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Hipogalaktozilacija imunoglobulina G (IgG) gingivalne tečnosti i pljuvačke u obolelih od parodontopatije

Hypogalactosylation of Immunoglobulin G (IgG) of Gingival Fluid and Saliva at the Patient with Periodontal Disease

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ORIGINALNI RAD (OR)
ORIGINAL ARTICLE

KRATAK SADRŽAJ

Promenjena glikozilacija imunoglobulina G (IgG), pre svega ekspresija terminalne galaktoze, utiče na brojne funkcije ovih imunoglobulina i korelira sa stepenom zapaljenja u mnogim bolestima.

***Cilj rada:** U ovom radu analizirana je distribucija IgG podklasa i sadržaj terminalne galaktoze u njima, u pljuvačci i gingivalnoj tečnosti bolesnika sa parodontopatijom različitog stepena inflamacije gingive.*

***Materijal i metod:** Kao materijal u ispitivanjima korišćena je gingivalna tečnost i pljuvačka 30 odraslih osoba sa kliničkom dijagnozom parodontopatija i 20 osoba sa zdravim parodoncijumom. Kvantifikacija IgG urađena je "dot-blot" postupkom, a određivanje terminalne galaktoze lektinskim imunoblot postupkom.*

***Rezultati**: Raspodela IgG podklasa u obe tečnosti se razlikovala kod parodontopatija i u kontrolnim uzorcima. I u pljuvačci i u gingivalnoj tečnosti obolelih, kvantitativno je dominirala IgG2 podklasa, nezavisno od parodontalnog statusa. U IgG obe oralne tečnosti terminalna galaktoza je bila eksprimirana kod osoba sa zdravim parodoncijumom (kontrola) i kod osoba sa početnom (inicijalnom) parodontopatijom, dok kod osoba sa uznapredovalom parodontopatijom ekspresija ovog šećera nije registrovana ni u jednoj od ove dve tečnosti.*

***Zaključak:** Rezultati ovih istraživanja ukazuju da postoji pomeranje prema hipogalaktozilovanim IgG glikoformama tokom procesa inflamacije gingive u obolelih od parodontopatije.*

Ključne reči: IgG, hipogalaktozilacija, gingivalna tečnost, pljuvačka, parodontopatija

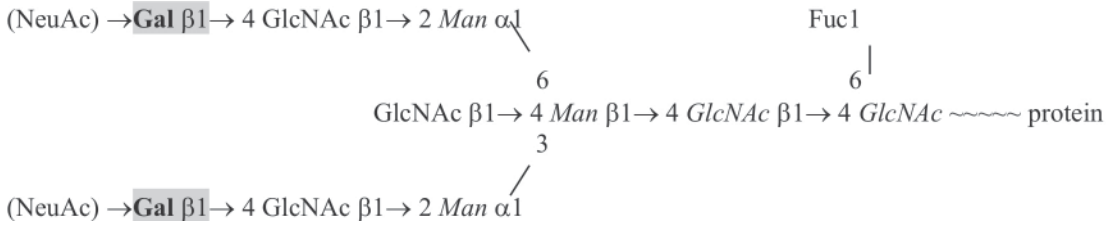
SUMMARY

*Changed glycosylation of immunoglobulin G (IgG), above all, the expression of thermal galactose, influence to numerous functions of those immunoglobulin and correlate with the inflammatory level in a number of diseases. **Aim**: This work analyses the distribution of IgG subclasses and the content of thermal galactose in them, in saliva and gingival fluid of the patients with periodontal disease and different gum inflammatory level. **Materials and methods:** It was used saliva and gingival fluid of 30 adults with clinical picture of periodontal disease and 20 persons with healthy periodontium. The qualification of IgG was done by "dot-blot" procedure and the, and thermal galactose was determined by lectin immunoblot procedure. **Results:** The division of IgG subclasses in both fluids was different in the patients with periodontal disease and in control samples. In saliva and gingival fluid of the diseased quantitatively dominated IgG2 subclasses, independently from periodontal status. In IgG of both fluids, thermal galactose was exprimated at the healthy periodontium persons (control) and with the person with initial periodontal disease, while at the person with increased periodontal disease the expression of this saccharide wasn't registered in neither of fluids. **Conclusion:** The results showed that there is a shift towards hypogalactosyled IgG glikoforms during the process of gum inflammation at the periodontal disease patients.*

Key words: IgG, hypo galactosylation, saliva, gingival fluid, periodontal disease

Imunoglobulini G (IgG) su jedna od pet klasa (izotipskih varijanti) imunoglobulinskog proteinskog sistema. Osim u serumu, gde ih ima najviše, IgG se javljaju i u drugim telesnim tečnostima kod ljudi, uključujući i oralne tečnosti kao što su pljuvačka i gingivalna tečnost. Pretpostavlja se da u oralnim tečnostima oni imaju, pored ostalog, i ulogu u imunološkoj zaštiti od parodontalnih patogena. Mehanizmi ove zaštite u patogenezi parodonatlnih bolesti, međutim, nisu poznati. Sposobnost imunoglobulina G da kontrolišu infekcije lokalnih oralnih mesta može zavisiti od distribucije molekulskih varijanti ove klase imunoglobulina (podklase, alotipovi) i njihovih glikoformi. Kod čoveka postoje četiri podklase IgG (IgG1, IgG2, IgG3, IgG4), preko 20 alotipskih varijanti (Gm faktori) i mnoštvo glikoformi. Glikoforme su određene sastavom ugljenih hidrata koji predstavljaju integralne strukturne elemente svakog IgG molekula, bitne za konformaciju i efektorne funkcije molekula (1). Ugljenohidratni sadržaj IgG konstituišu oligosaharidne jedinice (glikani) koje se formiraju povezivanjem različitih monosaharida (slika 1) i koji se kovalentno vezuju za evolutivno konzervirani Asn 297 u svakom CH2 domenu Fc regiona teških lanaca.

Immunoglobulines G (IgG) is one of the five classes (isotopic variables) of the immunoglobulin protein system. Besides the serum, where it could be found the most, IgG are found in other body fluids of the people, including oral fluids like saliva and gum fluid. It is assumed that, they have the role in immunology protection from the periodontal pathogens in oral fluids, besides the other roles they have. However, the mechanisms of this protection in the pathogeneses of the periodontal diseases are not known. The abilities of immunoglobulines G to control infections of the local oral places could depend on distribution of molecular variables of this class of mmunoglobulines (subclasses, alotypes) and their glycol-forms. The man possesses for subclasses of IgG (IgG1, IgG2, IgG3, IgG4), over20 alotypes variables (Gm factors) and lots of glycol-forms. Glycol-forms are determined by the structure of carbon hydrates that present the integral structural elements of each IgG molecule, which are important for conformation and effective function of the molecule (1). The carbon hydrate contents of IgG constitute oligo saccharide units (glycans) which are formed by bonding of the different mono saccharides (figure 1) and which are in covalent bonds with evolutionary conserved Asn 297 in each of CH2 domain of Fe heavy chains region.



Slika 1. Struktura N-vezanih oligosaharidnih lanaca u CH2 domenima Fc regiona IgG molekula.
Figure 1. Structure of the N-linked oligosaccharide chains in the CH2 domains of the Fc region in the IgG molecule.

Sadržaj pojedinih šećera koji ulaze u sastav glikana nije isti u svim molekulima IgG. Glikani vezani za Fc region IgG su uglavnom neutralni (bez sijalinske kiseline), a prema sadržaju terminalne galaktoze kojom se završavaju bočne, antenske grane oligosaharidnih lanaca, klasifikovani su u tri grupe: G0, G1 i G2, koje čine glikani bez galaktoze kao terminalnog šećera (agalaktozilovani), sa galaktozom vezanom za samo jednu od dve grane oligosaharidnog lanca (monogalaktozilovani) i sa galaktozom vezanom za obe grane (digalaktozilovani). Dodatna heterogenost rezultira iz prisustva ili odsustva fukoze (Fuc) i/ili bisektnog N-acetilglukozamina (GlcNAc), tako da je broj neutralnih asialoglikana koji mogu biti ugrađeni u IgG 16 (2). Svaki IgG, bez obzira na podklasu i alotip može sadržati bilo koju glikansku varijantu. Imunoglobulini G se, otuda, sagledavaju kao populacija različitih glikoformi od kojih svaka ima različite fizičke, biohemijske i funkcionalne karakteristike (3,4). Sadržaj IgG glikoformi varira pod fiziološkim, a naročito pod patološkim uslovima (5). Zapaženo je da kod nekih hroničnih zapaljenskih

Content of some of saccharides, which composed glycans, is not the same in all of the IgG molecules. Glycanes bonded to Fc region are mostly neutral (without sialinic acid), and according to the content of termal galactose which end side, antennae branches of oligosaccharide chains, they are classified into three groups: G0, G1 i G2, which consist of glycanes with no lactose as a thermal glucose (agalactosyled), with galactose bonded to only one, of the two branches of oligosaccharide chain (mono galactosyled) and with galactose bonded to both branches (digalactosyled). The additional heterogeneity results from the existence or absence of fucose (Fuc) and/or bisects N-acetyl glycosamine (GlcNAc), so the number of neutral asialoglycans, which can be embedded in IgG, is 16 (2). Each IgG, regardless its subclass and alotype can contain any glycolic variable. Immunoglobulins G are recognized as a population of different glycol forms, which, each of them has different physical, biochemical and functional characteristics (3,4). The content of IgG glycol form vary according to physiological, and specially, pathological conditions (5). It is noticed that,

bolesti ljudi dolazi do "pomeranja" u odnosu pojedinih glikoformi IgG, koje se najčešće manifestovalo povećanjem sadržaja agalaktozilovanih (G0) IgG u serumu. Štaviše, pokazano je da povećani sadržaj IgG kojima nedostaje terminalna galaktoza, korelira sa stepenom inflamacije kod nekih bolesti (6). To ukazuje da bi ugljeni hidrati mogli imati značaj za patogenezu bolesti (7). U skladu sa tim je izneta i pretpostavka da bi mogla postojati povezanost između glikozilacije imunoglobulina i oralnih disfunkcija (8). Međutim, iako se zna da u oralnim tečnostima mogu biti prisutne sve četiri IgG podklase (9), nema podataka o raspodeli glikoformi oralnih IgG podklasa, niti o mogućoj povezanosti nekih od njih sa stepenom zapaljenja u okviru parodontopatije.

Cilj ovog rada je bio da se analizira distribucija IgG podklasa i sadržaj galaktoze u njima, u pljuvačci i gingivalnoj tečnosti osoba obolelih od parodontopatije sa različitim stepenom inflamacije gingive.

at some chronic inflammatory diseases of people there happened some of the "shifts" in the relations of some IgG glycol forms, which is mostly manifested in increase of content of agalactosyled (G0) IgG-s in a serum. Furthermore, it is shown that the increased content of agalactosyled IgG-s that lack thermal galactose, correlates with the level of inflammation at some of diseases (6). That shows that carbon hydrates could have an importance for pathogeneses of a disease (7). According to that there is a hypothesis that there could be a connection between glycolysis of immunoglobulin and oral dysfunctions (8). However, although it is known that in oral fluids could exist all four IgG subclasses (9), there are no data about the division of glycol forms of oral IgG subclasses, or possible connection some of them with the level of inflammation at periodontal disease.

The aim of this work was to analyse the distribution of IgG subclasses and the content of galactose in it, in saliva and gum fluid at the patients with periodontal disease with different level of gum inflammation.

Materijal i metod

Pacijenti. Ispitivanja su obuhvatila 30 odraslih osoba (prosečna starost 43,6 godina) sa kliničkom slikom parodontopatije, lečenih na Klinici za parodontologiju i oralnu medicinu Stomatološkog fakulteta u Beogradu, gde je postavljena dijagnoza i određen klinički stadijumi bolesti. Na osnovu uobičajenih parametara (IKG – indeks krvarenja gingive, PI – plak indeks Silness Loe, DDž – dubina džepa, Nep – nivo pripojnog epitela) pacijenti su podeljeni u dve grupe: u grupu I (n=17) svrstane su osobe sa blagim zapaljenskim promenama gingive (početna parodontopatija), a u grupu II osobe sa izraženim zapaljenskim promenama (uznapredovala parodontopatija). Kontrolnu grupu je sačinjavalo 20 osoba sa klinički zdravim parodoncijumom (prosečna starost 26,7 g.).

Gingivalna tečnost. Gingivalna tečnost (GT) je sakupljana sa četiri mesta u najdubljim džepovima kod obolelih, dok su kod kontrola uzorci uzimani sa mesta na kojima je PI i IKG imali vrednost nula. Sakupljanje je vršeno pomoću traka od filter papira (Whatman, 4,2x13 mm) koje su umetane na dno parodontalnog džepa i posle 1 min prenošene u epruvete (Eppendorf) u koje je prethodno ubačen koktel inhibitora proteaza u 1 ml PBS, pH 7,2. Posle blagog mešanja, uzorci su zamrzavani na -70ºC do upotrebe. Težina svake epruvete i trake je merena pre i posle unošenja uzoraka.

Pljuvačka. Mešovita pljuvačka je sakupljana na način preporučen u Vodiču za nomenklaturu i sakupljanje pljuvačke (10), u epruvetama koje su bile obložene sa 1% rastvorom EDTA (da bi se umanjila aktivnost mikrokroorganizama proteaza). Uzorci su centrifugovani 15 min na 20000 x g, na 4ºC i, nakon zasićenja supernatanta sa 50% etilenglikolom, zamrzavani na -70ºC do upotrebe.

Materials and methods

Patients. The researches included 30 adults (the average age was 43,6) with the clinical picture of periodontal disease, that were treated on the Clinic for periodontal diseases and oral medicine of Dental faculty in Belgrade, where the diagnose and the clinical stadium of disease were established. Based on usual parameters (IBG – index of gum bleeding, PI – plaque index Silness Loe, DP-depth of the pocket, LEA- level of the epithelia attachment) the patients were divided into two groups: The patients with mild inflammatory changes of gum (initial periodontal disease) were put into the first group (n: 17), and the second group consisted of distinctive inflammatory changes (increasing periodontal disease). The control group consisted of 20 persons with clinically healthy periodontium (the average age was 26,7).

Gum fluid. Gum fluid (GF) was collected from four places in the deepest pockets of the diseased, while the control samples were taken from the places where PI and IBG were zero .The collecting was administered with the filter paper bands (Whatman, 4.2x13mm) which were implanted on the bottom of the periodontal pocket and after one minute transferred into the test tube (Eppendorf) in which the cocktail of protease inhibitors in 1ml PBS, pH 7.2, had already been put. After the mild stirring, the samples were frozen to –70°C till their use. The weight of each test tube and a band was measured before and after sample entry.

Saliva. The mixed saliva was collected in the way recommended in the Guide for nomenclature and saliva collection (10), in the test tubes overlain with 1% EDTA solution (so to lessen the activity of protease microorganisms). The samples were centrifuged for 15 minutes on 20000 x g, on 4C and, after the saturation of supernatant with 50% ethylene glycol; they were frozen to –70°C till their use.

Kvantifikacija IgG. Sadržaj IgG u pljuvačci i gingivalnoj tečnosti je određen "dot blot" postupkom. Po 50 µl pojedinačnih uzoraka ili njihovih mešavina je nanošeno na nitroceluloznu membranu (Amersham Biosciences), automatski, vakumskom aspiracijom u Bio Dot aparatu (Bio Dot Apparatus, Bio Rad). Svi uzorci, uključujući i kontrole, su analizirani istovremeno za datu IgG podklasu. Nakon sušenja, membrane su inkubirane mišjim monoklonskim antitelima specifičnim za humane γ1, γ2, γ3 i γ4 teške lance (Nordic, Holandija) i reakcije vizualizovane sekundarnim antitelom obeleženim peroksidazom. Koncentracije IgG podklasa su određivane denzitometrijskim trasiranjem vizualizovanih uzoraka. Za konstruisanje standardnih krivih korišćeni su homogeni, monoklonski IgG sve četiri podklase, izolovani i visoko prečišćeni iz seruma bolesnika sa multiplim mijelomom (banka uzoraka Instituta za medicinska istraživanja u Beogradu). Koncentracije su prikazane kao jedinice koncentracije (C.U.), u odnosu na kontrolne vrednosti koje su predstavljene kao 1.

Određivanje terminalne galaktoze

A) Terminalna galaktoza u ukupnim IgG pljuvačke i gingivalne tečnosti određena je lektinskim imunoblot postupkom (11), nakon elektroforeze uzoraka na poliakrilamid gelu (SDS-PAGE) pod redukujućim uslovima. Nakon elektroforetskog razdvajanja i elektrotransfera na nitroceluloznu membranu, membrana je inkubirana sa lektinima obeleženim biotinom (Vector, CA), RCA-I lektinom iz *Ricinus communis* i BS-II lektinom iz *Griffonia (Bandeiraea) simplicifolia*. RCA-I lektin specifično prepoznaje terminalnu galaktozu u oligosaharidnim lancima IgG kada u njima nema sijalinske kiseline, a BS-II lektin specifično prepoznaje GlcNAc kada nema terminalne galaktoze. Prečišćeni i redukovani normalni i monoklonski mijelomski IgG sve četiri podklase služili su kao markeri za molekulske mase teških i lakih lanaca IgG.

B) Ekspresija galaktoze u IgG podklasama prisutnim u pljuvačci i gingivalnoj tečnosti je procenjena pomoću "sendvič" ELISA testa u kome je na mikrotitarske ploče (E.I.A. II Plus, Libro Flow Laboratories Inc), nanošeno po 5 µl monoklonskih anti-γ1, γ2, γ3 i γ4 antitela i, posle inkubiranja na 4ºC preko noći i adekvatnog ispiranja, uzorci pljuvačke i gingivalne tečnosti u određenim razblaženjima. Na ploče je potom dodavan RCA-I lektin obeležen biotinom, a nakon inkubiranja 1^h na 37ºC i višestrukog ispiranja, i avidin-peroksidaza i hromogen (ABTS, Sigma Chemicals). Optička gustina (OD) je određivana kao absorbanca na 405 nm u ELISA čitaču (MULTISCAN PLUS reader, Labsystem Finland). Vrednosti optičke gustine registrovane u sistemu sa lektinom i mišjim antitelima nanetim na ploču, a bez uzoraka, su oduzimane od vrednosti optičkih gustina koje su dobijene kada je uzorak bio prisutan. Sve analize su rađene u duplikatu, a rezultati su prikazani kao srednja vrednost dve analize.

Qualification of IgG. The content of IgG in saliva and gum fluid was determined with "dot blot" procedure. 50 µl of individual sample or their mixture was put on nitrocellulose membrane (Amersham Biosciences), automatically, with vacuum aspiration in Bio Dot apparatus (Bio Dot apparatus, Bio Rad). All the samples, including controls, were analyzed at the same time for the assigned IgG subclass. After the drying, the membranes were incubated with mouse mono clone antibodies which were specific for humane heavy chains γ1, γ2, γ3 and γ4 (Nordic, Holland) and the reaction was visualized with the secondary antibody, which was marked with peroxidase. The concentrations of IgG subclasses were determined with densitometric tracing of the visualized samples. For the construction of the standard curves there were used homo genes, mono clone IgG with all four subclasses, isolated and highly refined from the serum of the diseased with multiple myelom (the sample bank in the Institute for medical research in Belgrade). The concentration was shown as the unit of concentration (C.U.) according to the control values shown as 1.

Determination of thermal galactose.

A) Thermal galactose in all IgG of saliva and gum fluid was determined with lectin immune blot procedure (11), after the electrophoresis of the samples on polyacrylamid gel (SDS-PAGE) under the reduced conditions. After electrophoretic partition and electro transfer into nitrocellulose membrane, the membrane was incubated with the lectins marked with biotin (Vector, CA), RCA-I lectin from *Ricinus communis* and BS-II lectin from *Griffonia (Bandeiraea) simplicifolia.* RCA-I lectin specifically recognizes thermal galactose in oligo saccharide chains of IgG when there is no sialic acid, and BS-II lectin specifically recognizes GlcNAc when there is no thermal galactose. Refined and reduced normal and mono clone myelo IgG of all four subclasses served as markers for molecular mass of the heavy and light IgG chains.

B) The expression of galactose in IgG subclasses present in saliva and gum fluid was estimated with the help of "sandwich" Elisa test in which 5 µl mono clone anti-γ1, γ2, γ3 and γ4 antibodies were put on micro titer plates (E.I.A II Plus, Libro Flow laboratories Inc), and after the incubation on 4°C during the night and adequate rinse, the samples of saliva and gum fluid in certain dilution. After that on the plates was added RCE-I lectin marked with biotin, and after incubation 1hour on 37°C and multiple rinse, avidin-peroxidase and chromogen (ABTS, sigma Chemicals) were added, too. Optical thickness (OT) was determined as an absorbance on 405 µm in ELISA reader (MULTISCAN PLUS reader, Labsystem Finland). The value of optical thickness was registered in a system with lectin and mouse antibodies that were put on a plate, but without the samples, was than subtracted from the values of optical thickness, which had been made when the sample was present. All of the analyses were made in duplicate, and the results were shown as a medium value of those two analyses.

Statističke analize. Za utvrđivanje linearne regresije standardnih krivih i ostale statističke analize korišćen je Prism Pad softver (Version 3.0, San Diego, CA, USA).

Statistical analyses. Prism Pad software was used to establish linear regression of the standard curve and other statistical analyses.

Rezultati

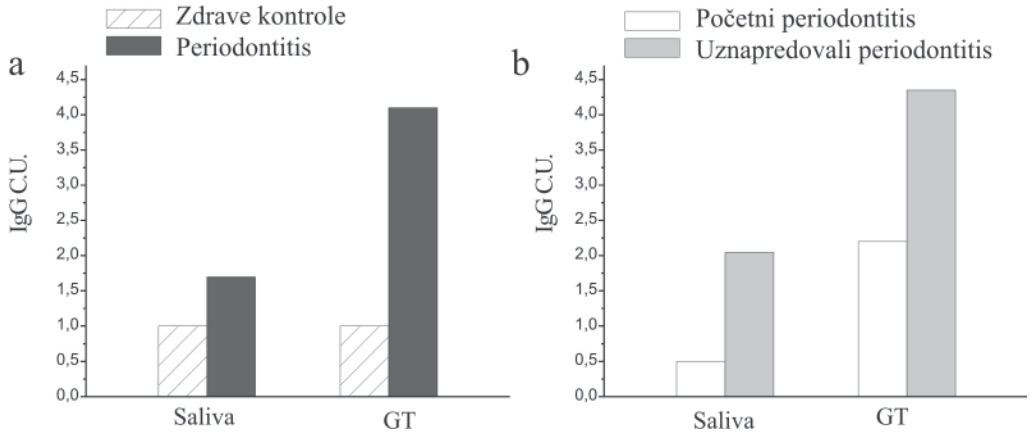
Sadržaj IgG u pljuvačci i gingivalnoj tečnosti

Srednja vrednost ukupnih IgG i u pljuvačci i u GT je bila veća kod obolelih od parodontopatije nego kod zdravih osoba kontrolne grupe, pri čemu su razlike bile upadljivije u GT (slika 2a). Nivo IgG u obe oralne tečnosti se povećavao progresivno kod osoba sa većim stepenom inflamacije gingive (slika 2b).

Results

The content of IgG in saliva and gum fluid

The medium value of total IgG in saliva and GF was larger at the patients that had periodontal disease than at healthy persons from the control group, and the differences were more obvious in GF (figure 2a). The level of IgG in both oral fluids was progressively increased at the diseased persons with higher level of gum inflammation (figure 2b).



Slika 2. Nivo IgG u pljuvačci i gingivalnoj tečnosti osoba bez periodontitisa (zdrave kontrole) i sa periodontitisom.
Figure 2. The level of IgG in saliva and GCF of healthy contols and periodontitis patients.

Tabela 1. Koncentracija i relativna zastupljenost IgG podklasa u pljuvačci i gingivalnoj tečnosti osoba bez periodontitisa (zdrave kontrole) i sa periodontitisom.
Table 1. The levels and the relative distribution of IgG subclasses in saliva and gingival fluid of healthy controls and periodontitis patients.

	IgG PODKLASA IgG1	IgG2	IgG3	IgG4
PLJUVAČKA				
Zdrave kontrole				
µg/ml	0,23	0,12	1,1	0,33
	(0,05-0,48)	(0,095-0,19)	(0,45-3)	(0,15-0,6)
%	12,9	6,7	61,7	18,5
Periodontitis				
I µg/ml	0,33	2,03	0,53	0,57
	(0,1-0,6)	(1,2-2,5)	(0,2-0,8)	(0,4-0,8)
%	9,7	58,6	15	16,4
II µg/ml	2,2	7,15	4,39	1,6
	(0,6-2,2)	(5-9)	(1,7-6,7)	(0,8-1,6)
%	14,3	46,6	28,6	10,4
GINGIVALNA TEČNOST				
Zdrave kontrole				
mg/ml	2,78	2,5	0,99	0,63
	(0,46-5,1)	(0,17-3,3)	(0,29-1,7)	(0,46-0,8)
%	40,2	36,2	14,3	9,1
Periodontitis				
I mg/l	2,43	13,49	3,02	0,72
	(1,1-4,4)	(9,9-16)	(1,7-6,7)	(0,4-0,8)
%	12,3	68,3	15,4	3,6
II mg/ml	9,9	44,9	2,55	1,95
	(2,2-17,6)	(19,8-70)	(1,7-3,4)	(0,8-3,1)
%	16,6	75	4,3	3,2

I u pljuvačci i u gingivalnoj tečnosti kvantitativno je dominirala IgG2 podklasa, nezavisno od parodontalnog statusa. Postojala je razlika u raspodela IgG podklasa u obe tečnosti između obolelih od parodontopatije i osoba kontrolne grupe, pri čemu su se ispoljile velike interindividualne razlike u nivou pojedinih podklasa (tabela 1). Prema dobijenim vrednostima, raspodela IgG podklasa za osobe sa zdravim parodoncijumom (kontrole) je bila: IgG3>IgG4>IgG1>IgG2 u pljuvačci i IgG1>IgG2≥IgG3>IgG4 u gingivalnoj tečnosti, dok je kod obolelih od parodontopatije ta raspodela bila: IgG2>IgG3>IgG1>IgG4 u pljuvačci i IgG2>>IgG1>IgG3>IgG4 u gingivalnoj tečnosti.

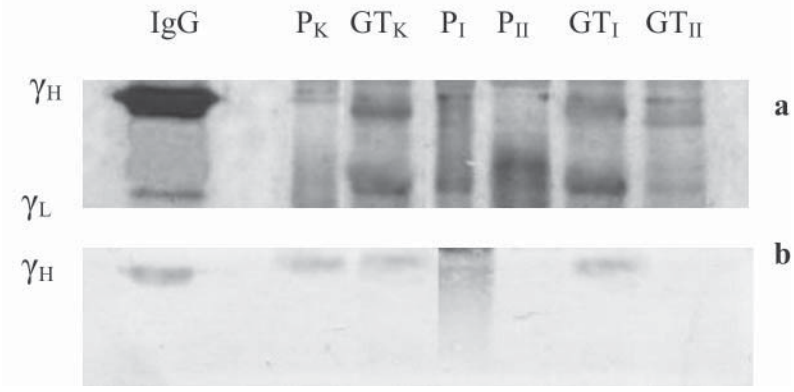
Sadržaj galaktoze

Elektroforeza na poliakrilamid gelu (SDS-PAGE) pljuvačke i gingivalne tečnosti je u svim ispitivanim uzorcima detektovala i teške i lake lance IgG (slika 3a). Pri tome su obe frakcije bile intenzivnije u GT nego u pljuvačci. Lektinski imunoblot izveden nakon SDS-PAGE sa RCA-I lektinom je pokazao da se lektin vezao za frakciju koja po molekulskoj masi odgovara teškim lancima IgG, bilo u pljuvačci ili gingivalnoj tečnosti. Međutim, reaktivnost RCA-I lektina sa teškim lancima IgG se ispoljila samo u pljuvačci i gingivalnoj tečnosti osoba kontrolne grupe i pacijenata sa početnom parodontopatijom, dok ta reaktivnost nije bila uočljiva sa IgG pacijenata sa uznapredovalom parodontopatijom (slika 3b). Ovakav nalaz pokazuje da u uslovima izraženog zapaljenja gingive, i u pljuvačci i u gingivalnoj tečnosti, preovlađuju IgG bez terminalne galaktoze. Kako nedostatak terminalne galaktoze rezultira u ekspresiji GlcNAc, sledećeg šećera u oligosaharidnom nizu, ispitano je da li IgG obe oralne tečnosti reaguju sa BS-II lektinom kojim se otkriva ovaj šećer. Pokazalo se da je reaktivnost sa ovim lektinom bila slaba (rezultati nisu prikazani).

IgG2 subclass, quantitatively dominated in saliva and in gum fluid, independently from periodontal status. There were the difference in distribution of IgG subclasses in both fluids between the periodontal disease patients and those from the control group, while there were shown large inter individual differences in the level of the certain subclasses (table 1). According to the values achieved, the distribution of IgG subclasses for the persons with healthy periodontium (control) was IgG3> IgG4> IgG1> IgG2 in saliva and IgG1> IgG2> IgG3> IgG4 in gum fluid, while at the patients with periodontal disease that distribution was: IgG2> IgG3> IgG1> IgG4 in saliva and IgG2> IgG1> IgG3> IgG4 in gum fluid.

Galactose contents

Electrophoresis on poly acrylic gel (SDS-PAGE) of saliva and gum fluid detected in all the samples heavy and light chains of IgG (figure 3a). Both fractions were more intensive in GF then in saliva. Lectine immunoblot reported after SDS-PAGE with RCA-I lectin showed that lectin had been bonded for fraction, which according to molecular mass belonged to heavy chains IgG, either in saliva or gum fluid. However, reactivity RCA-I lectin with heavy chains IgG were demonstrated only in saliva and gum fluid of the persons in the control group and the patients with initial periodontal disease, while that reactivity wasn't noticed at the IgG pf the patients with increasing periodontal disease (figure 3b). Those findings show that in the conditions of the outstanding gum inflammation, IgG without thermal galactose overcome, either in saliva or gum fluid. As the shortage of thermal galactose results in GlcNAc expression, the next saccharide in oligo saccharide line, it was examined if IgG of both oral fluids reacted with BS-II lectin, which was used to discover this saccharide. It was shown that the reactivity with this lectin was low (the results were irrelevant).



Slika 3. (a) Reprezentativni prikaz SDS PAGE: normalni IgG, pljuvačka osoba bez i sa periodontitisom i gingivalna tečnost osoba bez i sa periodontitisom. (b) RCA-I lektinski imunoblot IgG u pljuvačci i gingivalnoj tečnosti.
Figure 3. (a) Representative silver-stained SDS PAGE of normal IgG, normal and periodontitis saliva and GCF; (b) RCA-I lectin immunoblot of IgG in saliva and GCF.

Analiza galaktozilacionog statusa IgG podklasa ELISA postupkom je pokazala da je vezivanje RCA-I lektina za sve četiri podklase i u pljuvačci i u gingivalnoj tečnosti smanjeno u uznapredovaloj parodontopatiji, u poređenju sa početnim stadijumom parodontopatije. U pljuvačci, RCA-I lektin se vezivao slabije 7,2 puta za IgG1, 5,3 puta za IgG2, 6,0 puta za IgG3 i 4,7 puta za IgG4 u uznapredovaloj parodontopatiji nego što se vezivao za iste podklase u početnom stadijumu parodontopatije. U gingivalnoj tečnosti, RCA-I lektin se vezivao za sve podklase u početnom stadijumu parodontopatije, dok je u uznapredovaloj formi ova reaktivnost bila na granici detektabilnosti za sve četiri podklase.

Analyses galactozilative status of IgG subclasses with ELISA procedure showed that the bonding of RCA-I lectine for all of the four subclasses in both, saliva and gum fluid, was reduced in increasing periodontal disease, in comparison to initial stadium of periodontal disease. In saliva, RCA-I lectin was bonded 7.8 times weakly for IgG, 5.3 times for IgG2, 6.0 times for IgG3 and 4.7 times for IgG 4 in the increasing periodontal disease then it bonded for the same subclasses in the initial stadium of periodontal disease. In gum fluid, RCA-I lectin was bonded in all of the subclasses in the initial stadium of periodontal disease, while in increasing form this reactivity was on the edge of detectability for all of the four subclasses.

Diskusija

Parodontopatija je infektivno oboljenje praćeno lokalnim i sistemskim humoralnim imunim odgovorom na parodontalne patogene. Pretpostavlja se da imunoglobulini, pre svega IgG i IgA koji se stvaraju tokom tog odgovora, imaju protektivnu ulogu u patogenezi parodontopatije. Promene u specifičnom imunoglobulinskom odgovoru mogu imati odraza na progresiju bolesti (12). Te promene se mogu manifestovati kvantitativnim disbalansom pojedinih izotipskih varijanti, kao i raznim molekulskim abnormalnostima imunoglobulina. Time se stvaraju uslovi da dođe do poremećaja u nekoj od funkcija ovih odbrambenih proteina.

Glikozilacija je jedna od najvažnijih, strogo kontrolisanih, posttranslacionih modifikacija imunoglobulina, kojom se obezbeđuje strukturni osnov za različite efektorne aktivnosti njihovih molekula (2). Promene u obrascu glikozilacije, posebno imunoglobulina G, su zapažene u različitim patološkim stanjima kod ljudi i sagledavaju se ili kao uzrok ili kao posledica bolesti (7). Pokazalo se da su sa stanovišta kliničkog toka brojnih zapaljenskih bolesti najznačajnije promene u ekspresiji terminalne galaktoze, jednog od šećera iz oligosaharidnih lanaca vezanih za Fc region IgG molekula (slika 1). Pri tome je stepen zapaljenja gingive u tim bolestima najupadljivije korelirao sa porastom nivoa u serumu onih IgG koji sadrže malo galaktoze ili su bez nje (hipogalaktozilovani, agalaktozilovani IgG) (13,14).

O glikozilacionom statusu IgG prisutnih u oralnim tečnostima i povezanosti pojedinih IgG podklasa i njihovih glikoformi sa stepenom zapaljenja gingive nema podataka. Ovaj rad pokazuje da u parodontopatiji postoji pomeranje ka hipogalaktozilovanim glikoformama IgG, prisutnim u gingivalnoj tečnosti i pljuvačci obolelih. Galaktozilacioni status ukupnih IgG u ovim oralnim tečnostima je procenjen na osnovu njihove reaktivnosti sa RCA-I lektinom iz *Ricinus communis* koji specifično prepoznaje reziduum terminalne galaktoze unutar oligosaharidnih lanaca intaktnih IgG molekula. Postupak lektinskog imunoblota koji je

Discussion

Periodontal disease is an infective disease followed by local and systemic humoral immune answer to periodontal pathogens. It is assumed that immunoglobulins, above all IgG and IgA, which are made during the answer, have a protective role in pathogenesis of periodontal disease. The changes in a specific immunoglobulin answer could have a reflection in disease progression (12). Those changes could be manifested in quantitative misbalance of some of isotope variables as well as different immunoglobulin molecule abnormalities. That makes the conditions for the disturbance in some of the functions of those defensive proteins.

Glycosylation is one of the most important, severely controlled, post translatonal modifications of immunoglobulin, with which it is provided structural base for different effective activities of their molecules (2). The changes in glycosylation pattern, especially in immunoglobulin G, were noticed in different pathology states at people and they are recognized to be the reason or the consequence of a disease (7). It was shown that according to clinical course of the numerous inflammatory disease the most important changes were the expression of thermal galactose, one of the saccharides from oligosaccharide chains, bonded to Fc region of IgG molecules (figure 1). The level of gum inflammation was, in those diseases, the most obviously correlated with the rise of the level in serum of those IgGs, which contained a little of galactose or they were without it (hypo galatosyled, agalatosyled IgGs) (13, 14).

There are no data about the glicosylative status of IgGs present in oral fluids and the connection of some of IgG subclasses and their glycoforms with the level of gum inflammation. This work shows that there is a certain shift towards hypo galatosylated glycoforms of IgGs that are present in gum fluids and saliva of diseased. Galatosylative status of the total IgGs in those oral fluids is estimated on the base of their reactivity with RCA-I lectin from *Ricinus communis, which* specifically recognizes residuum of

primenjen pokazao se kao pogodan za praćenje promena u glikozilacionom profilu ukupnih oralnih IgG u uslovima zapaljenja gingive; njime je bilo moguće da se ustanovi razlika u tom profilu između obolelih i zdravih ispitanika. S obzirom da je koncentracija IgG u oralnim tečnostima obično niska, kao i da postoje brojne tehnički problem da se pojedine IgG podklase izoluju i prečiste u količinama dovoljnim za analize, za procenu stepena galaktozilacije IgG podklasa razvijena je posebna varijanta ELISA postupka, koja omogućava da se takva procena vrši u nefrakcionisanim oralnim tečnostima.

Dobijeni rezultati pokazuju da se, izgleda, sadržaj hipogalaktozilovanih glikoformi IgG povećava sa stepenom inflamacije gingive u okviru parodontopatije. Za razliku od osoba kontrolne grupe i obolelih sa blagom formom zapaljenja gingive koji su u gingivalnoj tečnosti ili pljuvačci imali galaktozilovane IgG i to u sličnom sadržaju, oboleli sa izraženim zapaljenskim promenama gingive su imali gotovo nedetektabilne količine takvih IgG. Drugim rečima, reaktivnost sa RCA-I lektinom koji detektuje terminalnu galaktozu nije bila manifestna u IgG oralnih tečnosti u uznapredovaloj parodontopatiji. Ovi rezultati ukazuju da tokom procesa inflamacije gingive može doći do promene kvantitativnog odnosa između pojedinih glikoformi tj. do pomeranja u njihovoj raspodeli prema hipogalaktozilovanim varijantama. Međutim, iako nedostatak galaktoze dovodi do povećane ekspresije N-acetilglukozamina, narednog šećera u oligosaharidnom nizu, nasuprot očekivanjima, nije uočeno povećano vezivanje GS-II lektina iz *Griffonia simplicifolia*, koji specifično prepoznaje ovaj šećer. Ovakav nalaz se može protumačiti činjenicom da postupak koji je primenjen za procenu ekspresije ispitivanih šećera nije uključivao denaturaciju IgG molekula, koja je ponekad neophodna da bi se lakše eksprimirali šećeri koji su zamaskirani konformacijom. Denaturacija je namerno izbegnuta kako bi se dobili podaci o ekspresiji šećera u nativnim, intaktnim IgG. U literaturi, inače, postoje podaci koji pokazuju da odnos galaktoze i N-acetilglukozamina nije uvek recipročan i da varira zavisno od bolesti kod koje se taj odnos ispituje (15).

Svaka glikoforma imunoglobulinskih molekula je, sa stanovišta oligosaharidnog sastava, strukturno jedinstvena i povezana sa određenom funkcijom. Promena u odnosu IgG glikoformi može dovesti do moduliranja funkcionalnih kapaciteta IgG u oralnim tečnostima. Za terminalnu galaktozu je pokazano da utiče na vezivanje IgG imunokompleksa za Fc receptore na fagocitima i, time, na klirens imunokompleksa iz lokalnog tkiva. Nemogućnost eliminacije imunokompleksa ovim putem može dovesti do akumulacije imunokompleksa i uticati na progresiju parodontalne lezije. Sposobnost agalaktozilovanih IgG da, preko eksponiranog N-acetilglukozamina, vežu manan-vezujući protein i tako efikasno aktiviraju komplement, može potencirati njihove patogene efekte i pojačati lokalne inflamatorne reakcije (16). Iz ovih istraživanja nije u potpunosti jasno kojoj od četiri podklase prisutnih

thermal galactose inside oligosaccharide chains of intact IgG molecules. The process of lectine immunoblot, which was applied, showed as a suitable device for tracing the changes in glicosyl profile of total IgGs in the state of gum inflammation; it provided establishment of the difference in that particular profile, between the healthy and the diseased. According to concentration of IgG in oral fluids, which is usually low, and that there are numerous technical problems to isolate certain IgG subclasses and refine them in the quantities enough for the analyses, for the estimation of the galactosylation level the special variant of ELISA proceeding was developed, that enabled such estimate to take place in infractioned oral fluids.

The results achieved show that the content of hypogalatosyled glycoforms of IgG increases with the level of gum inflammation in the scope of periodontal disease. In contrast to persons from the control group and with initial periodontal disease who in their gum fluid and saliva had galatosyled IgGs and they were in the similar contents, the diseased with outstanding gum inflammatory changes had almost undetectable quantities of such IgGs. In other words, the reactivity with RCA-I lectin, which is to detect thermal galactose, wasn't manifested in IgG of oral fluids in increasing periodontal disease. Those results show that during the inflammatory process of gums the change of quantitative relation among certain Glico forms could have happened, that is to say, there could be a shift in their distribution towards the hypo galatosyled variants.

However, although the lack of galactose leads to increased expression of N-acetilglycosamin, the next of the saccharide on oligosaccharide line, opposite to expectations, there was no increasing bonding of GS-II lectin from *Griffonia simplicifolia,* which specifically recognizes this saccharide. This kind of finding could be explained with the fact that the proceeding applied for the estimation of the expression examined saccharides didn't include denaturation of IgG molecule, which is sometimes necessary to ex primate saccharide easily that are masked with conformation. Denaturation was avoided on purpose, so that the data of saccharide expression in native, intact IgGs could be achieved. There are data in literature showing that, the relation of galactose and N-acetilglycosamin is not always reciprocal and that it is vary depending on a disease in which the relation is examined.

Each Glico form of immunoglobulin molecule is, according to its oligosaccharide content, structurally united and connected with a certain function. The change in IgG Glico form relations can lead to modulation of the functional capacity of IgG in oral fluids. For the thermal galactose it is shown that it influences on the bonding of IgG immune complex for Fc receptors on phagocytes, and eventually on clearance of immune complex from the local tissue. The impossibility of elimination of immune complex this way, could lead to accumulation of the immune complex and influence on a progression pf periodontal lesion. The ability of agalatosyled IgGs to,

u oralnim tečnostima osoba sa obolelim parodoncijumom bi se mogli pripisati takvi efekti, budući da su, u pogledu stepena hipogalaktozilacije, među njima detektovane samo neznatne razlike u uznapredovaloj parodontopatiji. S obzirom da je kod pacijenata sa parodontopatijom, koji su ispitivani, IgG2 podklasa bila kvantitativno dominantna i u gingivalnoj tečnosti i u pljuvačci, promene u galaktozilacionom profilu ove podklase bi, posebno, mogle biti od značaja, jer se IgG2 dominantno javlja u odgovoru na determinante ćelijskog omotača *Actinobacillus actinomycetemcomitans* i ispoljava opsonizirajući efekat na ovaj oralni patogen (17,18). Galaktozilacioni profil bi mogao biti bitan za opsonizirajuće karakteristike ove podklase, a promene u sadržaju galaktoze bi se, bez sumnje, mogle odraziti na sposobnost IgG2 da pojača fagocitno ugrađivanje mikroorganizama odgovornih za oboljenja parodoncijuma. Iako se tradicionalno smatra da su molekuli IgG1 i IgG3 podklase bolji opsonini od IgG2 molekula, jer se vezuju za Fcγ receptore na fagocitima, postoje podaci koji ukazuju da su IgG2 antitela efikasni opsonini kada interaguju sa fagocitima koji eksprimiraju H131 alotip FcγRIIa receptora (17). Međutim, potpuno je nejasno da li gubitak ili smanjenje takve interakcije korelira sa stepenom oštećenja parodoncijuma. Areaktivnost sa RCA-I lektinom, koja je u našim ispitivanjima bila povezana sa uznapredovalom formom parodontopatije, indirektno ukazuje da je smanjena galaktozilacija oralnih IgG mogla imati negativni efekat na opsonofagocitni proces, utičući možda na afinitet vezivanja takve glikoforme za Fcγ receptor.

Promene u glikozilacionom profilu IgG, povezane sa progresijom parodontalnog oboljenja, odražavaju dinamiku lokalne produkcije IgG podklasa i predstavljaju indikator efektornih funkcija podklasa. Poznato je da hipogalaktozilacija IgG korelira sa povećanim nivoom IL-6 (19), a povećan nivo ovog interleukina je registrovan u zapaljenom tkivu gingive (20,21). U svetlu ovakvih nalaza, ovi rezultati deluju logično, mada ostaje nejasno da li je hipogalaktozilovana forma IgG samo marker inflamacije ili je direktno uključena u patogenezu parodontopatije.

Kada se razmišlja o vakcini koja bi indukovala IgG antitela željenih efektornih potencijala u zaštiti od oboljenja parodoncijuma, podaci o glikoformama oralnih IgG bi mogli biti veoma značajni. Uz to, danas je sasvim sazrela predstava o neophodnosti da se *in vitro* biotehnološka priprema rekombinantnih glikoproteina, pre svega imunoglobulina, za *in vivo* terapijsku primenu u raznim oboljenjima ljudi, mora bazirati na saznanjima o glikozilacionom statusu IgG i promenama tog statusa u određenoj bolesti (22). U tom kontekstu su koncipirana i naša dalja istraživanja koja treba da definišu odnos oralnih IgG glikoformi i njihove specifičnosti za subgingivalne bakterijske vrste u parodontopatiji.

over expound N-acetilglycosamin, bond manna-bonding protein and so efficiently activate complement, could rise their pathogen effects and increase inflammatory reactions (16). This researches didn't make it clear which of the four subclasses, present in oral fluids at the persons with diseased periodontium, could be assigned those effects, since, according to the level of hypo galatosyled, among them detected only slight differences in increasing periodontal disease. While at the patients with periodontal disease who were examined, IgG2 subclass was quantitatively dominant in saliva and gum fluid, the changes in galatosyled profile of this subclass might be important, because IgG2 is dominant in the answer to determinants of the *Actinobacillus actinomycetemcomitans* cell cover and shows opsonic effect to this oral pathogen (17, 18). The changes in the galactose content could reflect on ability ofIgG2 to enforce phagocyte implementation of microorganisms that are responsible for periodontal disease. Although it is traditionally considered that molecules IgG1 and IgG3 subclasses are better opsonins from IgG2 molecules, because they bond for Fcγ receptors on phagocytes, there are data which show that IgG2 antibodies are effective opsonins when they inter react with phagocytes which ex primate H131 alotype FcγIia receptor (17). However, it is completely unclear if the loss or reduction of the interaction correlate with the level of periodontium destruction. A reactivity with RCA-I lectin, which is, in this research, connected with the level of increased form of periodontal disease, indirectly shows that the reduced galatosyled of oral IgG could have negative effect on opsonin phagocyte process, influenced maybe on the bonding affinity of such Glico form for Fcγ receptor.

The changes on glycosyled profile IgG, connected to the progression of periodontal diseases, keep the dynamic of the local production of IgG subclasses and they are indicators of the effective subclasses functions. It is known that hypo galatosyled of IgG correlate with increased level of IL-6, and the increased level of this inter leucine is registered in inflamed gum tissue (20,21). Those result seemed logic, although, it stays unclear if hypo galatosyled form of IgG only marker for inflammation or it is directly included in pathogenesis of periodontal disease.

When it is thought of vaccine, which could induce IgG antibodies of wanted potential effectors in the protection of periodontal diseases, the data of glycoforms of oral IgG might be significant. Besides, nowadays, the idea of necessity of *in vitro* biotechnology preparation of recombinant glycol proteins, above all immunoglobulin, for *in vivo* therapeutic application in different disease, have to be based on glycosyled status of IgG and the changes in that status in the specific disease (22). Our Further researches are formed in that context, so that they should define the relation of the oral glycoforms and their specifications and sub gingival bacteria spices in periodontal diseases.

Zaključak

Dobijeni rezultati ukazuju na postojanje pomeranja prema hipogalaktozilaciji IgG glikoformi tokom procesa inflamacije gingive u osoba obolelih od parodontopatije. Pormene u glikozilacionom profilu IgG vezane su za stepen inflamacije, odnosno progresiju parodontalnog oboljenja.

Conclusion

The results achieved during those researches showed that there are shifts towards hypo galatosyled IgG glycoforms during the process of gum inflammation at the patients with periodontal disease. The changes in galatosyled profiles IgG are connected with the level of inflammation, that is to say, the progression of periodontal disease.

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