

**THE IMPORTANCE OF HAEMATOLOGICAL AND BIOCHEMICAL FINDINGS
IN PATIENTS WITH WEST NILE VIRUS NEUROINVASIVE DISEASE****ZNAČAJ HEMATOLOŠKIH I BIOHEMIJSKIH NALAZA KOD BOLESNIKA SA
NEUROINVAZIVNIM OBLIKOM GROZNICE ZAPADNOG NILA**

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Summary

Background: West Nile virus neuroinvasive disease (WNND) occurs in less than 1% of infected people. Leukocytosis with lymphocytopenia, mild anaemia, thrombocytopenia, elevated liver and muscle enzymes and hyponatremia are occasionally present in patients with WNND. Cerebrospinal fluid (CSF) findings resemble other viral neuroinfections. The purpose of this study is to present some of the most important laboratory findings of our patients with WNND and to evaluate their correlation with fatal outcome.

Methods: The study included 161 patients with WNND. Their blood and CSF samples were cytochemically analysed and the obtained variables were then tested for predictive significance of the disease outcome, or used for differentiation between two clinical syndromes (encephalitis vs meningitis).

Results: West Nile encephalitis was present in 127 (78.9%) patients and West Nile meningitis was diagnosed in 34 (21.1%) cases. Leukocytosis was found in 45.9% patients. CRP level higher than 100 mg/L was registered only in those with encephalitis ($p=0.020$). CSF leukocyte count was 146 ± 171 per microlitre, with slight lymphocytic predominance (mean 52%). Hypoglycorrhachia was registered in 9.3% of our patients with WNND. Twenty-eight (17.4%) patients died and all of them had encephalitis. Independent predictors of fatal outcome in WNND were serum CRP > 100 mg/L ($p=0.011$) and CSF proteins > 1 g/L ($p=0.002$).

Kratak sadržaj

Uvod: Do neuroinvasivnog oblika groznice Zapadnog Nila (WNND) dolazi kod manje od 1% zaraženih osoba. Kod bolesnika sa WNND se mogu registrovati leukocitoza sa limfocitopenijom, laka anemija, trombocitopenija, povišene vrednosti enzima jetre i mišića i hiponatrijemija. Nalaz u cerebrospinalnoj tečnosti (CST) sličan je kao kod drugih virusnih neuroinfekcija. Cilj rada je prikaz laboratorijskih parametara kod naših bolesnika sa WNND i određivanje njihove korelacije sa smrtnim ishodom.

Metode: U studiju je uključen 161 bolesnik sa WNND. Uzorci njihove krvi i CST su citobiohemijski analizirani i potom je testiran prediktivni značaj dobijenih varijabli na ishod bolesti ili značajnost razlike u zavisnosti od kliničkog sindroma (meningitis ili encefalitis).

Rezultati: West Nile encefalitis je dijagnostikovao kod 127 (78,9%) bolesnika, a West Nile meningitis kod 34 (21,1%) bolesnika. Kod 45,9% bolesnika je registrovana leukocitoza. Vrednosti CRP-a veće od 100 mg/L su zabeležene samo kod bolesnika sa encefalitisom ($p=0,020$). Zabeleženo je prosečno 146 ± 171 leukocita po mikrolitru CST, sa lakom predominacijom limfocita (prosečno 52%). Hipoglikorahija je registrovana kod 9,3% bolesnika. Umrlo je 28 (17,4%) bolesnika, svi sa encefalitisom. Vrednosti serumskog CRP-a > 100 mg/L ($p=0,011$) i vrednosti proteina u CST > 1 g/L ($p=0,002$) bile su nezavisni prediktori smrtnog ishoda kod bolesnika sa WNND.

Zaključak: Od WNND najčešće obolevaju stariji muškarci. Prolongirana predominacija neutrofila u CST i hipogliko-

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Conclusions: WNND usually affects older males. Prolonged neutrophilic predominance in CSF can occasionally be present, as well as hypoglycorrhachia. Patients with encephalitis, high serum CRP and high CSF protein level have a higher risk of fatal outcome.

Keywords: CSF, encephalitis, leukocytes, meningitis, West Nile

Introduction

West Nile virus (WNV) is a neurotropic, mosquito-borne, single-stranded positive-sense RNA virus. Humans are accidental hosts involved in an enzootic cycle between birds and mosquitoes. WNV as a human infection was acknowledged back in 1937 but the first cases in Serbia were not identified until a recent outbreak in 2012 (1). It is considered that WNV infection is asymptomatic in almost 80% of humans, and in symptomatic cases WNV mostly causes mild febrile illness. Approximately 1 in 150 of those infected develop West Nile virus neuroinvasive disease (WNND), which usually presents as aseptic meningitis, encephalitis and/or acute flaccid paralysis (AFP) syndrome (2).

Complete blood count of patients with WNND is mostly without major abnormalities, but leukocytosis with lymphocytopenia, mild anaemia and thrombocytopenia also may occur (3). Elevated aspartate aminotransferase (AST), alanine aminotransferase (ALT) and creatine kinase (CK) levels could be present in a certain number of WNND cases, as well as hyponatremia (1).

Cerebrospinal fluid (CSF) findings in WNND are similar to those found in central nervous system (CNS) infections caused by other viruses: mild to moderate pleocytosis, with predominance of lymphocytes, elevated protein levels and normal glucose concentrations (4).

The purpose of this study is to present the most important laboratory findings in our patients with WNND, compare them with the previously reported results, and to evaluate their correlation with fatal outcome.

Materials and Methods

Patients' data were collected from the medical records of 161 cases with WNND who had been treated during 2012 and 2013 at the Clinic for Infectious and Tropical Diseases, Clinical Centre of Serbia, Belgrade. We used the European Centre for Disease Prevention and Control (ECDC) case definition criteria (5). Any person meeting the clinical criteria (at least one of the following: viral encephalitis, viral meningitis, AFP) and laboratory criteria (WNV-specific IgM class antibody response in CSF) was considered a confirmed case of WNND. Any person meeting the abovementioned clinical criteria and with

rahija se mogu povremeno registrovati. Rizik od smrtnog ishoda povećan je kod bolesnika sa encefalitisom, visokim vrednostima serumskog CRP-a i proteinorahije.

Ključne reči: CST, encefalitis, leukociti, meningitis, Zapadni Nil

WNV-specific antibody response (IgM or IgG) in serum was considered a probable case of WNND (5). Standard clinical criteria for encephalitis were the presence of fever, and at least one of the following: acutely altered mental status (quantitative and/or qualitative), focal neurological signs, epileptic seizures or neuroimaging findings consistent with cerebral inflammation. Meningitis was defined as combination of fever, pleocytosis in CSF and at least one meningeal sign or symptom (nuchal rigidity, headache, photophobia). Pleocytosis was defined as 5 or more leukocytes per microlitre of CSF. AFP was defined as asymmetric flaccid paresis or paralysis of one or more limbs without the sensory loss, and it could include the presence of meningitis/encephalitis.

The study was approved by the local ethics committee, and it was conducted appreciating the principles of Helsinki declaration and its amendments (6). The written consent was signed by all the patients included in the study, or their closest relatives.

Blood and CSF samples collected from our patients were analysed in The National Laboratory for viral hemorrhagic fevers and ARBO viruses, which is placed in the Institute of Virology, Vaccines and Sera »Torlak« in Belgrade, using enzyme linked immunosorbent assay (anti-West Nile virus ELISA IgG and anti-West Nile virus ELISA IgM, EUROIMMUN Medizinische Labordiagnostika AG). Bacterial and common viral causes of CNS infections (other than WNV) were ruled out using the routine CSF examination (bacterial cultures and virologic tests). Presence of pneumonia, urinary tract infection and/or bacteraemia was used as an exclusive criterion because of their potential effect on inflammatory markers elevation. Cranial computerized tomography was used to exclude noninfectious causes of altered mental status. Not a single patient had a history of vaccination against flaviviruses (e.g. yellow fever vaccine), recent blood transfusion or organ transplantation.

For the purpose of this study, cytological and biochemical analyses were conducted in patients' blood and CSF samples during the first 24 hours from admission to the hospital. All of the samples were analysed in the Centre for Medical Biochemistry, Department of the Clinic for Infectious and Tropical Diseases, Clinical Centre of Serbia, Belgrade. Haematology analyser Coulter HmX, Beckman Coulter was used for the determination of complete blood count. Erythrocyte sedimentation rate (ESR) was calculated

using the Westergreen method. Fibrinogen level was determined using the Clauss method with Multifibren U reagent on a Sysmex® CA-1500 System, Siemens. We used the Siemens Dimension Xpand biochemistry analyzer and the reagents were supplied by the same manufacturer. The serum levels of C-reactive protein (CRP) were determined using the turbidimetric method, AST, ALT, CK using the IFCC enzyme method and sodium levels using indirect sample sensing with the QuikLITE® Integrated Multisensor Technology. Glycorrhachia was determined with the hexokinase method and proteinorrhachia using a colorimetric method with pirogallol. CSF cytology analysis was performed manually using the Samson solution and the Nageotte counting chamber.

Statistical Package for Social Sciences (SPSS®, version 11.5) was used for statistical analysis of all gathered data. Cases were grouped according to the clinical syndrome (meningitis, encephalitis and/or AFP) and according to the overall outcome (survival or death). We used methods of descriptive and analytical statistics. Parametric variables were analysed using Student T test and nonparametric variables were analysed using Mann-Whitney U test or χ^2 test. Values at the $p \leq 0.05$ level were considered statistically significant and the confidence interval (CI) was 95%. The correlation between the variables that were proven significant in univariate analysis and the fatal outcome was established by performing the multivariate

logistic regression and the results were presented as odds ratios (OR) with a CI of 95%.

Results

A total of 161 patients with WNND, who met the earlier defined criteria, were included in the study. Among them, 104 (64.6%) were male and 57 (35.4%) female patients ($p < 0.001$). Mean age of our patients was 65.7 ± 14.0 years (range, 21–88). Age of 60 or higher (73.9%) was more common ($p < 0.001$). West Nile encephalitis (WNE) was present in 127 (78.9%) cases and it was more frequent than West Nile meningitis (WNM) which was diagnosed in 34 (21.1%) cases ($p < 0.001$). Among those with WNE, 18 (11.2%) patients also showed signs of AFP. The average length of symptom duration prior to blood and CSF sampling and laboratory diagnostics was 5.4 ± 3.0 days (range, 1–14).

The mean of white blood cells (WBC) count in WNND cases was $10.6 \times 10^9/l$ (Table I). Leukocytosis was found in 74 (45.9%) patients, among which 64 (50%) had WNE and 10 (29%) had WNM ($p = 0.029$). The average leukocyte levels in WNE and WNM groups were 10.9 ± 4.3 and 9.3 ± 3.8 , respectively ($p = 0.056$). In WBC differential, the mean percentage of lymphocytes was $16.0 \pm 8.5\%$. Lymphocytopenia was common, since it was found in 90 (55.9%)

Table I Haematological and biochemical parameters in patients with West Nile virus neuroinvasive disease (n=161).

	Mean \pm SD	Minimum – Maximum	Reference range
Haematological analysis			
Leukocyte count ($\times 10^9/L$)	10.6 ± 4.2	3.4–32.2	3.4–9.7
Lymphocytes (%)	15.9 ± 8.5	3.0–55.0	20.0–46.0
Erythrocyte count ($\times 10^{12}/L$)	4.35 ± 0.58	2.10–5.95	4.34–5.72 (M) 3.86–5.08 (F)
Haemoglobin (g/L)	131.6 ± 19.8	133–179	138–175 (M) 119–157 (F)
Platelets ($\times 10^9/L$)	184.7 ± 70.9	53–584	158–424
Biochemical analysis			
Sedimentation rate (mm/h)	42.1 ± 24.3	5–120	≤ 20 mm/hr (M) ≤ 30 mm/hr (F)
Fibrinogen (g/L)	4.2 ± 0.9	2.0–7.5	2.0–4.0
C-reactive protein (mg/L)	38.1 ± 47.6	1.1–274.0	< 5 mg/L
Sodium (mmol/L)	139.1 ± 5.4	125–159	135–148
AST (U/L)	53.5 ± 60.2	11–575	< 37
ALT (U/L)	46.6 ± 44.5	8–355	< 40
Creatine kinase (U/L)	812.5 ± 1225.9	16–7855	< 200 (M) < 150 (F)

patients. Mild anaemia was registered in 64 (39.7%) cases, and moderate to severe anaemia was present in only 4 (2.5%) patients. Thrombocytopenia was found in 46 (28.6%) patients.

The levels of inflammatory markers at the time of admission fluctuated widely (Table I). ESR and fibrinogen levels did not differ significantly between the both types of disease, while the mean CRP level was higher in WNE group (50.7 ± 4.9) compared with WNM (25.2 ± 4.7) ($p=0.001$). CRP level higher than 100 mg/L was present in 18 (14.2%) patients with WNE while all the patients with WNM had CRP level 100 mg/L and less ($p=0.020$).

Hyponatremia was found in a total of 23 (14.2%) patients with WNND. Abnormal levels of AST and ALT were recorded in 78 (48.4%) and 63 (39.1%) patients, respectively. Only two of those

patients have had previous liver disease (chronic hepatitis C and alcohol abuse). Increased CK levels were common and present in 108 (67.1%) patients ($p<0.001$). Among those with CK elevation, only four patients have had epileptic seizures and none have previously used statins, nor have had elevated cardiospecific enzymes. We have not found statistically significant difference when comparing the mean values of the aforementioned biochemical analysis between encephalitis and meningitis groups.

The examination of CSF cytology in our study showed a mean leukocyte count of 146 per microlitre with slight lymphocytic predominance (mean 52%) (Table II). Pleocytosis was present in 97% of WNE group CSFs, with a mean of 143 ± 159 per microlitre, and in every (100%) WNM CSF, with a mean cell count of 153 ± 210 per microlitre ($p=0.766$). High pleocytosis (more than 500 leukocytes per microlitre) was found in 5% and 9% of patients with WNE and WNM, respectively. Examining the CSF's white blood cells differential, we calculated that the mean percentage of lymphocytes was $53 \pm 28\%$ in WNE, and $50 \pm 27\%$ in WNM group ($p=0.567$). The neutrophil predominance (more than 50% neutrophils) was present in 52 (41%) cases with WNE and 17 (50%) with WNM ($p=0.343$). In patients whose symptoms started less than 3 days prior to CSF analysis, neutrophils were present with a mean of approximately $48 \pm 25\%$, while the others had a mean of $47 \pm 29\%$ neutrophils ($p=0.957$).

CSF protein level higher than 1 g/L was more frequent in patients with WNE (51%) than in those with WNM (26%) ($p=0.010$). Mean proteinorrachia level registered in WNE group (1.1 ± 0.53 g/L) was also significantly higher compared to WNM group (0.86 ± 0.48 g/L) ($p=0.015$). Hypoglycorrachia, with a glucose level of less than 2.7 mmol/L, was reg-

Table II Cerebrospinal fluid findings in patients with West Nile virus neuroinvasive disease (n=161).

	Mean \pm SD	Minimum – Maximum	Reference range
Haematological analysis			
Leukocytes ($\times 10^6/L$)	145.6 ± 170.9	0–882	$< 5 \times 10^6/L$
Lymphocytes (%)	52.0 ± 27.8	1–100	/
Neutrophils (%)	47.6 ± 27.7	0–99	/
Biochemical analysis			
Glucose (mmol/L)	4.0 ± 1.4	1.7–9.9	2.7–4.1
Proteins (g/L)	1.05 ± 0.53	0.36–3.07	0.15–0.45

Table III Haematological and biochemical parameters as risk factors for fatal outcome in patients with West Nile virus neuroinvasive disease (n=161).

	Survived (n=133)	Died (n=28)	p-value	Multivariate analysis odds ratio (95% CI)
Blood				
Leukocytosis (%)	58 (43.6)	16 (57.1)	0.192	/
Anaemia (%)	57 (42.8)	11 (39.3)	0.728	/
Thrombocytopenia (%)	36 (27.1)	10 (35.7)	0.357	/
CRP > 100 mg/L (%)	8 (6.0)	10 (35.7)	<0.001	7.21 (1.56–33.28)
Cerebrospinal fluid				
WBC < $5 \times 10^6/L$ (%)	2 (1.5)	2 (7.1)	0.081	/
WBC > $500 \times 10^6/L$ (%)	7 (5.2)	2 (7.1)	0.694	/
Neutrophils > 50 (%)	59 (44.3)	10 (35.7)	0.401	/
Hypoglycorrachia (%)	13 (9.8)	2 (7.1)	0.663	/
Proteins > 1 g/L (%)	49 (36.8)	25 (89.3)	<0.001	12.07 (2.43–60.02)

istered in 15 (9.3%) of our patients with WNND. Twelve of them had WNE and three of them had WNM. Their means of CSF findings are the following: leukocytes 243 ± 245 per microlitre, lymphocytes $52 \pm 27\%$, glycorrachia 2.2 ± 0.3 mmol/L and proteinorrachia 1.49 ± 0.68 g/L. All of them had normal glycaemia levels at the time of CSF analysis.

Twenty-eight (17.4%) patients with WNND in our study have died and 133 (82.6%) survived. All of the patients with fatal outcome had WNE. Using a univariate statistical analysis, we found out that serum CRP level higher than 100 mg/L and CSF protein level higher than 1 g/L on admission have statistical significance ($p < 0.001$, both) (Table III). In a multivariate logistic regression analysis this significance was retained, even when advanced age (≥ 60 years) and respiratory failure, as strong predictors of fatal outcome ($p = 0.012$ and $p < 0.001$, respectively), were included in the analysis. Among haematological and biochemical parameters, independent predictors of fatal outcome in WNND were serum CRP > 100 mg/L ($p = 0.011$; OR 7.21; 95% CI 1.56–33.28) and CSF proteins > 1 g/L ($p = 0.002$; OR 12.07; 95% CI 2.43–60.02).

Discussion

Despite the fact that WNV is neurotropic for less than 1% of persons infected, WNND is a severe disease, with significant lethality, unpredictable long term outcome, and no currently available vaccine or virus-specific therapy. These facts oblige us to use rapid and accurate laboratory diagnostics, based on reliable clinical suspicion. Fundamental tasks for the clinicians are to recognise all patients with suspicious WNND, provide supportive care, as well as to rule out treatable CNS infections (primarily bacterial and herpesviral infections) (4). Blood and CSF cytochemical analyses are the most valuable tools for the first-line diagnostics and etiology differentiation in patients with CNS infection.

Elevated WBC in WNND were reported in several previous studies, mostly up to $20 \times 10^9/L$. The maximum of a $30.8 \times 10^9/L$ was recorded by the scientists in New York during the outbreak in 1999, which is similar to what we have found (7–9). In our study, almost half of the patients have had leukocytosis, while it was revealed in only about a third of the patients in the study of an outbreak in Israel in 2000 (8). In a small series of 16 cases with WNND, Sejvar et al. found out that patients with WNV meningitis had a higher mean of leukocyte level, compared with those with encephalitis and AFP (10). Quite opposite to these results, our patients with WNE had a higher mean of leukocyte level than those with WNM, but the result was on the edge of statistical significance. However, leukocytosis at the time of admission was more frequently found in our patients with WNE than

in those with WNM. All the patients with suspected WNV infection and leukocytosis should be carefully monitored, since there is a possibility for them to subsequently develop WNE. In two studies, lymphocytopenia was present in 11/18 (38.8%) patients and the mean of lymphocytes in WBC differential was 14.1% (7, 9). More than half of our patients have had lymphocytopenia, with a close mean of lymphocytes to those in aforementioned studies. Anaemia at the time of admission was detected in 41% of Israeli patients with WNND and its presence was one of the factors associated with death (8). We did not find any correlation between the presence of anaemia and fatal outcome. Thrombocytopenia was present in a few of our patients and it did not have any major clinical significance.

There is not much data concerning the serum inflammatory markers levels in WNND patients available in the literature. Serum CRP levels were higher in WNE group compared to WNM group. CRP level higher than 100 mg/L at the time of admission represented a significant factor that strongly correlates with the presence of encephalitis, and it was an independent predictor of fatal outcome.

Hyponatremia can be found in 33 to 50% of neuroinvasive WNV infections, and it is more often present in patients with encephalitis (8, 9, 11). In our patients, hyponatremia was rare and equally present among groups. Elevated liver enzymes in our study were registered more frequently than 20% to 24%, which are the percentages that other authors have noted (8, 9). Liver injury was mild and not clinically significant, although WNV might cause severe liver damage, even with acute liver failure (12). Elevated CK can occur as a result of rhabdomyolysis associated with WNV, suggesting viral myositis as one of the extraneural manifestations (13, 14). A significant portion of our patients had high CK levels, which could, in the presence of signs and symptoms of neuroinfection, indicate an association with WNV.

In a large series (250 cases) of serologically confirmed WNND, Tyler et al. reported the presence of CSF pleocytosis in 97% of patients with meningitis with a mean cell count of $226 \times 10^6/L$ and in 95% of those with encephalitis (mean $227 \times 10^6/L$). Only 8% of patients in both groups had pleocytosis of $500 \times 10^6/L$ or greater (15). Other studies reported pleocytosis with leukocyte counts ranging from 0 to 2317 (7–10). In general, our results were similar to those of Tyler et al. with the presence of CSF pleocytosis up to $500 \times 10^6/L$ in the majority of cases, although the mean cell count was lower in our study. Absence of CSF pleocytosis occurred rarely, only in patients within the WNE group, as a reminder that normal cell count in CSF is not a useful criterion for ruling out viral encephalitis.

Lymphocytic predominance in CSF is typical for viral CNS infections, although it may not be present

during the first 24–36 h after symptom onset (16, 17). Bai et al. corroborated that neutrophils, as a key figure of innate immunity, have a paradoxical role in the pathogenesis of WNV infection. They found rapid expression of neutrophil attracting chemokines (CXCL1 and CXCL2) in macrophages during WNV infection (18). Some of the authors reported neutrophilic predominance in subsets of patients with WNND (9, 10). Others postulated that neutrophilic predominance is more likely early in the course of the disease (19, 20). Rawal et al. (21) suggested that the mean of neutrophil fractions was higher in patients whose CSF was sampled within the first 3 days of WNND onset.

In our study, we noted the lymphocytic predominance in CSF within WNE group, but in patients with WNM the mean percentages of lymphocytes and neutrophils were even. Tyler et al. found that 45% of WNM patients and 37% of WNE patients had predominance of neutrophils, which is similar to our results (15). Nevertheless, we demonstrated that the length of illness in our patients with neutrophilic predominance could be more than 3 days, so their CSF cell counts resemble those in bacterial CNS infection.

Results of previous studies suggest that CSF protein levels in WNND are ranging from normal to 8.99 g/L (7–10, 19, 21). We demonstrated that protein levels greater than 1 g/L are more likely present in patients with encephalitis than in those with meningitis, which is consistent with the data published by Tyler et al. (15). The same study failed to show a significant correlation of proteinorrhachia and the disease outcome in a multivariate analysis, while in our study protein level higher than 1 g/L was found to be an independent predictor of fatal outcome.

The level of glycorrhachia is the most important CSF finding in distinguishing the viral from other potentially treatable causes of CNS infection, having in mind that low levels are the common feature of

bacterial, mycobacterial and fungal CNS infections (22). It is unusual, but low CSF glucose levels can occasionally also be noted in viral CNS infections caused by mumps, enteroviruses, lymphocytic choriomeningitis virus, herpes simplex and herpes zoster viruses, but it is not characteristic of arboviral neuroinvasive diseases, including West Nile virus (23). Researching the available literature, we found data about a single case with WNND and hypoglycorrhachia (2). In this study, we revealed that hypoglycorrhachia was present in almost 10% of our patients. Most of them had WNE with lymphocytic predominance in the CSF cell count. All of these patients were treated with i.v. antibiotics for as long as it took us to get negative bacterial CSF cultures and confirm WNND (according to the case definition criteria) due to the fact that these CSF findings could initially also suggest bacterial etiology of the disease.

In conclusion, WNV has become an important threat to public health in Serbia since its emergence in 2012. WNV should be considered as a neuro-pathogen in the late summer and early fall seasons. WNND usually affects older males and it is characterised by mild to moderate pleocytosis and lymphocytic predominance in CSF, although prolonged neutrophilic predominance could occasionally be present. WNV should be recognised as a potential viral cause of hypoglycorrhachia. Patients with WNE, high serum levels of CRP and high CSF protein levels should be carefully monitored because these findings could anticipate a fatal outcome (24).

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Conflict of interest statement

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