Clinical Research

Med Sci Monit, 2000; 6(6): 1136-1141

Metabolic activity of neutrophils in patients suffering from chronic viral hepatitis

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key words: liver disease, neutrophils, NBT reduction test, metabolic activity

SUMMARY

The aim of the study was to evaluate the functional state of peripheral blood neutrophils in patients with chronic active viral hepatitis. Twenty-six patients with HBV, HCV or CMV in different clinical status were included in the analysis. In the study, the number of leukocytes and neutrophils was determined. The metabolic activity of neutrophils was examined in NBT reduction tests i.e. spontaneous (NBTsp) test and the one stimulated with LPS E. coli (NBTst). The results were analysed in relation to disease advancement and the type of viral infection. The data obtained from the affected patients were compared with the results from 46 healthy subjects. Most patients displayed neutropenia. It was found that the number of NBT positive cells and the coefficient of neutrophil metabolic activity (CNMA) in NBT sp test were highly significantly increased. The majority of patients had reduced values in NBTst test, which suggested lack of response to LPS E. coli by neutrophils. These findings may reflect the state of inflammation in the body. NBT reduction tests may be useful in monitoring metabolic activity of neutrophils in patients with chronic active viral hepatitis.

INTRODUCTION

Among viral hepatitis with progressing changes in hepatocytes, the term 'chronic' applies to those cases of hepatitis when virus is not eliminated within 6 months. Viral hepatitis include HAV, HBV, HCV, HDV, CMV, EBV and HSV. The pathogenic factors responsible for hepatocyte damage in chronic hepatitis are specific and non-specific mechanisms, mediated by the immune system and immune reactivity of the affected patient is strictly related to the development of chronic infection. The changes observed in the liver after infection result both from local and general response to infection and are manifested in clinical symptoms and laboratory parameters. People with chronic hepatitis display hypergammaglobulinaemia with respect to the levels of IgG, IgA and IgM in blood serum and hepatocytes [1–4].

In liver diseases, neutrophils, lymphocytes and many other cells cumulating at the site of inflammation induce vascular and parenchymal damage of the liver. They are also present in blood stream. The infected cells are eliminated if they are recognised by natural killer cells and cytotoxic T cells [1–3,5].

Neutrophils, representing the most numerous group of leukocytes in peripheral blood, are involved in a variety of pathophysiological processes in the body and due to their metabolic, synthetic and secretory activity they play an important role in immunoregulation. Their stimulation as a result of inflammation has a beneficial effect if it intensifies the inactivation of infection factors, but it may well have negative consequences, when neutrophils destroy host's cells. Qualitative and quantitative neutrophil dysfunction is observed in various disease entities [6–13].

Received: 2000.04.04
Accepted: 2000.06.16

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The aim of the study was to evaluate the functional status of neutrophils in peripheral blood of patients with chronic viral hepatitis. It was important to establish whether pathological status was reflected in metabolic activity of peripheral blood neutrophils, determined in NBT reduction tests.

MATERIAL AND METHODS

The analysis was based on 26 patients, both males and females, aged 22–75 years, with the diagnosis of chronic active viral hepatitis. In the analysis, the type of virus (HBV, HCV, CMV) and clinical status (a – light, b – moderate, c – severe) based on Child-Pough's score were taken into account. This punctual score includes such parameters as albumin and bilirubin levels, prothrombin index, hydroperitoneum and hepatic encephalopathy. HBV was diagnosed in 19 patients, HCV – in 5, cytomegaly – in 2. Clinical status was light in 5 subjects, moderate in 9 and severe in 12. The patients underwent diagnostic investigations and treatment in Chair and Department of Gastroenterology, Collegium Medicum, Jagiellonian University in Cracow.

The number of leukocytes, proportional content and absolute value of neutrophils were calculated. Metabolic activity of neutrophils was determined with the use of nitroblue tetrazolium spontaneous (NBTsp) and stimulated (NBTst) reduction tests, following the methodology described by Park et al. [14], Park and Good [15], with our own modifications.

The results in 46 healthy subjects were considered normal reference values for peripheral blood neutrophils [16].

NBT-sp reduction test

0.1 ml of 0.1% NBT solution was added to 0.1 ml heparinised venous blood (50 u/ml). After 15 minute incubation at 37°C and 15 minute incubation at room temperature, the smears were prepared. After May-Grünwald Giemza staining, the percentage of NBT-positive cells with black formazan inside them was evaluated.

NBT-st reduction test

Lipopolysaccharide (LPS) E. coli – Pyrogen manufactured by Biomed, Cracow – was used for the stimulation of metabolic activity of peripheral blood neutrophils.

4 μg LPS E. coli in 0.02 ml Krebs-Ringer solution was added to 0.1 ml heparinised venous blood. After 10-minute incubation at room temperature, 0.1 ml of 0.1% NBT solution was added, and the procedure was repeated as in NBT-sp test.

Statistical analysis

The results are presented as mean absolute values with standard deviations and with the coefficient of neutrophil metabolic activity (CNMA), calculated from the proportion of percentage values of NBT-positive cells observed in the examined subjects to NBT-positive values in control group.

T-Student test and Statistica for Windows were used for the comparison of study results.

RESULTS

Table 1 presents the number of leukocytes and neutrophils in peripheral blood of patients with hepatitis, with respect to virus type and clinical status and in relation to normal values. Fifty-three per cent of patients with HBV had leukopenia and 63% displayed neutropenia. This group included the most patients with severe clinical condition. The analysis of 26 patients without reference to virus type revealed that 46% of them had reduced leukocyte count and 58% – reduced neutrophil count.

Table 2 demonstrates the results of metabolic activity of peripheral blood neutrophils in NBT-sp test with respect to normal values. The former were shown as absolute values of NBT positive cells and relative CNMA values, with regard to virus type and clinical status. Elevated values of NBTsp test and relative CNMA test were found in most patients with HBV, among whom subjects with moderate and severe clinical status prevailed. Higher values of NBTsp and CNMA tests were also observed in patients with HCV. In total, the majority of 26 subjects displayed 2 – 6 times higher values of NBT-positive cells and CNMA.

Table 3 provides data on NBT test stimulated with LPS E. coli (NBTst LPS). In HBV patients, lower values of NBT-positive cells were found in 15 subjects and lower CNMA values – in 10. Reduced values of the test and CNMA were observed in patients with CMV. Out of all the subjects with chronic active viral hepatitis, 73% had lower values of NBT positive cells and 54% – lower CNMA values.

Table 1. Peripheral blood neutrophils in patients with chronic, viral hepatitis.
The relationship between clinical status, viral infection and normal values.

Patients		Number of leukocytes							
		N		N	decreased	N	normal	N	increased
		26	**4965±3078^{NS}**	**12**	**3217±579**	**12**	**5242±616**	**2**	**13800±5940**
HBV	a	4	4262±736***	1	3200	3	4617±247		
	b	6	6875±5605^{NS}	2	3050±212	3	5717±29	1	18000
	c	9	3633±1085****	7	3193±718	2	5175±460		
	Σ	19	4789±3394^{NS}	10	3165±594	8	5169±554	1	18000
HCV	a	1	4700^{NS}			1	4700		
	b	3	5117±1308^{NS}	1	3900	2	5725±1096		
	c	1	9600****					1	9600
	Σ	5	5930±2258^{NS}	1	3900	3	5383±975	1	9600
CMV	c	2	4225±1662	1	3050	1	5400		
Control		46	5826±987						

Patients		Number of neutrophils							
		N		N	decreased	N	normal	N	increased
		26	**3020±2972^{NS}**	**15**	**1773±671**	**9**	**3191±404**	**2**	**11604±6500**
HBV	a	4	2718±574**	2	2305±274	2	3130±482		
	b	6	4362±5838^{NS}	5	1995±753			1	16200
	c	9	2163±1199****	5	1241±526	4	3315±525		
	Σ	19	2974±3339^{NS}	12	1732±717	6	3254±470	1	16200
HCV	a	1	2773NS			1	2773		
	b	3	2552±555**	2	2236±129	1	3185		
	c	1	7008****					1	7008
	Σ	5	3488±2009^{NS}	2	2236±129	2	2979±291	1	7008
CMV	c	2	2291±1342**	1	1342	1	3240		
Control		46	3696±781						

** p 0.02–0.01, *** p 0.005–0.001, **** p 0.0005–0.0000...1, a, b, c – degree of viral advances

Table 2. Metabolic activity of neutrophils in spontaneous NBT test (NBTsp).
Mean absolute number of NBT positive cells, depending on degree of disease advances and types of virus infection and the coefficient of neutrophil metabolic activity (CNMA) in NBTsp test were expressed.

Patients		NBT positive cells in NBT sp test								CNMAsp
		N		N	decreased	N	normal	N	increased	
		26	**1082±1770*****	**3**	**126±11**	**5**	**370±83**	**18**	**1439±2039**	**3.56±1.93******
HBV	a	4	644±480***	1	127			3	817±409	2.39±1.40****
	b	6	1996±3637***	1	114	1	328	4	2884±4346	3.24±1.79****
	c	9	637±359****	1	136	3	417±66	5	869±299	3.24±1.79****
	Σ	19	1068±2047**	3	126±11	4	395±70	12	1527±2494	3.21±1.50****
HCV	a	1	1747****					1	1747	6.93****
	b	3	1535±575****					3	1535±575	6.49±1.05****
	c	1	701***					1	701	1.10^{NS}
	Σ	5	1411±576****					5	1411±576	5.50±2.58****
CMV	c	2	393±177^{NS}			1	268	1	518	1.98±0.31****
Control		46	309±129							1.0±0.21

** p 0.02–0.01, *** p 0.005–0.001, **** p 0.0005–0.0000...1, a, b, c – degree of viral advances

DISCUSSION

The analysis of patients with the diagnosis of chronic, active, viral hepatitis revealed reduced number of neutrophils in peripheral blood in most subjects, particularly in patients whose clinical status was moderate or severe. Neutrophils as the first defence line play an important role in the body,

Table 3. Effect of LPS E. coli on metabolic activity of neutrophils in NBT stimulated test (NBTst LPS) expressed in mean absolute number of NBT positive cells and the coefficient of neutrophil metabolic activity (CNMA).

Patients		NBT positive cells in NBT st test								CNMAst				
		N		N	decreased	N	normal	N	increased		N	decreased	N	normal
		26	**1022±1155^{NS}**	**19**	**583±237**	**6**	**1559±350**	**1**	**6156**	**0.82±0.32****	**14**	**0.58±0.10**	**12**	**1.01±0.15**
HBV	a	4	873±474	3	667±285	1	1493			0.74±0.27	2	0.56±0.24	2	0.93±0.17
	b	6	803±1745^{NS}	4	440±251	1	1534	1	6156	0.77±0.36	4	0.56±0.12	2	1.17±0.35
	c	9	723±398****	8	612±237	1	1606			0.88±0.28^{NS}	4	0.62±0.07	5	1.09±0.17
	Σ	19	1024±1312^{NS}	15	577±247	3	1544±57	1	6156	0.82±0.30**	10	0.59±0.12	9	1.07±0.20
HCV	a	1	1081^{NS}			1	1081			0.96^{NS}			1	0.96
	b	3	1195±847^{NS}	2	710±148	1	2166			1.08±0.53^{NS}	1	0.64	2	1.30±0.52
	c	1	1472^{NS}			1	1472			0.51	1	0.51		
	Σ	5	1228±616^{NS}	2	710±148	3	1573±550			0.94±0.45^{NS}	2	0.57±0.09	3	1.19±0.42
CMV	c	2	497±305**	2	497±305					0.52±0.02**	2	0.52±0.02		
Control		46	1385±451							1.0±0.24				

** p 0.02–0.01, *** p 0.005–0.001, **** p 0.0005–0.0000...1, a, b, c – degree of viral advances

their deficiency manifested in neutropenia or dysfunction may have serious clinical consequences. Patients with cirrhosis often display neutropenia and reduced cell viability, which makes them vulnerable to bacterial infections. In these patients, neutropenia may result partly from markedly elevated apoptosis in vitro [9–11,17,18].

NBT tests which are widely used in laboratory diagnostics of various diseases, allow for the determination of phagocytosis and metabolic activity of neutrophils [10,16,19–22]. In 69% analysed patients, the values of NBTsp test and CNMAsp were 2–6 times higher. The clinical status of 78% of these subject was moderate or severe. Elevated values in NBTsp test might suggest an ongoing inflammatory process, induced by viruses, their products or other micro-organisms. In HCV infected patient, the sequences of viral genome may be detected in medullary haematopoietic cells and mononuclear cells in peripheral blood, i.e. phagocytes (neutrophils, monocytes/macrophages) and B cells. In patients with disseminated human cytomegalovirus, the infectious virus, viral DNA and HCMVpp65 antigen were found in neutrophils. Possible penetration and replication of viruses in immune system cells has a detrimental effect on normal cell function [23,24].

The liver is the main organ involved in endotoxaemia and responding to LPS. Patients with chronic viral hepatitis often display increased blood levels of endotoxins and systemic endotoxaemia as well as elevated concentration of soluble receptor sCD14 for LPS. This contributes to the disorders accompanying these diseases. It was observed that intravenous LPS administration to experimental animals is the cause of pathological changes in the liver characterised by the infiltration of platelets and neutrophils, sequestration of inflammatory cells in vessels, migration through endothelium and adhesion to hepatocytes as well as the release of cytotoxic mediators responsible for hepatocyte necrosis [5,25–29]. Peripheral blood neutrophils isolated from the majority of patients did not respond to LPS E. coli stimulation. It was manifested in reduced NBTst and CNMAst values, particularly in patients with moderate or severe condition. The impairment of neutrophil response to LPS E. coli stimulation might result from the activation of these cells before extravasation. Neutrophils fully activated in vivo did not respond to the stimulant (LPS) in vitro. This would suggest lack of 'functional reserve of the cells' able to fulfil additional function, thus making the patient's body susceptible to further infections.

The evaluation of the function of peripheral blood neutrophils seems significant in the assessment of chronic active viral hepatitis. The sequence of phenomena taking place in the body affected by inflammation usually lead to the impairment of immune system reactivity. It was manifested in neutropenia or disturbances in metabolic activity of neutrophils diagnosed in the analysed group of patients. The complexity of chronic active hepatitis is further enhanced by possible development of autoimmune diseases, when auto-antibodies may bind with antigens in situ in hepatocytes and cause further destruction [1,2,4]. It seems important to start adequate treatment with special reference to biologically active drugs/preparations, which have

a stimulating/inhibiting effect on immune system and a regenerating or adjuvant action, e.g. ubiquitin complex, interferon alpha [2,30–33].

CONCLUSIONS

1. The following were observed in most patients with chronic active viral hepatitis: a) neutropenia; b) elevated NBTsp and CNMAsp values; c) reduced NBTst and CNMAst values, which indicates the absence of 'functional reserve of the cells', capable of additional response to LPS stimulation measured in vitro.

2. Our findings confirm the presence of inflammation and reflect the involvement of peripheral blood neutrophils in this process.

3. Monitoring functional status of peripheral blood neutrophils in NBT reduction tests is an easy-to-use method which may be employed during the treatment of patients with chronic active viral hepatitis.

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