgG positivities were confirmed after 12 weeks, at variance of innocent aPL transiently occurring as immune epiphenomenon during infectious diseases. Altogether these data do suggest that anti-DI antibodies may cluster in patients with systemic autoimmune diseases and in particular in patients with a high risk profile (22).

However, up to one third of patients carrying anti-β2GPI antibodies were negative for anti-DI IgG in a large multi-center study (22). Whether or not epitopes other than DI can be targeted by pathogenic anti-β2GPI antibodies is still matter of research. Nevertheless, this finding may explain why inhibition studies with the CH2-deleted anti-DI monoclonal antibody or with the DI peptide never reached a complete inhibition of polyclonal anti-β2GPI IgG fractions from patients (15, 24). The main conclusions up to now are that anti-DI assay cannot replace the test with the whole molecule because of possible misdiagnosis although it may provide a promising second-line test for a better risk-stratification of clinical events.

e) Assays for anti-β2GPI-D1 antibodies.

In an attempt to improve the clinical relevance of the assay for the detection of anti-β2GPI antibodies, methods for the determination of antibodies that have specific reactivity against DI of β2GPI have been described. Currently, the anti-DI β2GPI assay is mainly used in research-based settings, and more prospective data are needed before antibodies recognized by these assays can be added to international classification criteria.

A two-step technique was initially used to characterize β_2GPI-DI aPL (18). In detail, β_2GPI coated to hydrophilic but not to hydrophobic microtiter plates displays conformational changes that expose DI to the surface, making it more accessible for autoantibody binding. The clinical utility of the assay was validated in two studies, one of which carried out among several centers. An association with LA and mainly venous thrombosis was reported in the first study and was extended also to obstetric events in the second one (18, 19). However, the study did not find any correlation between LA and miscarriages, in clash with several previous publications and the known clinical predictive value of LA for miscarriages.

Using a novel method of production and purification of human DI by Escherichia coli multiple mutants of DI have been obtained and tested for aPL reactivity in both solid phase and fluid assays (20). The solid phase assay was recently validated in one study on seronegative APS (29). An additional inhibition ELISA has been described that uses a synthetic DI (30). A novel β2GP1-D1 chemiluminescence immunoassay (CIA, INOVA Diagnostics, San Diego, USA) based on the BIO-FLASH system (Biokit, Barcelona, Spain) and using recombinant β2GP1-D1 coupled to paramagnetic particles has been established (23). The CIA assay has been recently evaluated in a large series of APS patients (28).

There are no published studies aimed to correlate the results of the different tests for anti-DI β2GPI. We recently carried out a comparative study by using two of the four anti-β2GPI DI assays reported in the literature. Preliminary results from the international group show that the peptide ELISA and the CIA assay display a good correlation. Although concordance beteween the two assays is good, few sera resulted discordant suggesting that the different coating systems may affect the conformation of the coated molecules, so explaining the few differences at least in part (Willis et al. in preparation).

Conclusions

- Characterization of the epitope specificity of anti-β2GPI antibodies may offer new tools for improving the diagnostic/prognostic power of the assay.