INTERNATIONAL JOURNAL OF IMMUNOPATHOLOGY AND PHARMACOLOG Vol. 21, no. 2, 267-278 (2008)

ARTICLE

ANTIBODIES AS PREDICTORS OF COMPLEX AUTOIMMUNE DISEASES

A. VOJDANI

Immunosciences Lab., Inc., Beverly Hills, CA, USA

Received February 13, 2008– Accepted April 28, 2008

Emerging evidence has suggested environmental factors such as infections and xenobiotics and some dietary proteins and peptides in the pathogenesis of many autoimmune diseases. Considering the fact that autoantibodies can often be detected prior to the onset of a disease, in this study an enzyme immunoassay was used for measurement of antibodies against different highly purified antigens or synthetic peptides originating not only from human tissue, but also from cross-reactive epitopes of infectious agents, dietary proteins and xenobiotics. The measurement of antibodies against a panel of antigens allows for identification of patterns or antibody signatures, rather than just one or two markers of autoimmunity, thus establishing the premise for increased sensitivity and specificity of prediction, as well as positive predictive values. This panel of different autoantibodies was applied to 420 patients with different autoimmune diseases, including pernicious anemia, celiac disease, thyroiditis, lupus, rheumatoid arthritis, osteoarthritis, Addison's disease, type 1 diabetes, cardiovascular disease and autoimmunity, which are presented in this article. In all cases, the levels of these antibodies were significantly elevated in patients versus controls. Antibody patterns related to neuroautoimmune disorders, cancer, and patients with somatic hypermutation will be shown in a subsequent article. We believe that this novel 96 antigen-specific autoantibody or predictive antibody screen should be studied for its incorporation into routine medical examinations. Clinicians should be aware that the detection of antibodies should not automatically mean that a patient will definitely become ill, but would rather give a percentage of risk for autoimmune disease over subsequent months or years.

Autoimmune diseases arise when an environmental insult superimposed on genetic susceptibility disrupts normal immune regulation. Autoimmune diseases afflict most organ systems and impose an enormous burden on the health care system (1).

The predictive onset of immunologic changes or the outcome of autoimmune disease is increasingly being employed by academic and industrial investigators. These biomarkers identify usually susceptible individuals or populations, facilitate prognosis, and aid in developing improved treatments or preventive measures (2).

In relation to specificity of humoral immune response, it is known that with increased aging up to one third of the general population have some reactive antibodies against self-antigens (3). Yet systemic autoimmune disease such as lupus develops in about 1%. Therefore, while autoimmunity or the presence of self-reactive T and B cells and autoantibodies is common in the general population, autoimmune disease is relatively rare. However, some of these individuals develop a more persistent but mild and localized autoimmune disease which is referred

Key words: predictive antibodies, autoimmune diseases, environmental triggers, ELISA

Mailing address: A. Vojdani, Ph.D., M.T.
Immunosciences Lab., Inc.
8693 Wilshire Blvd., Ste. 200
Beverly Hills,
CA 90211, USA
Tel: ++1 310 657 1077 Fax: ++1 310 657 1053
e-mail: immunsci@ix.netcom.com

0394-6320 (2008)

to as undifferentiated autoimmune or connective tissue disease. Finally, a small percentage of patients develop a full-blown autoimmune disease.

This full-blown autoimmune disease development depends on the interplay of qualities and quantities of MHC Class II (4), the loss of inhibitory and suppressor mechanisms (5), and the presence of pathogenic factors such as infections and xenobiotics (6-7). This suggests that autoimmunity contributes to, but might not be sufficient by itself to cause the development of tissue lesions, and that local factors such as cytokines and chemokines might have crucial roles in the development of tissue lesions. Therefore, biomarkers for autoimmune diseases should be designed not only based on antibodies against tissue antigens, but also on environmental factors as causative agents, and other immune markers which might enable the prediction of disease development. Indeed, in an earlier study, by applying a panel of antibodies against neural antigens we confirmed a diagnosis of multiple sclerosis with a sensitivity of 75% and specificity of 70%. By the addition of other immune markers such as antigen-specific lymphocyte activation and cytokine production, we were able to increase the sensitivity and specificity of these immune parameters to 95-100% (8).

The standardized quantitative measurement of disease-associated autoantibodies to autoantigens as well as environmental factors, such as infections, xenobiotics and discrete autoantigen epitopes, is currently one of the few surrogate markers that can reliably detect changes in autoimmune pathogenesis. Measurements of antibodies against autoantigen epitopes and factors inducing autoimmune diseases should, therefore, be included in studies that aim to track pathogenic events leading to autoimmune disease or changes that follow immune intervention (9-15).

There would therefore be many advantages in using a panel of different autoantibodies, some of which would be related to autoantigens and others to causative agents. In the first place, the sensitivity of these assays would be increased. Additionally, in a modality that prescribes the removal of causative factors from the patient's environment, assays using these autoantibodies may allow the monitoring of the progress of the disease. The disappearance of these autoantibodies upon therapy might also indicate a beneficial response. Further, simultaneous measurement of antibodies against 96 pure antigens, performed in our laboratory for research only and presented in these articles, makes possible a rapid screening for dozens of diseases. Our overall goal is to make this high throughput assay a routine part of medical examinations in the near future.

MATERIALS AND METHODS

Antigens and peptides

Parietal cell, intrinsic factor, tropomyosin, dipeptidyl peptidase IV (DPP IV), trypsin, motilin, wheat gluten, thyroglobulin (TG), thyroid peroxidase (TPO), thyroid stimulating hormone receptor (TSHR), ssDNA, phosphorylcholine, cow's milk protein, aggregated IgG, streptococcal enzymes, adrenal gland, glomerular basement protein, insulin, oxidized-low density lipoprotein (o-LDL), α-B-crystallin, ganglioside GM_1, sulfatide, aflatoxin fumonisin, ganglioside GD3 and secretin were purchased from Sigma-Aldrich (St. Louis, MO). Platelet Glycoprotein, β2-glycoprotein, and elastin were purchased from EMD Bioscience (La Jolla, CA). The following peptides: transglutaminase (tTG), vasoactive intestinal peptide (VIP), gliadin, gluteomorphin, glutamic acid decarboxylase (GAD), cerebellar, synapsin-I, Rotavirus, *Saccharomyces*, thyroid binding globulin, thyroid binding prealbumin, *Yersinia enterocolitica*, heat shock protein-60 (HSP-60), *Borrelia, Bartonella*, lupus peptide, SmD3, laminin, poly (ADP-ribose) polymerase (PARP), fibrillarin, chromatin, rheumatic fever peptide, streptococcal M5, M12 and M19 proteins (SMT6P), *Mycoplasma arthritidis, Mycobacterium avium,* human HSP-60, myelin basic protein (MBP), *Chlamydia pneumoniae*, fibulin-4, 21-hydroxylase, islet cell antigen-2 (IA-2), tyrosine phosphatase, *Coxsackievirus*, myosin-α, myelin oligodendrocyte glycoprotein (MOG), proteolipid protein (PLP), transaldolase, herpes type-6 (HHV-6), acinetobacter, myelin associated glycoprotein (MAG), *Campylobacter jejuni* toxin, glutamate receptor, ion channel, superoxide dismutase-1 (SOD1), blood-brain barrier protein (BBB protein), B-cell D8/17, tubulin, pyruvate dehydrogenase, cytochrome P-450, matrix metalloproteinase (MMP), folate receptor, *Borrelia burgdorferi*, OsPA, OsPC, OsPE, LFA, C2, C6, *Babesia, Ehrlichia, P53, HER-2/neu*, prostatic antigen, serotonin receptor, neuropeptides-y, and neurofilament protein peptides were synthesized by Biosynthesis (Lewisville, TX). Anti-C1q complement was purchased from Jackson Immune Research, West Grove, PA. Albumin-mercury complexes were prepared according to methods described by Thierse et al. (14) and modified in our laboratory.

Albumin-isocyanate complexes were prepared according to methods described by Zeiss et al. (15). PDH-octynoic acid was prepared according to Leung et al. (12).

Human sera

Pooled normal human serum and disease state human plasmas from patients with lupus, arthritis, cardiovascular disease and biliary cirrhosis were purchased from Innovative Research, Southfield, MI. Human serum from patients with celiac disease, coagulation problem, thyroid disease, type-1 diabetes and Addison's disease were obtained from Inova Diagnostics, Inc., San Diego, CA. Sera from patients with different autoimmune diseases were sent by different clinicians to our laboratory, and, after removal of their identification, were kept in the freezer at –70° until used.

Enzyme-linked immunosorbent assay (ELISA) procedure

ELISA was used for measurement of antibody in different sera against 96 antigens simultaneously (Fig. 1). Ninety-six different antigens and peptides were dissolved in methanol at a concentration of 1.0 mg/mL and then diluted 1:100 in 0.1 M carbonate-bicarbonate buffer (pH 9.5), and 50 µl was added to each well of a polystyrene flat-bottom ELISA plate. The configuration of antigens on the ELISA plate is shown in Fig. 1. Plates were incubated overnight at 4°C and then washed three times with 200 µl of Tris-buffered saline (TBS) containing 0.05% Tween 20, pH 7.4. The non-specific binding of immunoglobulins (Igs) was prevented by adding a mixture of 1.5% bovine serum albumin (BSA) and 1.5% gelatin in TBS and then incubating the mixture for 2 h at room temperature and then overnight at 4°C. Plates were washed as described above, and then serum samples diluted 1:200 in 1% HSA in TBS containing –1 mg/mL IgG Fc fragments (to avoid reactivity of specific antibodies with rheumatoid factors) were added to duplicate wells and incubated for 1 h at room temperature. This IgG Fc fragment was added to TBS for measurement of all antibodies except for measurement of rheumatoid factor and immune complexes. Plates were washed, and then alkaline-phosphatase-conjugated goat anti-human IgG or IgA $F(ab')_2$ fragments (KPI, Gaithersburg, MD) at an optimal dilution of 1:400 to 1:2000 in 1% HSA-TBS was added to each well; the plates were then incubated for an additional 1 h at room temperature. After washing five times with TBS-Tween buffer, the enzyme reaction was started by adding 100 µl of paranitrophenylphosphate in 0.1 mL of diethanolamine buffer (1 mg/mL) containing 1 mM $MgCl_2$ and sodium azide, pH 9.8. The reaction was stopped 45 mins later with 50 µl of 1 N NaOH. The optical density was read at 405 nm (OD_{405}) with a microtiter reader. To detect non-specific binding, several plates containing all reagents except human serum were used for color development. Pooled normal human serum was examined on additional plates coated with the same 96 wells for the purpose of specificity of reaction and calculation of indices. For examination of assay reproducibility, each serum (patient and healthy control) was run on duplicate plates, each coated with 96 antigens. Index was calculated according to the following formula:

$$\text{Index} = \frac{\text{mean optical density of clinical specimen - optical density of blank}}{\text{mean optical density of normal human serum - optical density of blank}}$$

RESULTS

In this antibody array, 96 different pure or synthetic antigens or peptides were bound to a solid matrix for measurement of disease-specific antibodies and etiologic agents or cross-reactive antigens associated with them. This autoimmune reaction may occur against any tissue antigen, including gastrointestinal, peripheral, or even central nervous system.

Association between parietal cell and intrinsic factor antibody has been previously reported in patients with pernicious anemia, which is an organ-specific autoimmune disease (16-17). Sera from 3 patients with pernicious anemia were tested against parietal cell, intrinsic factor, tropomyosin and many other non-related antigens and peptides. We found that patients #1 and #2 showed a significant elevation in IgG antibody against parietal cell and intrinsic factor, with indices ranging from 3.3-5.9, while in patient #3 antibodies were detected only against parietal cells. Interestingly, patients with elevation in parietal cells plus intrinsic factor antibody also reacted against tropomyosin (Table I). Antibodies

Table I. *Detection of antibodies against different antigens in 3 representative patients with GI autoimmunity expressed by index.*

Antigen	Patient # 1	Patient # 2	Patient # 3
Parietal Cell	5.9	3.5	7.6
Intrinsic Factor	5.7	4.3	1.8
Tropomyosin	4.1	3.3	1.3
Transglutaminase	3.8	1.2	4.8
DPP IV	2.9	2.0	1.6
Trypsin	4.3	1.8	0.9
Motilin	4.0	1.4	1.4
VIP	3.6	1.5	0.8

Table II. *Detection of antibodies against different antigens in 3 representative patients with celiac disease and gluten intolerance expressed by index.*

Antigens & Peptides	# of Patients with Celiac Disease						# of Patients with Gluten Intolerance					
	1		2		3		1		2		3	
	IgG	IgA	IgG	IgA	IgG	IgA	IgG	IgA	IgG	IgA	IgG	IgA
Wheat Gluten	4.6	3.5	1.5	4.3	2.9	4.6	5.5	1.6	4.3	1.9	3.7	1.0
Gliadin Peptide	4.2	3.1	1.2	3.8	2.5	3.9	6.4	1.8	4.7	1.3	4.1	1.5
Gluteomorphin	1.3	2.8	1.0	2.2	1.7	1.2	2.1	1.4	5.8	1.1	1.7	1.4
Transglutaminase	2.4	4.6	1.4	2.6	2.8	3.7	1.8	1.2	1.6	1.0	1.5	1.1
Glutamic Acid Decarboxylase	1.1	0.9	0.7	1.9	3.4	3.8	4.1	0.8	1.5	0.7	1.4	1.2
Cerebellar	1.8	1.5	0.9	1.7	2.7	3.2	1.0	0.9	1.2	1.5	0.9	1.3
Synapsin	3.3	2.8	1.4	2.2	2.3	2.8	4.7	1.6	3.8	1.8	3.1	0.8
Rotavirus	1.3	1.4	1.6	1.9	3.6	2.4	5.1	2.0	4.7	1.4	4.5	1.2
Saccharomyces	3.5	3.1	1.8	2.7	3.1	4.3	4.8	1.3	3.9	1.5	3.1	0.9
Dipeptidyl Peptidase IV	2.6	2.5	1.4	2.5	2.3	3.5	4.1	1.5	3.2	1.7	5.4	1.1

Table III. *Detection of antibodies against different antigens in 3 representative patients with thyroid autoimmunity expressed by index.*

Antigen	Patient # 1	Patient # 2	Patient # 3
Thyroglobulin	3.0	0.9	1.6
Thyroid Peroxidase	8.5	1.5	1.3
TSH Receptor	2.4	5.6	1.6
Thyroid Binding Globulin	1.3	1.8	4.9
Thyroid Binding Prealbumin	1.0	1.2	5.7
Glutamic Acid Decarboxylase	3.5	0.8	1.1
Y. enterocolitica HSP-60	1.2	1.0	4.9
Borrelia	2.7	1.8	1.3
Bartonella	3.0	1.6	0.7
Gliadin Peptide	4.8	3.3	0.9

Table IV. *Detection of antibodies against different antigens in 3 representative patients with lupus expressed by index.*

Antigen	Patient # 1	Patient # 2	Patient # 3
DNA	5.8	2.3	1.8
Lupus Peptide SmD3	5.6	3.7	4.2
Phosphorylcholine	4.6	3.5	1.5
Laminin Peptide	4.8	4.0	3.5
ADP-Ribose Polymerase	3.7	2.4	1.2
Fibrillarin & Chromatin	3.9	3.2	1.7
Mercury-HSA	4.1	3.6	1.9
Immune Complex	2.2	5.4	4.3
Wheat Gliadin	3.8	2.0	1.4
Cow's Milk Protein	3.3	2.9	1.2
Rheumatoid Factor	3.5	4.2	2.6
Rheumatic Fever Peptide	4.0	3.8	1.7
Streptozyme	2.8	3.6	1.8
Strep M Protein	3.1	4.1	1.3
Mycoplasma arthritidis	2.4	2.6	1.1
HSP-60	2.6	2.1	1.9
MBP	3.7	1.2	0.9

against cytoskeletal protein tropomyosin have been demonstrated in patients with ulcerative colitis and in patients with colorectal cancer (18-19).

In addition, antibodies against 5 out of 93 other antigens, including tTG, DPP IV, trypsin, motilin and VIP were detected in 2 out of 3 tested patients' sera (Table I). These assays were applied to the blood samples of 50 healthy controls and 50 patients with GI autoimmunity. In 46-68% of the patients versus 6-12% of controls, elevation in antibodies was demonstrated against these antigens ($p < 0.001$) (Fig. 2).

In celiac disease, another autoimmune disorder of the gut, antibodies against tTG, an enzyme that modifies many proteins, is detected. In these individuals, gliadin peptide found in gluten extracted from wheat, rye and barley by binding to tTG incites the immune system to attack the lining of the small intestine, resulting in autoimmunity or enteropathy (20). However, since 1966, scientific evidence

Table V. *Detection of antibodies against different antigens in 3 representative patients with rheumatoid arthritis expressed by index.*

Antigen	Patient # 1	Patient # 2	Patient # 3
Rheumatoid Factor	4.8	5.6	6.9
Arthritis Peptide	2.7	2.1	1.3
Citrullinated Peptide	5.4	4.5	2.0
Collagen	6.2	4.9	1.2
Mitochondrial Dehydrogenase	1.3	5.3	2.4
Mycobacterium avium	2.6	1.9	1.7
Mycoplasma arthritidis	3.5	3.2	2.6
Chlamydia pneumoniae	2.9	2.0	3.9
HSP-60	3.8	1.5	4.5
Rheumatic Fever Peptide	4.1	1.3	3.8
Streptozymes	3.8	1.0	2.7
Strep M Protein	3.2	0.8	2.5
D8/17	4.6	1.2	3.2
Ganglioside	3.4	0.9	2.4
LFA	4.7	2.9	1.6
Borrelia b. Antigen	3.5	2.4	1.2
Immune Complexes	4.3	3.8	4.7
Y. enterocolitica HSP-60	2.9	3.5	1.1

Table VI. *Detection of antibodies against different antigens in 3 representative patients with osteoarthritis expressed by index.*

Antigen	Patient # 1	Patient # 2	Patient # 3
Rheumatoid Factor	2.4	4.8	3.6
Rheumatic Fever Peptide	4.4	3.2	1.0
Fibulin	3.7	6.5	3.4
Y. enterocolitica HSP-60	0.9	1.8	4.7
Streptozyme	3.3	2.6	1.5
Strep M Protein	2.8	2.1	1.2
D8/17	2.5	2.7	0.8
Ganglioside	3.2	1.5	1.0
LFA	4.4	1.8	1.4

Table VII. *Detection of antibodies against different antigens in 3 representative patients with Addison's disease expressed by index.*

Antigen	Patient # 1	Patient # 2	Patient # 3
Adrenal Gland	4.9	2.7	4.1
21-Hydroxylase	3.8	2.8	4.5
Glomerular Basement Membrane Protein	1.2	2.5	1.7
Thyroglobulin	0.9	2.3	1.6
Thyroid Microsomal	1.5	0.7	3.3
Transglutaminase	3.1	1.4	2.5
MMPs	2.4	2.0	1.9

Table VIII. *Detection of antibodies against different antigens in 3 representative patients with Type-1 diabetes expressed by index.*

Antigen	Patient # 1	Patient # 2	Patient # 3
Insulin	1.6	5.3	1.2
Islet-Cell Antigen-2	4.5	2.4	3.9
Glutamic Acid Decarboxylase	5.1	4.8	3.5
Coxsackievirus	4.7	3.9	4.2
Cow's Milk Protein	3.6	4.0	3.3
HSP-60	1.4	3.7	0.9
Gliadin Peptide	4.4	1.8	1.1
Chlamydia pneumoniae	1.6	4.1	1.2

Table IX. *Detection of antibodies against different antigens in 3 representative patients with cardiovascular and autoimmune disease expressed by index.*

Antigen	Patient # 1	Patient # 2	Patient # 3
Myosin-α	5.3	4.8	1.9
o-LDL	5.8	3.3	6.2
Platelet Glycoprotein	4.6	1.7	1.4
β2-Glycoprotein	3.5	1.1	1.8
Endothelial Cell	1.9	3.5	4.1
Phosphorylcholine	5.0	0.8	1.3
HSP-60	1.6	3.7	4.6
C. pneumoniae	1.4	4.2	5.4
Tropomyosin	3.6	4.5	1.6
Immune Complex	1.1	3.9	4.3

has been accumulated demonstrating that gluten sensitivity or intolerance and antibody production against gliadin can exist in the absence of tTG antibody and enteropathy (21-22). For this reason we measured IgG and IgA antibodies in patients with classic celiac disease with enteropathy and in patients with gluten intolerance. Results depicted in Table II show that in addition to wheat gluten, gliadin peptide, gluteomorphin and tTG, both IgG and IgA antibodies were detected in patients with celiac disease against DPP IV, GAD, cerebellar, synapsin, Rotavirus and *Saccharomyces* peptides. Interestingly, in patients with gluten intolerance, IgG but not IgA antibodies were detected against all the above-mentioned antigens or peptides (Table II). Antibodies against other antigens, including heat shock protein, *Chlamydia pneumoniae*, myosin-α, milk proteins and MBP were detected at a significant level (data not shown). Percentage elevation in IgA antibodies against these major antigens was tested

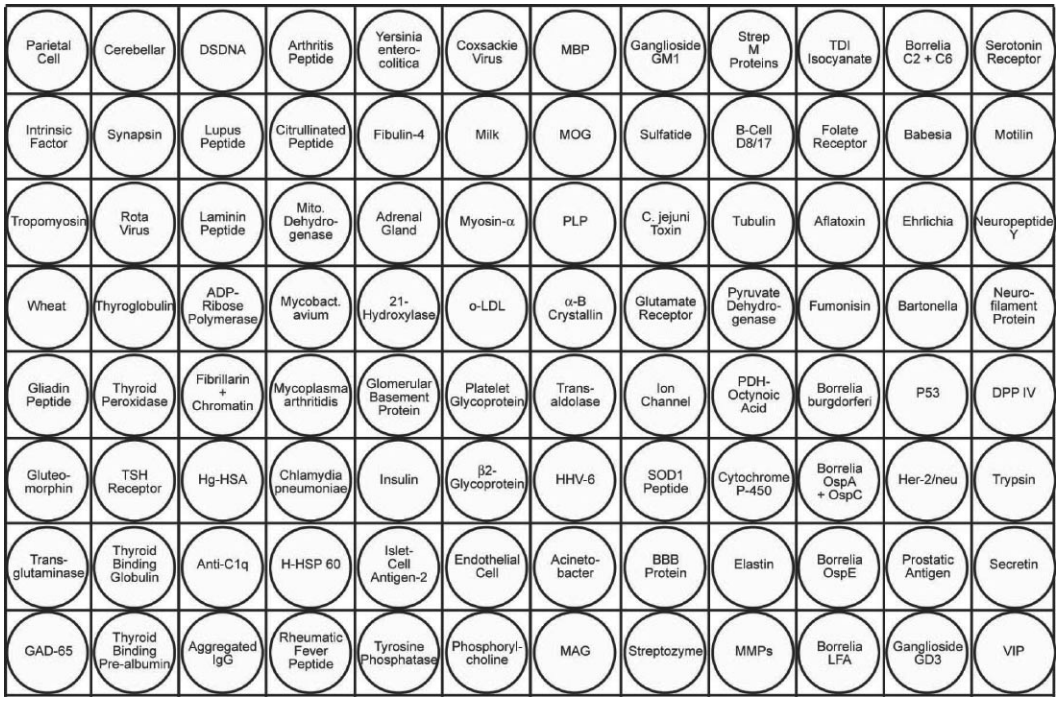

Fig. 1. *Format for antigen coating plate used in ELISA antibody array.*

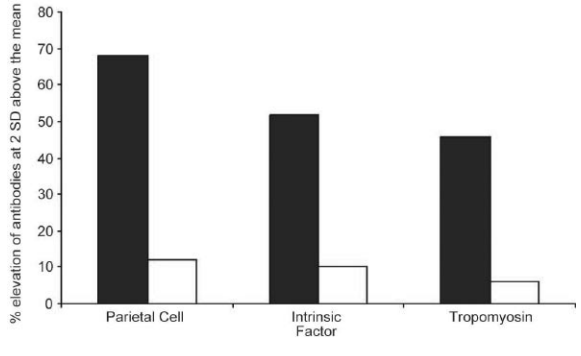

Fig. 2. *Elevation in IgG antibody in healthy controls* □ *compared to afflicted patients* ■ *with GI autoimmunity; n = 50 for each group, p < 0.001.*

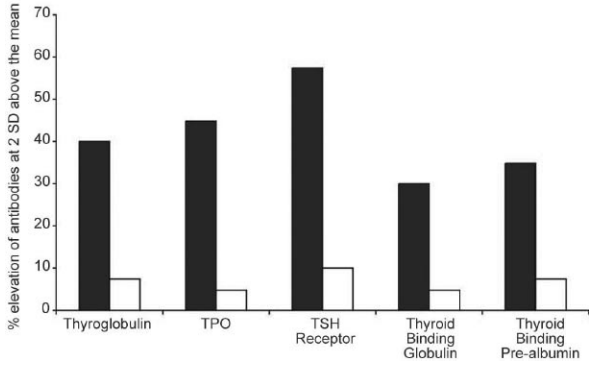

Fig. 4. *Elevation in IgG antibody in healthy controls* □ *compared to afflicted patients* ■ *with thyroid autoimmunity; n = 40 for each group, p < 0.001.*

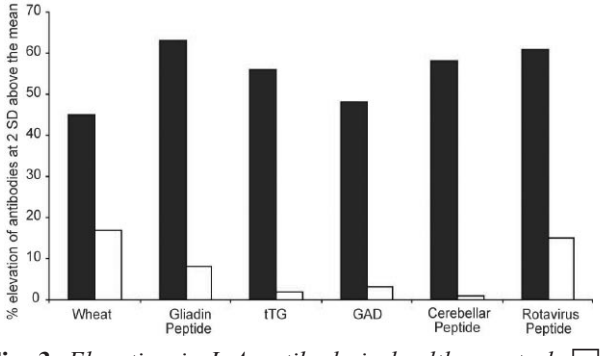

Fig. 3. *Elevation in IgA antibody in healthy controls* □ *compared to afflicted patients* ■ *with celiac disease; n = 50 for each group, p < 0.001.*

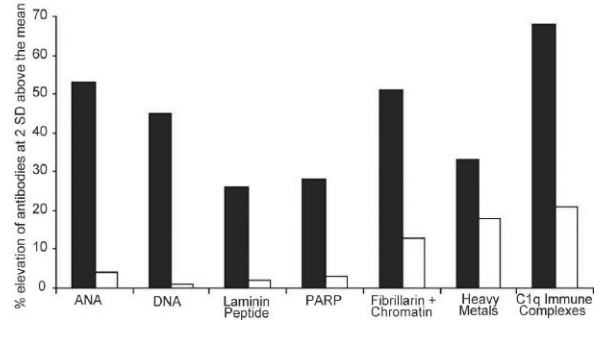

Fig. 5. *Elevation in IgG antibody in healthy controls* □ *compared to afflicted patients* ■ *with lupus; n = 100 for each group, p < 0.001.*

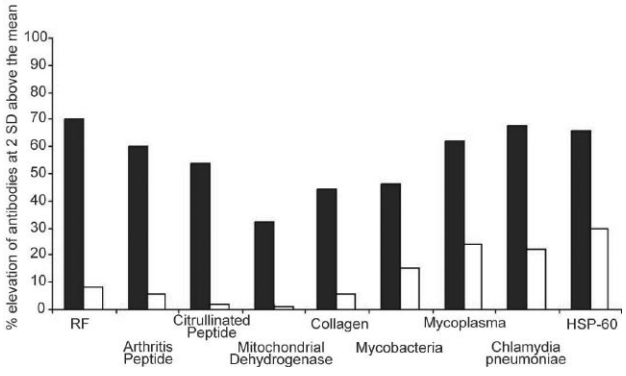

Fig. 6. *Elevation in IgG antibody in healthy controls* □ *compared to afflicted patients* ■ *with rheumatoid arthritis; n = 50 for each group, p < 0.001.*

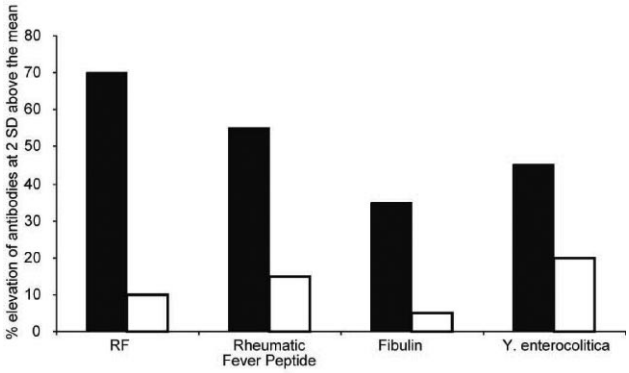

Fig. 7. *Elevation in IgG antibody in healthy controls* □ *compared to afflicted patients* ■ *with osteoarthritis; n = 20 for each group, p < 0.001.*

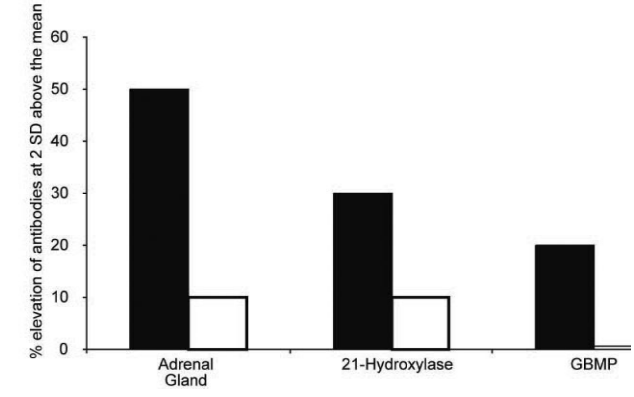

Fig. 8. *Elevation in IgG antibody in healthy controls* □ *compared to afflicted patients* ■ *with Addison's disease; n = 10 for each group, p < 0.05.*

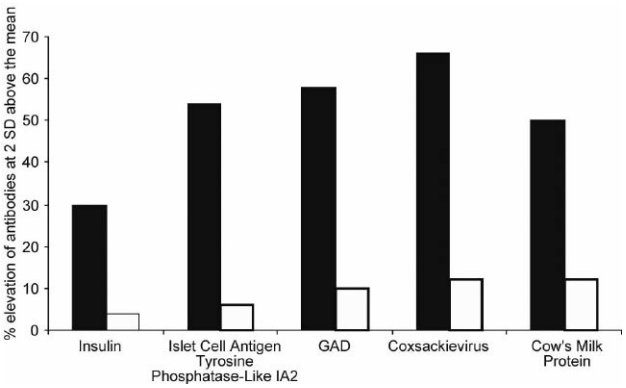

Fig. 9. *Elevation in IgG antibody in healthy controls* □ *compared to afflicted patients* ■ *with type-1 diabetes; n = 50 for each group, p < 0.001.*

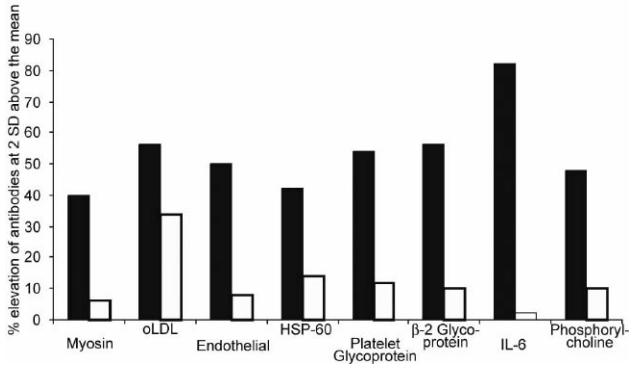

Fig. 10. *Elevation in IgG antibody in healthy controls* □ *compared to afflicted patients* ■ *with cardiovascular disease and autoimmunity; n = 50 for each group, p < 0.001.*

in controls and patients. Data presented in Fig. 3 shows that IgA antibody elevation in patients with $p < 0.001$ was significantly different from controls.

Antibodies against the two major antigens TG and TPO reflect disease activity and progression (23). While clinicians rely on the antibody level and elevation in TSH, little emphasis has been put on the other enzymes, binding proteins and receptors involved in thyroiditis (24-25). This includes TSHR, thyroxine-binding globulin, thyroxine-binding prealbumin and thyroxine deiodinase. As shown in Table III, patients may have no or low levels of antibody against TG or TPO but have a significant elevation in antibody against TSH-R, thyroid binding globulin or thyroid binding prealbumin. Furthermore, these assays were applied to 40

controls and 40 patients with thyroid autoimmunity. A significant percentage of patients showed elevation against different thyroid antigens ($p < 0.001$) (Fig. 4). In addition, sera from these patients reacted with GAD, *Yersinia enterocolitica, Borrelia, Bartonella* and gliadin peptide (Table III).

Systemic lupus erythematosus (SLE) is an autoimmune disease affecting different organs accompanied by the production of antibodies against ssDNA and extractable nuclear antigens, including Sm, RnP, Ro, La and phospholipids (26). Recently, highly specific peptides such as SmD3 and laminin, enzymes such as PARP, metal binding proteins and antibody production against them have been reported (27). The pattern of antibodies measured against ssDNA, lupus SmD3 peptide, cardiolipin, laminin peptide, PARP, fibrillarin, chromatin, mercury-HSA and immune complexes in three patients with negative or positive for ssDNA is shown in Table IV. In addition, rheumatoid factor (RF) and antibodies against rheumatic fever peptide, Streptozyme, strep-M-protein, *Mycoplasma arthritidis*, HSP-60 and MBP were detected in these individuals (Table IV). Antibodies against these antigens were measured in 100 controls and 100 patients with lupus. The percentage elevation of these antibodies is presented in Fig. 5 ($p < 0.001$).

Rheumatoid arthritis (RA) is another complex autoimmune disease. Its characteristic feature is a chronic destructive inflammation that is primarily localized in the synovial lining of the joints. RA can also cause inflammation in organs, including skin, lungs and heart, and in peripheral nerves (28). Serum antibodies specific for modified self proteins are a hallmark for many complex autoimmune disorders, including RA. These disease-specific antibodies predominantly react with modified self proteins such as IgG, citrullinated protein, collagen and mitochondrial dehydrogenase, and several mimic peptides (9, 29-30). Antibodies against aggregated IgG, arthritis peptide, citrullinated peptide, collagen type 2 peptide, mitochondrial dehydrogenase, *Mycobacterium avium, Mycoplasma arthritidis, Chlamydia pneumoniae* and HSP-60, which are detected frequently in patients with RA are shown in Table V and Fig. 6. Furthermore, antibodies against some or all of the following antigens or peptides were detected: rheumatic fever peptide, streptozyme, strep-M-protein, B cell D8/17, Ganglioside GM_1, leukocyte function associated antigen, *Borrelia burgdorferi* antigen, *Yersinia enterocolitica* and total immune complexes.

Autoimmunity to chondrocyte-producing proteins such as fibulin-4 has been reported in patients with osteoarthritis (31). In reactive arthritis Yersinia heat shock protein has been identified as the target of HLA-B27-restricted CTL response (32). That is why in addition to RF and rheumatic fever peptide, antibodies against fibulin and Yersinia HSP-60 were measured and detected in a significant percentage of patients with osteoarthritis (Table VI, Fig. 7). Additionally, antibodies against streptococcal antigen, B cell D8/17, ganglioside and LFA were detected. Multiple cross-reactivities between tissue-specific expressed autoantigens might explain the effect on multiple tissues or organs in some patients with autoimmune diseases.

Adrenalitis is another organ-specific autoimmune disease that can lead to adrenal gland failure or Addison's disease (33). Adrenalitis-associated antibodies were measured against adrenal gland antigens, 21-hydroxylase and glomerular basement membrane protein. Results depicted in Table VII and Fig. 8 show that adrenal gland antigen and 21-hydroxylase were most commonly detected in patients with Addison's disease.

In type I diabetes the immune system attacks the beta cells in the pancreas and manufactures antibodies against different tissue antigens (34). The measurement of antibodies against insulin, GAD and IA-2 is shown in Fig. 9. IgG antibody against these antigens, Coxsackievirus and milk protein was detected in the majority of tested patients' sera (Table VIII, Fig. 9). Furthermore, antibodies against tyrosine phosphatase, HSP-60, gliadin peptide and *Chlamydia pneumoniae* were detected in about one third of patients positive for insulin, GAD and IA-2 antibodies (Table VIII).

In cardiac autoimmunity, antibodies are produced against heart myosin, vascular endothelial cells, platelet glycoprotein, β2-glycoprotein, phosphorylcholine, HSP-60, or against modified low o-LDL (35-37). Table IX and Fig. 10 show immune response and antibody production in up to 56% of patients with cardiovascular disease against the specific and non-specific antigens *Chlamydia*

pneumoniae and tropomyosin. Furthermore, in two out of three patients very high levels of total immune complexes were detected (Table IX). Data related to neuroautoimmune disorders and cancer will be presented in a subsequent manuscript.

DISCUSSION

In writing this manuscript, we were inspired by the pioneering work of Dr. A. L. Notkins, who, in "New Predictors of Diseases," emphasized that "one day Y-shaped molecules called autoantibodies in a patient's blood may tell doctors whether a patient is 'brewing' a certain disease and may even indicate roughly how soon the individual will begin to feel symptoms (13)." Furthermore, the article states "molecules called predictive autoantibodies appear in the blood years before people show symptoms of various disorders. Tests that detected these molecules could warn of the need to take preventive action (13)."

During the past five years we have applied hundreds of sera obtained from patients with twenty different disorders to a microtiter plate coated with 96 different highly pure antigens or peptides originating from human tissue or infectious agents.

In gastrointestinal autoimmunity such as pernicious anemia, celiac disease and inflammatory bowel disease, immune reaction against common mucosal tissue antigens, dietary proteins or peptides and commensal bacteria have been reported. A breakdown in immune tolerance and the induction of T_H1/T_H2 imbalance could be due to change in production of TGF-beta and IL-17, which contributes to inflammation and autoimmunity.

Similar to findings in an earlier report, in patients with pernicious anemia (17) we detected antibodies against parietal cells and intrinsic factor. However, in this study we found that up to 68% of sera from patients with PA versus 12% from controls reacted with cytoskeletal protein, tropomyosin, DPP IV, trypsin, motilin and VIP. Antibodies against tropomyosin have been reported in patients with ulcerative colitis and colorectal cancer (18-19). Based on these findings, the inclusion of antibody measurements against tissue-specific antigens, enzymes, receptors or regulators of the GI tract, in future studies of PA is likely to contribute to the sensitivity and predictive values of laboratory examinations.

Celiac disease is another autoimmune disease associated with tissue antibodies against endomysial and reticulin, and antibodies against gliadin (20-22). Both IgG and IgA antibodies against these antigens have been used in patients with classic celiac disease with sensitivity, specificity and predictive values of 70-100% (20). Since 1966, in both patients with celiac disease and gluten intolerance, antibodies against a variety of tissue antigens, including thyroid, joints, bone, heart, pancreas, brain and even synapses have been reported (21-22). From these reports and our findings summarized in Table II and Fig. 3, testing for celiac disease and gluten intolerance should not only include antibodies against gliadin, gluteomorphin, parietal cell and transglutaminase, which limit their finding only to the gut, but should also emphasize the importance of the measurement of antibodies against TPO, HSP, MBP, neurofilaments, cerebellar and many other antigens.

Thyroid autoantibodies TG and TPO can also reflect disease activity and progression and are valuable in disease prediction and the classification of Hashimoto and Graves disease (23). Data presented in Table III and Fig. 4 further support measurements of antibodies against TSH-receptor, thyroxine-binding albumin, and thyroxine-binding prealbumin to increase the sensitivity of prediction in thyroid autoimmunity (24-25).

Lupus and RA are autoimmune diseases affecting different tissues and organs, accompanied by the production of antibodies against modified nucleic acids, nucleoproteins and self proteins (38). In this regard, while our data support the inclusion of newly reported antibodies against SmD3, laminin, PARP for lupus (38), arthritis-induced peptide and mitochondrial dehydrogenase for arthritis (28-30), and chondrocyte-producing protein such as fibulin-4 for patients with osteoarthritis (31-32), factors responsible for the direct induction of autoimmune disease or self protein modifications and antibodies against them may be considered in future studies of predictive antibodies. For example, despite the fact that it is very well accepted that injection of $HgCL_2$ can induce scleroderma and lupus in animal models (14, 39-40), no attempt was made to measure antibodies against Hg-HSA or Hg-binding

nucleoproteins, such as fibrillarin and chromatin, in human lupus (11). Similarly, in the case of infections with *Mycoplasma, Chlamydia* and *Mycobacteria*, their heat shock proteins or mimic peptides are considered as the most likely triggering factors for RA (9, 32, 41). To our knowledge, this is the first report showing elevation in antibodies against protein-modifying factors in clinical specimens from lupus and arthritis (see Tables IV, V, VI and Fig. 5, 6 and 7).

These findings also apply to type-1 diabetes. In the sera of these patients, almost every patient with elevation of IgG antibodies against GAD and IA-2 was either positive for cow's milk, *Coxsackievirus*-B antibody or both (Table VIII, Fig. 9) (42-43).

In cardiovascular autoimmunity, based on earlier reports (37), we detected significant elevation of IgG antibodies against heart myosin, vascular endothelial cells, platelet glycoprotein, β2-glycoprotein, phosphorylcholine, HSP-60, or against modified low density lipoprotein (o-LDL) (Table IX, Fig. 10). Antibodies against some or all of these antigens were detected in a significant percentage of healthy controls.

In conclusion, environmental factors such as bacterial and viral infections, toxic chemicals, and some dietary proteins and peptides are major instigators of autoimmunity. Without identification of the factors responsible for specific autoimmune diseases, one would not be able to take preventive action. Moreover, the inclusion of antibody measurements against tissue-specific epitopes and environmental factors can increase the clinical sensitivity, clinical specificity and clinical predictive values for these antibodies.

REFERENCES

1. Brady GF, Congdon KL, Clark AG, Sackey FNA., Rudolph EH, Radic MZ, Foster MH. Editing rescues autoreactive B cells destined for deletion in mice transgenic for a dual specific anti-laminin Ig. J Immunol 2004; 172:5313-21.
2. Rose NR. New principles of autoimmunity prediction and prevention of autoimmune diseases part I. Ann NY Acad Sci 2007; 1109:117-28.
3. Teubner A, Tillmann HL, Schuppan D, Gericke G, Manns MP, Stolzel U. Prevalence of circulating autoantibodies in healthy individuals. Med Klin 2002; 97:645-649.
4. Friese MA, Jones EY, Fugger L. MHC II molecules in inflammatory diseases: interplay of qualities and quantities. Trends Immunol 2005; 26(11):559-561.
5. Deocharan B, Marambio P, Edelman M, Putterman C. Differential effects of interleukin-4 in peptide-induced autoimmunity. Clin Immunol 2003; 108: 80-88
6. Maitre A, Hours M, Bonneterre V, Arnaud J, Arslan MT, Carpentier P, Bergeret A, de Gaudemaris R. Systemic sclerosis and occupational risk factors: role of solvents and cleaning products. J Rheumatol 2004; 31:2385-2401.
7. Hamandzic D, *Kasman LM, LeRoy EC*. The role of infectious agents in the pathogenesis of systemic sclerosis. Curr Opin Rheumatol 2002; 14:694-698.
8. Vojdani A, Vojdani E, Cooper EL. Antibodies to myelin basic protein, myelin oligodendrocytes peptides, α-B-crystallin, lymphocyte activation and cytokine production in patients with multiple sclerosis. J Internal Med 2003; 254:363-374.
9. Wildner G, Diedrichs-Moehring M. Multiple autoantigen mimotopes of infectious agents induce autoimmune arthritis and uveitis in Lewis rats. Clin Diag Lab Immunol 2005; 12:677-9.
10. Griem P, Wulferink M, Sachs B, Gonzalez JB, Gleichmann E. Allergic and autoimmune reactions to xenobiotics: how do they arise? Immunol Today 1998; 19:133-141.
11. Takeuchi K, Turley SJ, Tan EM, Pollard KM. Analysis of the autoantibody response to fibrillarin in human disease and murine model of autoimmunity. J Immunol 1995; 154:961-71.
12. Leung PSC, Park O, Tsuneyama K, *Kurth MJ, Lam KS, Ansari AA, Coppel RL, Gershwin ME*. Induction of primary biliary cirrhosis in guinea pigs following chemical xenobiotic immunization. J Immunol 2007; 179:2651-7.
13. Notkins AL. New predictors of disease. Sci Am 2007; 296:72-79.
14. Thierse H-J, Moulon C, Allespach Y, *Zimmermann B, Doetze A, Kuppig S, Wild D, Herberg F, Weltzien HU*. Metal-protein complex-mediated transport and delivery of Ni^{2+} to TCR/MHC contact sites in nickel-specific human T cell activation. J Immunol 2004; 172:1926-34.
15. Zeiss CR, Kanellakes TM, Bellone JD, Levitz D, Pruzansky JJ, Patterson R. Immunoglobulin E-mediated asthma and hypersensitivity pneumonitis with precipitating anti-hapten antibodies due to diphenylmethane di-isocyanate (MDI) exposure. J Allergy Clin Immunol 1980; 65:346-52.
16. James D, Asherson G, Chanarin I, Coghill N, Hamilton S, Himsworth RL, Webster D. Cell-

mediated immunity to intrinsic factor in autoimmune disorders. Br Med J 1974; 4:494-6.
17. Gleeson PA, van Driel IR, Toh B. Parietal cell autoantibodies. In: Autoantibodies. Peter JB, Shoenfeld Y (Eds), Elsevier, Amsterdam, The Netherlands,1996; 600-6.
18. Das KM, Dasgupta A, Mandal A, Geng X. Autoimmunity to cytoskeletal protein tropomyosin: a clue to the pathogenic mechanism for ulcerative colitis. J Immunol 1993; 150:2487.
19. Nakamura H, Hinoda Y, Nakagawa N, Makiguchi Y, Itoh F, Endo T, Imai K. Detection of circulating anti-MUC1 mucin core protein antibodies in patients with colorectal cancer. J Gastroenterol 1998; 33:354-61.
20. McMillan SA, Haughton DJ, Biggart JD, Edgar JD, Porter KG, McNeill TA. Predictive value for celiac disease of antibodies to gliadin, endomysium, and jejunum in patients attending for jejunal biopsy. Brit Med J 1991; 303:1163-5.
21. Vojdani A, O'Bryan T, Kellermann GH. The immunology of immediate and delayed hypersensitivity reaction to gluten. Eur J Inflamm 2008; 6:1-10.
22. Vojdani A, O'Bryan T, Kellermann GH. The immunology of gluten sensitivity beyond the intestinal tract. Eur J Inflamm 2008 (In Press).
23. Rapoport B, McLachlan SM. Thyroid autoimmunity. J Clin Invest 2001; 108:1253-9.
24. Moreno M, Berry MJ, Horst C, et al. Activation and inactivation of thyroid hormone by type I iodothyronine deiodinase. FEBS Lett 1994; 344: 143–146.
25. Szkudlinski MW, Fremont V, Ronon C, Weintraub BD. Thyroid-stimulating hormone and thyroid-stimulating hormone receptor structure-function relationship. Physiol Rev 2002; 82:473-502.
26. Hochberg MC. Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum 1997; 40:1725.
27. Grader-Beck T, Casciola-Rosen L, Lang TJ, *Puliaev R, Rosen A, Via CS*. Apoptotic splenocytes drive the autoimmune response to poly (ADP-ribose) polymerase 1 in a murine model of lupus. J Immunol 2007; 178:95-102.
28. Girbal-Neuhauser E, Durieux J-J, Arnaud M, Dalbon P, Sebbag M, Vincent C, Simon M, Senshu T, Masson Bessiere C, Jolivet Reynaud C, Jolivet M, Serre G. The epitopes targeted by the rheumatoid arthritis-associated antifilaggrin autoantibodies are posttranslationally generated on various sites of (pro)filaggrin by deamination of arginine residues. J Immunol 1999; 162:585-94.
29. van Gaalen F, Ioan-Facsinay A, Huizinga TWJ, Toes REM. The devil in the details: the emerging role of anticitrulline autoimmunity in rheumatoid arthritis. J Immunol 2006; 175:5575-80.
30. Cook AD, Gray R, Ramshaw J, Mackay IR, Rowley MJ. Antibodies against the CB10 fragment of type II collagen in rheumatoid arthritis. Arthritis Res Ther 2004; 6:R477-83.
31. Xiang Y, Sekine T, Nakamura H, Imajoh-Ohmi S, Fukuda H, Yudoh K, Masuko-Hongo K, Nishioka K, Kato T. Fibulin-4 is a target of autoimmunity predominantly in patients with osteoarthritis. J Immunol 2006; 176:3196-204.
32. Sanja U, Metrz A, Wu P, Braun J, Sieper J. A single nonamer from the Yersinia 60-kDA heat shock protein is the target of HLA-B27-restricted CTL response in Yersinia-induced reactive arthritis. J Immunol 1997; 159:5715-23.
33. Betterle C, Volpato M, Rees Smith B, Furmaniak J, Chen S, Zanchetta R, Greggio NA, Pedini B, Boscaro M, Presotto F. Adrenal cortex and steroid 21-hydroxylase autoantibodies in children with organ-specific autoimmune diseases: markers of high progression to clinical Addison's disease. J Clin Endocrinol Metab 1997; 82:939-942.
34. Leslie RDG, Atkinson MA, Notkins AL. Autoantigens IA-2 and GAD in Type I (insulin-dependent) diabetes. Diabetologia 1999; 42:3-14.
35. Shaw PX, Goodyear CS, Chang M, Witztum JL, Silverman GJ. The autoreactivity of anti-phosphorylcholine antibodies for atherosclerosis-associated neo-antigens and apoptotic cell. J Immunol 2003; 170:6151-7.
36. Danese S, Dejana E, Fiocchi C. Immune regulation by microvascular endothelial cells: directing innate and adoptive immunity, coagulation and inflammation. J Immunol 2007; 178:6017-22.
37. Kornberg A, Renaudineau Y, Blank M, Youinou P, Shoenfeld Y. Anti-beta-2-glycoprotein-1 antibodies and anti-endothelial cell antibodies induce tissue factor in endothelial cells. IMAJ 2000; 2(S):27-31.
38. Arbuckle MR, McClain MT, Rubertone MV, Scofield RH, Dennis GJ, James JA, Harley JB. Development of autoantibodies before the clinical onset of systemic lupus erythematosus. N Engl J Med 2003; 349:1526-33.
39. Reuter R, Tessars G, Vohr HW, Gleichmann E, Luhrmann R. Mercuric chloride induces autoantibodies against U3 small nuclear ribonucleoprotein in susceptible mice. Proc Natl Acad Sci USA 1989; 86:237-41.
40. Hultman P, Bell LJ, Enestrom S, Pollard KM. Murine susceptibility to mercury. I. Autoantibody profiles

and systemic immune deposits in inbred, congenic and intra-H-2 recombinant strains. Clin Immunol Immunopathol 1992; 65:98-109.
41. Carty SM, Snowden S, Silman AJ. Should infection still be considered as the most likely triggering factor for rheumatoid arthritis? Ann Rheum Dis 2004; 63(S2):ii46-9.
42. Virtanen SM, Laara E, Hypponen E, Reijonen H, Rasanen L, Aro A, Knip M, Ilonen J, Akerblom HK. Cow's milk consumption, HLA-DQB1 genotype, and type 1 diabetes. A nested case-control study of siblings of children with diabetes. Diabetes 2000; 49: 912-7.
43. Clements GB, Galbraith DN, Taylor KW. Coxsackie B virus infection and onset of childhood diabetes. Lancet 1995; 346:221-3.