

IMMUNOLOGICAL STUDIES IN BALKAN ENDEMIC EPHROPATHY

Polenakovic M.,¹ Cukuranovic R.,² Savic V.,²
Jovanovic I.,³ Stefanovic V.,²

¹*Macedonian Academy of Sciences and Arts, Skopje, Macedonia*

²*Institute of Nephrology and Haemodialysis, Faculty of Medicine, Niš, Serbia*

³*Institute of Anatomy, Faculty of Medicine, Niš, Serbia*

Abstract: *Aim.* To re-examine serum complement and immunoglobulin levels in patients with Balkan Endemic Nephropathy (BEN) in the early stage of the disease; to study autoantibodies (anti-thyroid, anti-smooth muscle, anti-mitochondrial, anti-parietal, anti-nuclear, and anti-DNA) in these patients; and, finally, to re-assess the immunohistology in kidney biopsies from BEN patients. A review of the immunological studies in BEN will be presented.

Methods. Forty-five BEN patients in the early stage of disease, from the South Morava Region, were included in this study. Fifty-five apparently healthy controls, matched for age and sex, from the neighbouring control villages were selected. Serum complement and immunoglobulin levels were determined by the Immunochemistry system of Beckman. Auto-antibodies were detected in the sera by the indirect fluorescence method or by radio-immunoassay (anti-single stranded DNA). Immunofluorescent microscopy was used for kidney samples treated with FITC conjugated antisera for IgG, IgM, IgA, C3, C1q, C4 and fibrin/fibrinogen.

Results. Serum C3 was decreased in the sera of patients with BEN compared to healthy controls ($p < 0.001$), serum IgM was also decreased ($p < 0.05$). Anti-thyroid antibodies were detected in 5/45 BEN patients, anti-parietal in 7/45 patients and ANA in 2 BEN patients. No anti-smooth muscle, anti-mitochondrial, or anti-DNA antibodies were detected in any BEN patient. Immunofluorescent studies of 10 kidney biopsy specimens showed rare, unspecific, focal, mesangial deposits of C3 and IgM in some glomeruli, and rare deposits, mostly of C3, in tubuli and extraglomerular vessels.

Conclusion. Humoral immune mechanisms do not appear to play a pathogenic role in BEN. A few studies on cell-mediated immunity in BEN were performed, and further studies are needed on patients in the early stage of the disease.

Key words: Balkan Endemic Nephropathy (BEN), immunological study, serum complement, immunoglobulins, autoantibodies, immunohistology.

Introduction

Balkan Endemic Nephropathy (BEN) is a chronic tubulointerstitial nephritis (TIN) with an insidious onset and slow progression to terminal renal failure. It was first described in Serbia [1] and in Bulgaria [2]. It affects people living in the alluvial plains along the tributaries of the River Danube in Serbia, Bosnia, Croatia, Bulgaria and Rumania [3].

In TIN an immune response to either foreign antigens or auto-antigens may cause renal damage by predominantly humoral or cell-mediated reactions [4]. In contrast with glomerular injury, cell-mediated reactions predominate over humoral mediated mechanisms in the pathogenesis of tubulointerstitial lesions [5]. Tubulointerstitial infiltrates are the hallmark of any form of TIN. They include, to variable degrees, neutrophils, T- and B-lymphocytes, macrophages, natural killer and plasma cells.

The predominant pathology of BEN is that of a chronic, multifocal interstitial nephritis, with rare infiltrates [6, 7]. Multifocal chronic inactive sclerosing non-specific lesions regularly found in the kidneys of earlier stages of BEN may suggest accelerated ageing triggered by long-lasting low-dose exposure to an unknown environmental nephrotoxic and mutagenic agent. The findings are non-specific and similar to those associated with ageing [8–10]. Similar nonspecific findings are associated with nephrotoxic metals such as lead [11], cadmium [12] and lithium [13]. Histological changes in BEN share similarities with renal damage caused also by ochratoxin A [14], aristolochic acid [15] and polycyclic aromatic hydrocarbons [16].

Few observations on the deposition of immunoglobulins (Igs) and complement components in kidney biopsies of BEN patients have been reported. Glomerular and vascular staining for immune reactants has been mainly negative or insignificant [6,17]. Deposits of C3 along the tubular epithelium correlated with the intensity of tubulo-interstitial changes and the degree of proteinuria. An immunohistological investigation of renal tissue from six patients with BEN was performed [17]. Focal deposits of C3 and IgM were found in some glomeruli, but they were not uniform and could not be placed in any form of glomerulonephritis. In ten cases with pyelonephritis, including cases with renal calculosis, glomerular deposits of C3 (positive in 60% of cases), IgG (positive in 20% of cases), and IgM (40%) were often found. In some tubules positive protein

cylinders for IgG (30%), IgM (40%) and IgA (50%) were found. Small deposits could also be found in some tubular cells. Discrete deposits of IgM and IgA were found in interstitial blood vessels. By direct or indirect immunofluorescence, Vizjak *et al.* [18] tested deposition of IgA, IgG, IgM, C3, C1q, C4 fibrin/fibrinogen, albumin, B2-microglobulin (beta 2m) and Tamm-Horsfall glycoprotein (THG) on frozen renal tissue sections in 52 BEN patients. Glomerular findings were negative or mostly insignificant, with a mild or moderate mesangial deposition of IgM in 16, IgA in 11, IgG in three, C3 in 15, C1q in two, C4 in one and fibrin/fibrinogen in two cases, respectively. The predominance of mesangial IgA deposits in five cases suggested IgA glomerulonephritis (GN) concomitant with BEN. Homogeneous lumpy or granular deposits in small extraglomerular vessels contained IgM in nine, C3 in 45, C1q in three cases, and C4 in one case. Focal linear or granular C3 was noted along the tubular basement membrane in eight cases.

In 1975 Radonić *et al.* reported on tubular antibasement membrane antibodies in sera of patients with BEN [19]. Serum of 21 BEN patients from the endemic region west of Slavonski Brod was tested by the indirect fluorescence method. None of 21 sera showed positive antibodies against tubular basement membranes. In the study by Macanovic *et al.*, anti-glomerular and anti-tubular basement membrane antibodies were not detected by the indirect immunofluorescence method [20].

Any significant activation of either the classical or the alternative pathway of the complement system in BEN. The minor modifications observed in some patients with BEN may be a result of the pathological process rather than being directly associated with their cause [21]. Serum immunoglobulins in BEN patients were in the normal range. Cryoglobulins were not found in the early stages of BEN [22].

The aim of this study was to reexamine serum complement and immunoglobulin levels in patients with BEN in the early stage of the disease; to study autoantibodies (anti-thyroid, anti-smooth muscle, anti-mitochondrial, anti-parietal, anti-nuclear, and anti-DNA) in these patients; and, finally, to reassess the immunohistology in the kidney biopsies. A review of the immunological studies in BEN will be presented.

Participants and Methods

Study subjects

Forty-five BEN patients in the early stage of disease, from the South Morava Region, were included in this study. Diagnosis of BEN was made according to criteria previously described [23]. Fifty-five apparently healthy controls, matched for age and sex, from the neighbouring control villages, were selected.

Methods

Serum complement and immunoglobulin levels were determined by the Immunochemistry system of Beckman. Autoantibodies were detected in the sera by the indirect fluorescence method or by radioimmunoassay (anti-single stranded DNA). Immunofluorescent microscopy was used for 10 kidney biopsy samples treated with FITC conjugated antisera for IgG, IgM, IgA, C3, C1q, C4 and fibrin/fibrinogen.

Statistical analysis

Data were analysed using Jandel SigmaStat[®] statistical software for Windows Version 2.0, and expressed as means \pm SD. Unpaired Student's t-test was used to test differences between the groups. A P value of less than 0.05 was considered statistically significant.

Results

Serum complement and immunoglobulin levels

The C3 complement component in BEN patients was significantly decreased ($P < 0.001$) compared to the healthy control level (Table 1). C4 was within the limits of normal. The IgM level in BEN patients was significantly ($P < 0.05$) lower than in the healthy controls, IgG and IgA were within the limits of normal.

Table 1 – Tabela 1

Serum complement and immunoglobulin levels in patients with BEN
Ниво на комплемент и имуноглобулини кај пациенти со БЕН

	Control	BEN
C3, g/L	1.00 \pm 0.24(0.93–1.06) 0.94 (0.92–0.94)	0.81 \pm 0.25(0.73–0.89) 0.78 (0.76–0.85)*
C4, g/L	0.42 \pm 0.12 0.42 (0.35–0.45)	0.46 \pm 0.14 0.45(0.41–0.50)
IgG, g/L	14.14 \pm 2.47 (13.47–14.81) 14.38 (13.21–15.18)	14.93 \pm 3.92 (13.70–16.15) 14.38 (13.21–16.00)
IgA, g/L	2.49 \pm 0.85 (2.26–2.72) 2.46 (2.06–2.71)	2.8 \pm 1.04 (2.51–3.15) 2.71 (2.30–3.15)
IgM, g/L	1.54 \pm 0.47 (1.41–1.66) 1.45 (1.38–1.59)	1.40 \pm 0.62 (1.20–1.59) 1.25 (1.06–1.38)**

Mean values \pm SD are given, with 5–95% value in parenthesis. Median with 5–95% value in parenthesis.

* $P < 0.001$ vs. control

** $P < 0.05$ vs. control

Autoantibodies

Anti-thyroid antibodies were detected in 5/45 BEN patients, anti-parietal in 7/45 patients and ANA in 2 BEN patients. No anti-smooth muscle or anti-mitochondrial antibodies were detected in any BEN patient (Table 2). Anti-DNA antibodies in a titer higher than 25 units/ml were not detected in BEN patients.

Table 2 – Tabela 2

*Autoantibodies in the sera of patients with BEN**Аутоантитела во серум на пациент со БЕН*

Antibodies	Grade 1	Grade 2	Grade 3
Antii-thyroid	1	2	2
Anti smooth-muscle	0	0	0
Anti-mitochondrial	0	0	0
Anti-parietal	2	2	3
Anti-nuclear	0	2	0

The presence of autoantibodies was graded from 1 (small) to 3 (high).

Kidney immunofluorescent studies

Glomerular findings were negative or insignificant, with mild mesangial deposition of IgM and C3 in some glomeruli. Focal linear or granular C3 deposits were noted along the tubular basement membrane in two cases. Weak granular deposits of C3 were noted in the small interstitial vessels of six patients.

Discussion

Previous studies on humoral immunity in BEN have shown no major role of immunity in the pathogenesis of BEN. We re-examined serum complement and serum immunoglobulin levels in 45 patients in the early stage of BEN. Some minor decrease of C3 complement component was demonstrated, similar to that in the previous study [21]. Studies of various complement components with appropriate controls were performed on 150 sera from the South Morava River region. Five groups of subjects were analysed: patients with BEN at the initial stage, patients with suspected BEN, BEN patients on haemodialysis, persons from endemic families but without any kidney disease, and healthy individuals living in this region. A complete complement study of 4 families with BEN was also performed. Several complement components were studied: CH50, C3, C4, C3 Activator, C1sINA, C1q, C5 components and C3 Nef activity. No consistent alteration of the complement system was found in any group. However, BEN patients on haemodialysis exhibited a moderate decrease of CH50 and

C3. In patients with BEN at the initial stage and patients with suspected BEN, 17% and 12% of patients respectively had a mild decrease of CH50 only. Six out of 18 patients with BEN at the initial stage had a decreased C3 component. Groups 4 and 5 were completely comparable to the control group outside of the BEN area. These results do not reveal any significant activation of either the classical or the alternative pathway of the complement system in BEN. The minor modifications observed in some patients with BEN may be a result of the pathological process rather than being directly associated with their cause.

A few studies on cell-mediated immunity in BEN were performed, with no definitive conclusion. Belovezhdov has studied a delayed type of hypersensitivity in BEN patients [24]. Three groups of patients were studied: 31 BEN patients, 30 patients with chronic pyelonephritis and a control group of 30 healthy individuals, using antigens from renal tubular cells and from glomerular basement membrane (GBM). A positive result indicating cell-mediated autoimmunity to renal tubular antigen (RTA) was obtained in 22/31 (70.9%) of patients with BEN. Three BEN patients (9.6%) gave a positive result with GBM antigen. Positive results either with RTA or GBM were not obtained in patients with chronic pyelonephritis and healthy controls. BEN patients showed a significantly decreased response to phytohemagglutinin transformation in the peripheral lymphocyte culture, in contrast to patients with chronic pyelonephritis and healthy controls.

Twelve BEN patients from the Vitina area, all but two in advanced renal failure, were studied by Polenakovic and Karanfilski [25]. Normal human renal tissue antigen (RTA) was used as a specific antigen. In 5 patients with BEN, RTA inhibited leukocyte migration, i.e. the test was positive. In all 12 patients lymphocyte migration was inhibited by phytohemagglutinin.

Savic *et al.* studied cell-mediated immunity in 19 BEN patients by using an E-rosette forming test and a leukocyte migration inhibition test (LIF) [26]. Only 3 patients had serum creatinine in the normal range, 16 had some degree of chronic renal failure. The number of E-rosette forming cells was 3.8 times lower in BEN patients compared to healthy controls; the mean LIF index for PPD and Candida antigen was, however, similar in BEN patients and healthy controls. From this study, any pathogenic role of cell-mediated immunity in BEN could be established requiring patients in the early stage of the disease, and testing with several *in vitro* tests.

The features of BEN, such as unknown physiopathogenicity with an unknown causal agent, hereditary but weak penetrance with no simple Mendelian segregation, and a chronic course, are the common features of all diseases associated with histocompatibility antigens (HLA) and therefore having a substantial immunogenetic background. This prompted a study on BEN in two different ways: to compare the frequencies of HLA antigens in a group of unrelated patients with the group of healthy controls, and second, to study families in order to see whether affected relatives share HLA haplotypes more often than expected

[27]. Two groups of patients from two distinct endemic areas (Slavonski Brod and Niš), three control groups (two from the endemic area and one common country control) and seven families of patients were studied for HLA-A, -B and -DR antigens. No significant correlation of HLA-A, -B and -DR antigens was found. The segregation of HLA haplotypes in families of patients suggests some interesting hypotheses on inheritance and etiology but needs confirmation in a larger number of informative families.

Kidney immunofluorescent studies in BEN are nonspecific. Glomerular findings were negative or insignificant, with mild mesangial deposition of IgM and C3 in some glomeruli. Focal linear or granular C3 deposits were noted along the tubular basement membrane in some cases. Week granular deposits of C3 were noted in the small interstitial vessels of some patients. The predominance of mesangial IgA deposits in five cases by Ferluga *et al.* [7] and two cases by Dojcinov *et al.* [6] suggested IgA glomerulonephritis (GN) concomitant with BEN. Although humoral immune mechanisms would not appear to play a pathogenetic role in BEN, immunohistologic examinations are important in the recognition of possible concomitant immune complex-mediated GN.

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Резиме

ИМУНОЛОШКИ СТУДИИ КАЈ БАЛКАНСКА ЕНДЕМСКА НЕФРОПАТИЈА (БЕН)

Поленаковиќ М.,¹ Чукурановиќ Р.,² Савиќ В.,²
Јовановиќ М.,³ Стефановиќ В.²

¹Македонска академија на науките и уметностите, Скопје, Р. Македонија

²Институт за нефрологија и хемодијализа, Ниш, Србија

³Институт за анатомија, Медицински факултет, Ниш, Србија

Цел. Да се преиспита нивото на комплементот и на имуноглобулините во серумот кај пациенти со балканска ендемска нефропатија (БЕН) во раниот стадиум на болеста; кај овие пациенти да се иследат автоантителата (анти-тиреоидни, антителата спрема мазните мускули, анти-митохондријални, анти-париетални, анти-нуклеарни и анти-ДНК); и конечно, да се процени имунохистологијата на бубрежните биопсии од пациентите со БЕН. Ќе се прикаже ревија на имунолошки студии кај пациенти со БЕН.

Методи. Во оваа студија беа вклучени четириесет и пет пациенти во раниот стадиум на болеста (БЕН) од регионот на Јужна Морава. Беа селектирани педесет и пет здрави контроли, со соодветна возраст и пол од соседните контролни села. Нивото на комплемент и имуноглобулините во серумот беа одредени со имунохемискиот систем на Beckman. Автоантителата беа одредувани во серумот со индиректен флуоресцентен метод или со радиоимуноесеј (anti-single stranded DNA). Имуофлуоресцентен микроскоп беше користен за преглед на бубрежното ткиво боено со FITC конјугирани антисеруми за IgG, IgM, IgA, C3, C1q, C4 и фибрин/фибриноген.

Резултати. C3 во серумот на пациенти со БЕН беше намален споредено со здрави лица како контрола ($p < 0.001$), IgM во серумот беше исто така намален ($p < 0.05$). Антитиреоидните антитела беа откриени кај 5/45 пациенти со БЕН, антипариетални кај 7/45 пациенти и ANA кај два пациенти со БЕН. Не се најдени антитела кон мазните мускули, анти-митохондријални и антитела кон ДНК кај ниеден пациент со БЕН. Имуофлуоресцентните иследувања на 10 парчиња бубрежно ткиво добиено со биопсија покажале ретко, неспецифично, фокално, мезенгијално паталожување на C3 и на IgM во некои гломерули и ретки депозити (паталожување), главно на C3, во тубулите и во екстрагломеруларните садови.

Заклучок. Хуморалните имуни механизми изгледа дека не играат патогенетска улога во БЕН. Малку студии се направени за целуларниот имунитет кај БЕН и потребни се понатамошни иследувања кај пациенти во раниот стадиум на болеста.

Клучни зборови: балканска ендемска нефропатија (БЕН), имунолошки студии, комплемент во серумот, имуноглобулин, автоантитела, имунохистологија.

Corresponding Author:

Polenakovic Momir
Macedonian Academy of Sciences and Arts
Krste Misirkov 2
1000, Skopje, Republic of Macedonia
Tel. ++ 389 2 3235 400
Fax. ++ 389 2 3235 500

E-mail: maknefro@mt.net.mk

Table 1. Serum complement and immunoglobulin levels in patients with BEN
Ниво на комплемент и имнуоглобулини кај пациенти со БЕН

	Control	BEN
C3, g/L	1.00±0.24(0.93-1.06) 0.94 (0.92-0.94)	0.81±0.25(0.73-0.89) 0.78 (0.76-0.85)*
C4, g/L	0.42±0.12 0.42 (0.35-0.45)	0.46±0.14 0.45(0.41-0.50)
IgG, g/L	14.14±2.47 (13.47- 14.81) 14.38 (13.21-15.18)	14.93±3.92 (13.70- 16.15) 14.38 (13.21-16.00)
IgA, g/L	2.49±0.85 (2.26-2.72) 2.46 (2.06-2.71)	2.8±1.04 (2.51-3.15) 2.71 (2.30-3.15)
IgM, g/L	1.54±0.47 (1.41-1.66) 1.45 (1.38-1.59)	1.40±0.62 (1.20-1.59) 1.25 (1.06-1.38)**

Mean values ± SD are given, with 5-95% value in parenthesis. Median with 5-95%

value in parenthesis.

*P< 0.001 vs. control

**P< 0.05 vs. control

Table 2. Autoantibodies in the sera of patients with BEN
Аутоантитела во серум на пациент со БЕН

Antibodies	Grade 1	Grade 2	Grade 3
Antii-thyroid	1	2	2
Anti smooth-muscle	0	0	0
Anti-mitochondrial	0	0	0
Anti-parietal	2	2	3
Anti-nuclear	0	2	0

The presence of autoantibodies was graded from 1 (small) to 3 (high)

Резиме

ИМУНОЛОШКИ СТУДИИ КАЈ БАЛКАНСКА ЕНДЕМСКА НЕФРОПАТИЈА (БЕН)

Поленаковиќ М.,¹ Чукурановиќ Р.,² Савиќ В.,² Јовановиќ М.,³ Стефановиќ В.²

¹ Македонска академија на науките и уметностите, Скопје, Р. Македонија

² Институт за нефрологија и хемодијализа, Ниш, Србија

³ Институт за анатомија, Медицински факултет, Ниш, Србија

Цел. Да се преиспита нивото на комплементот и на имуноглобулините во серумот кај пациенти со Балканска Ендемска Нефропатија (БЕН) во раниот стадиум на болеста; кај овие пациенти да се иследат автоантителата (анти-тиреоидни, антителата спрема мазните мускули, анти-митохондријални, анти-париетални, антинуклеарни и анти-ДНК); и конечно, да се процени имунохистологијата на бубрежните биопсии од пациентите со БЕН. Ке се прикаже ревија на имунолошки студии кај пациенти со БЕН.

Методи. Во оваа студија беа вклучени четириесет и пет пациенти во раниот стадиум на болеста (БЕН) од регионот на Јужна Морава. Беа селектирани педесет и пет здрави контроли, со соодветна возраст и пол од соседните контролни села. Нивото на комплемент и имуноглобулините во серумот беа одредени со имунохемискиот систем на Beckman. Автоантителата беа одредувани во серумот на индиректен флуоресцентен метод или со радиоимуноесеј (anti-single stranded DNA). Имуофлуоресцентен микроскоп беше користен за преглед на бубрежното ткиво боено со FITC коњуигирани антисеруми за IgG, IgM, IgA, C3, C1q, C4 и фибрин/фибриноген.

Резултати. C3 во серумот на пациенти со БЕН беше значајно намален споредено со здрави лица како контрола ($p < 0.001$), IgM во серумот беше исто така намален ($p < 0.05$). Анти-тиреоидните антитела беа откриени кај 5/45 пациенти со БЕН, антипариетални кај 7/45 пациенти и ANA кај два пациенти со БЕН. Не се најдени антитела кон мазните мускули, анти-митохондријални и антитела кон ДНК кај ниеден пациент со БЕН. Имуофлуоресцентните иследувања на 10 парчиња бубрежно ткиво добиено со биопсија покажале ретко, неспецифично, фокално, мезенгијално исталожување на C3 и на IgM во некои гломерули и ретки депозити (исталожување), во главно на C3, во тубулите и во екстрагломеруларните садови.

Заклучок. Хуморалните имуни механизми изгледа дека не играат патогенетска улога во БЕН. Малку студии се направени за целуларниот имунитет кај БЕН и потребни се понатамошни иследувања кај пациенти во раниот стадиум на болеста.

Клучни зборови. Балканска Ендемска Нефропатија (БЕН), имунолошки студии, комплемент во серумот, имуноглобулино, автоантитела, имунохистологија.

Corresponding author:

Polenakovic Momir
Macedonian Academy of Sciences and Arts
Krstе Misirkov 2
1000, Skopje, Republic of Macedonia
Tel. ++ 389 2 3235 400
Fax. ++ 389 2 3235 500
E-mail: maknefpo@mt.net.mk