

U. GRATA-BORKOWSKA<sup>1</sup>, A. STECIWKO<sup>1,2</sup>, M. POKORSKI<sup>3</sup>, J. DROBNIK<sup>1,2</sup>,  
K. GAŚSIOROWSKI<sup>4</sup>, I. PIROGOWICZ<sup>1,2</sup>, E. CIEŚLAR-MARCZAK<sup>4</sup>

## EFFECTS OF NEURAMINIDASE ON APOPTOSIS OF BLOOD LYMPHOCYTES IN RATS WITH IMPLANTED MORRIS TUMOR

<sup>1</sup>Department of Family Medicine, Wrocław Medical University, Wrocław, Poland; <sup>2</sup>Public Medical Vocational School, Opole; <sup>3</sup>Medical Research Center, Polish Academy of Sciences, Warsaw, Poland; <sup>4</sup>Department of Basic Science, Wrocław Medical University, Wrocław, Poland; <sup>5</sup>Department of Biochemistry, Wrocław Medical University, Wrocław, Poland.

In this study we examined the influence of neuraminidase on apoptosis of peripheral blood lymphocytes in rats with an implanted Morris tumor. The main objectives of the study were to determine whether the percentage of apoptotic blood lymphocytes would depend on the dosing regimen of neuraminidase and whether neuraminidase would affect caspase-3 activity, a marker of the apoptosis, in blood lymphocytes. A total of 51 rats were used for the study. In three groups, totalling 39 animals, Morris tumor was implanted and neuraminidase was injected intravenously using two dosing regimens: 10 units three times on Day 4, Day 7, and Day 14 and 5 units as a single dose on Day 4 of the experiment or was skipped (control). The remaining 12 rats constituted a reference group of healthy animals. At the end of the experimental period on Day 21, blood was drawn from the heart, and mononuclear cells were separated and cultured. Apoptosis of blood lymphocytes was assessed in cell cultures from fluorescence spectra generated by a Sybr Green I dye forming bonds with nuclear DNA. Caspase-3 activity was measured colorimetrically in homogenates of lymphocyte cultures using a CASP-3-C kit (Sigma, St. Louis, MO). On the whole, the results demonstrate that the bigger, but not the smaller, dose of neuraminidase was markedly effective in preserving the vitality of blood lymphocytes and in decreasing both the number of apoptotic lymphocytes and caspase-3 activity in the rats with Morris tumor. Neuraminidase treatment failed, however, to lessen the tumor size. In conclusion, the study demonstrates that neuraminidase caused an appreciable decline in apoptosis of blood lymphocytes in rats with the Morris tumor; the effect was dose-dependent. Although neuraminidase failed to influence the local cancer development in terms of tumor size, its anti-apoptotic effect toward the cells of the immune system of a cancer host is of research interest as it may potentially offer a way to strengthen the host's immune response.

**Key words:** *apoptosis, lymphocytes, Morris tumor, neuraminidase*

## INTRODUCTION

The pathomechanism of tumor development is still an up-to-date topic in modern medicine. The apoptotic process, which is the carefully programmed and controlled suicide of a cell, is closely connected with tumor development. Disturbance of this process is seen in both tumor cells (apoptosis inhibition) and in cells of the immune system of a tumor host (apoptosis stimulation).

In experimental research, an increase in the rate of apoptosis of immune system cells of the tumor host has been shown (1-3). Some of the authors show that neuraminidase, an enzyme produced by bacteria, viruses (e.g., influenza virus), and protozoa, stimulates the apoptotic process (4, 5). On the other hand, other authors suggest that the enzyme can have an apoptosis inhibiting influence (6, 7). Since the views concerning the influence of this enzyme on cell apoptosis are ambiguous, we set out to study the influence of different doses of neuraminidase on apoptosis of peripheral blood lymphocytes in an experimental model consisting of rats with an implanted Morris tumor. The aim of this research was answering the following questions:

1. Does neuraminidase given to rats with the Morris tumor affect apoptosis of blood lymphocytes?
2. Does the proportion of blood lymphocytes having apoptotic characteristics depend on the dosing regimen of neuraminidase?
3. Does neuraminidase treatment affect the activity of caspase-3, a marker of the apoptosis, in blood lymphocytes?
4. How does neuraminidase treatment affect the tumor size?

## MATERIAL AND METHODS

The study was approved by a local Ethics Committee for Animal Experiments in Wrocław, Poland, and a permission to conduct the experiments was granted to the authors by Vice President for Scientific Matters of the Wrocław Medical University in Wrocław, Poland.

*Animals and experimental protocol*

Fifty one adult female Buffalo rats 12 weeks old and weighing *ca* 200 g were used for the study. The animals were kept in an air-conditioned room at  $21 \pm 1^\circ\text{C}$  and 50% humidity under a 12-h light/dark cycle (lights on at 6:00 a.m.). They obtained commercial Labofeed H feed and had water *ad libitum*.

The pulp of Morris hepatoma obtained by passaging was implanted intramuscularly into the left thigh region of animals from all of the groups on the first day of the experiment (except for the control group consisting of healthy rats). Enzymatically active neuraminidase (Sigma; St. Louis, MO), isolated from the *Clostridium perfringens* (*Clostridium welchii*) strain, was used in the study. Neuraminidase was dissolved *ex tempore* in phosphate buffer and was injected into the tail vein, starting as of the fourth day after implanting the Morris tumor. The animals were divided into the following groups:

Group I - 18 rats with an implanted Morris tumor, exposed to a 10 unit dose of neuraminidase three times (applied on the fourth, seventh, and fourteenth day of the experiment).

Groups II - 13 rats with a Morris tumor, exposed to a single 5 unit dose of neuraminidase given once on the fourth day of the experiment.

Group III - 8 control rats with an implanted Morris tumor, but no injection of neuraminidase

Group IV - a reference group consisting of 12 healthy rats not subjected to any experimental maneuvers.

On the 21<sup>st</sup> day of the experiment all of the rats were sacrificed by an overdose of anesthetics and blood samples were withdrawn from the heart onto an anticoagulant (10% edetate disodium - 1 mg/ml) for laboratory analyses.

### *Assessment of apoptotic cells*

Withdrawn blood was separated in a density gradient ficol/metrizamide (Histopaque 1077; Sigma, St. Louis, MO) centrifuged at 1400 x g at 20°C for 15 min. A layer of mononuclear cells was collected, washed twice in Eagle basal medium, and centrifuged 400 x g at 6°C for 10 min. After assessing the lymphocytes vitality (using the cell dyeing method with a 0.4% solution of trypan blue), cell culturing was initiated. Cell cultures were collected after 48 h and centrifuged. The obtained cell pellet was suspended in 2 ml of PBS, of which 0.5 ml was transferred to separate test-tubes and secured for further microscopic examination of cells undergoing the apoptotic process.

From the 0.5 ml pool of cells meant for the microscopic examination, 50 µl from each culture was taken and a culture was initiated on a 96-well plate to assess cell numbers by a spectrophotometric dyeing method with the use of Sybr Green I fluorochrome. The culture was kept for 1 h in a CO<sub>2</sub> incubator (5% CO<sub>2</sub> atmosphere and a temperature of 37°C) and it was then centrifuged. The supernatant was collected and cells were dried for 2 h at 37°C with blowing air. 50 µl of a Sybr Green I dye solution (1:10 000 concentration) was added and the cells were left in it at 37°C for 16 h to allow Sybr Green I forming bonds with nuclear DNA. Afterward, fluorescence spectra were measured with an induction wavelength of 485 nm and emission at 530 nm, with the use of a Victor 2 spectrofluorimeter.

The number of cells in a culture was estimated by comparison with a standard curve of lymphocytes. The standard curve was constructed from one culture that was precisely counted under a microscope in the Bürker grid with the use of Türk dye. A number of cell dilutions were formed from 1x10<sup>3</sup> to 1x10<sup>5</sup> and subsequently dyed in the above mentioned method. That allowed obtaining a standard relationship between the fluorescence emitted by the bound dye and the number of cells.

### *Measurement of caspase-3 activity*

Caspase-3 activity was measured colorimetrically in homogenates of lymphocyte cultures with the use of a commercial kit (CASP-3-C; Sigma, St. Louis, MO). The analysis was performed in the same lymphocyte cultures, in which the microscope evaluation of cells in apoptosis was performed with the use of an annexin-FITC/PI kit. 50 µl of lytic solution containing: non-ionized detergent 0.1% CHAPS, 50 mM HEPES, 10 mM dithiotreitol (DTT), 100 mM NaCl, 1mM disodium versenate, and 10% sucrose, pH of 7.4, was added to the palette of cells. Cell lysis was conducted at 4°C in an ice-cold bath in Eppendorf test-tubes for 15 min. At first, cells were vigorously pipetted out in a lytic buffer. Cells were then centrifuged at 17000 x g at 4°C for 15 min and the supernatant containing caspase-3 was safeguarded for further analysis.

To perform colorimetric measurements, a 96-well, flat-bottomed culture plate was used, to which 85  $\mu\text{l}$  of a measurement buffer, 5  $\mu\text{l}$  of the supernatant from the culture, in which caspase-3 was to be measured, and 10  $\mu\text{l}$  of the specific substrate for caspase-3 Ac-DEVD-pNA (acetyl-Asp-Glu-Val-Asp p-nitroaniline) were added. This four-aminoacid peptide contains a covalently connected p-nitroaniline (chromogen dye). When caspase-3 recognizes the substrate, the enzyme induces substrate proteolysis with the release of p-nitroaniline, characterized by a high absorption factor at a wavelength of 405 nm. Samples were incubated at 37°C for 90 min and then the absorption of released p-nitroaniline was assessed spectrophotometrically.

In each culture, a trial with a protease inhibitor, which inhibits caspase-3 activity, was conducted. Each supernatant was studied in octuple on a 96-well plate. A significant decline of p-nitroaniline absorption in these samples was observed as opposed to the samples that did not contain an inhibitor.

Results of caspase-3 activity were standardized, converted to  $1 \times 10^4$  cells. Caspase-3 activity was calculated in the following way. A standard curve of p-nitroaniline absorption was used for determining what micromolar concentration of p-nitroaniline corresponds to a given unit value of absorption at a wavelength of 405 nm. p-nitroaniline concentration was expressed in nmoles per 100  $\mu\text{l}$  incubation mixture in the 96-well plate and later converted to nmol per 1 ml of supernatant per min, as the absorption was measured after 90-min incubation at 37°C. Caspase-3 activity in each culture was expressed as an arithmetical mean  $\pm$ SD from octuple measurements.

### *Data analysis*

All data are expressed as mean values  $\pm$ SD. Normality of data distribution of each variable was checked with the Shapiro-Wilk test. In case of normally distributed data, differences between the mean values were assessed with a two-tailed unpaired *t*-test. Otherwise, a non-parametric Kolmogorov-Smirnov analysis was applied. A value of  $P < 0.05$  was taken as indicative of statistically significant differences. A commercial Statistica PL 6.0 package was used for all statistical data elaboration.

## RESULTS

### *Proportions of live, apoptotic, and dead lymphocytes*

Proportions of live, apoptotic, and dead lymphocytes in the blood of rats stratified into the experimental groups studied are depicted in *Table 1*. The highest mean percentage of live lymphocytes was found in the rats with an implanted Morris tumor which were exposed to the influence of a bigger and repeatedly administered 10 unit dose of neuraminidase (Group I). The mean percentage amounted to  $68 \pm 14.6\%$  and was significantly higher than the  $59 \pm 12.3\%$  in the reference group of healthy rats (Group IV) ( $P < 0.05$ ) (*Table 1, Column A*). Noteworthy, the mean percentage of live cells in the rats with Morris tumor exposed to a smaller, single 5 unit dose of neuraminidase (Group II), although significantly lower than that observed after the bigger neuraminidase dose, was still about 3-fold higher than that in rats with Morris tumor which were remained unexposed to neuraminidase (Group III) ( $P < 0.05$ ) (*Table 1, Column A*). The latter percentage amounted to  $10 \pm 3.4\%$  and was the lowest of all the experimental groups studied.

Table 1. Proportions of live, apoptotic, and dead lymphocytes in peripheral blood in the experimental groups studied after 21 days of the experiment.

	A	Live Lymphocytes (%)	B	Apoptotic Lymphocytes (%)	C	Dead Lymphocytes (%)
Group I (n=18)		68 ±14.6		26 ±7.1		6 ±2.0
Group II (n=13)		30 ±8.1		63 ±18.1		7 ±1.2
Group III (n=8)		10 ±3.4		78 ±13.6		12 ±1.1
Group IV (n=12)		59 ±12.3		35 ±10.1		6 ±1.0

Values are means ±SD. Group I – Morris tumor treated with 10 units of neuraminidase administered 3 times; Group II – Morris tumor treated with a single 5 unit dose of neuraminidase; Group III – Morris tumor - untreated control; Group IV – reference healthy rats without Morris tumor. The statistically significant differences among the mean values given in the table are the following. Column A - Live lymphocytes – each group mean is different from one another; Column B - Apoptotic lymphocytes – Group II mean and Group III mean each is different from Group I mean and Group IV mean; Column C - Dead lymphocytes – Group III mean is different from the other three group means.  $P < 0.05$  for all differences.

With regard to apoptosis, the results appeared converse to those of the above mentioned for live cells. The lowest mean percentage of lymphocytes displaying apoptotic features, which corresponded closely to that present in the reference healthy rats (Group IV), was found in rats with Morris tumor which were treated with the bigger 10 unit dose of neuraminidase (Group I) and the highest was in those which remained unexposed to neuraminidase (Group III);  $26 \pm 7.1\%$  vs.  $78 \pm 13.6\%$ , respectively ( $P < 0.05$ ). The smaller, single 5 unit dose of neuraminidase (Group II) had a contrastingly smaller antiapoptotic effect on lymphocytes compared with the bigger dose of neuraminidase (Group I). The percentage of apoptotic lymphocytes in Group II, although tending to decline, remained indistinctly different from that in the rats with Morris tumor which were untreated with neuraminidase (Group III) ( $P > 0.05$ ) (Table 1, Column B).

The implanted Morris tumor apparently fostered the propensity for premature death of blood lymphocytes. The mean percentage of dead lymphocytes was highest in rats with the tumor which remained unexposed to neuraminidase (Group III), amounting to  $12 \pm 1.1\%$ . This figure was nearly twice as high as in the other experimental groups, i.e., in the rats with the tumor which were treated with neuraminidase and in the healthy ones (Table 1, Column C). Concerning the proportion of dead lymphocytes, no significant differences were noted between the effects of the two dosing regimens of neuraminidase used.

### Caspase-3 activity

Distribution of caspase-3 activity assessed in homogenates of cultures of peripheral blood lymphocytes in the experimental groups studied was somehow

similar to the proportion of apoptotic cells described above. The lowest mean caspase-3 activity was found in the rats with Morris tumor treated with the bigger 10 unit dose of neuraminidase (Group I), whereas the highest was in those with the tumor that remained unexposed to neuraminidase (Group III);  $0.41 \pm 0.11$  nmol/ml/min vs.  $2.26 \pm 0.59$  nmol/ml/min, respectively. Therefore, the treatment with the bigger dose of neuraminidase caused more than a 5-fold decrease in caspase-3 activity ( $P < 0.05$ ) (Table 2). The decreased caspase-3 activity after the bigger dose of neuraminidase tended to reach the level of caspase-3 activity present in the healthy untreated rats (Group IV); the difference between the two was insignificant. The smaller dose of neuraminidase (Group II) also tended to decrease caspase-3 activity, but the effect did not reach statistical significance compared with the neuraminidase-untreated tumors of Group III (Table 2). These results show that the level of caspase-3 activity in rats with the Morris tumor changed in response to neuraminidase treatment in concert with the proportion of apoptotic lymphocytes found in the blood; the fewer apoptotic cells the lower was the caspase-3 activity.

### *Tumor size*

The mean values of the final volume of the tumor were similar for all groups with implanted Morris tumors, irrespective of whether or not the rats were

Table 2. Caspase-3 activity in homogenates of peripheral blood lymphocytes in the experimental groups studied after 21 days of the experiment (nmol/ml/min).

Group I (n=15)	$0.41 \pm 0.11$
Group II (n=9)	$1.99 \pm 0.26$
Group III (n=6)	$2.26 \pm 0.59$
Group IV (n=7)	$0.19 \pm 0.04$

Values are means  $\pm$ SD. Group I – Morris tumor treated with 10 units of neuraminidase administered 3 times; Group II – Morris tumor treated with a single 5 unit dose of neuraminidase; Group III – Morris tumor - untreated control; Group IV – reference healthy rats without Morris tumor. The statistically significant differences among the mean values given in the table are the following. Group I mean is different from both Group II and Group III means; Group II and Group III means are both different from Group IV mean.  $P < 0.05$  for all differences.

Table 3. Size of Morris tumor in the experimental groups subjected to the action of neuraminidase after 21 days of the experiment (cm<sup>3</sup>).

Group I (n=15)	$9.3 \pm 2.01$
Group II (n=9)	$9.2 \pm 3.94$
Group III (n=6)	$10.5 \pm 3.48$

Values are means  $\pm$ SD. All groups had an implanted Morris tumor. Group I – treated with 10 units of neuraminidase administered 3 times; Group II – treated with a single 5 unit dose of neuraminidase; Group III – untreated control. There were no statistically significant differences in Morris tumor size among the three groups.

exposed to neuraminidase and its dose. The volume ranged between 9-11 cm<sup>3</sup>, and no statistically significant differences in the tumor size could be substantiated (*Table 3*).

#### DISCUSSION

The major finding of this study was that neuraminidase administered to rats with an implanted Morris tumor had, on the whole, a positive effect on the sustainment of the vitality of peripheral blood lymphocytes in that they were less prone to enter the process of apoptosis and the number of dead lymphocytes was smaller. Dampened process of apoptosis in neuraminidase-treated rats was accompanied by a decline in the activity of its marker, caspase-3. The effect of neuraminidase treatment was, however, dose-dependent. Neuraminidase was highly effective in the bigger dose used, which was 10 units administered on three occasions over a 10-day period, as opposed to a single 5 unit dose. The latter dose exerted only borderline effects on blood lymphocytes. Despite a positive effect on the survival of blood lymphocytes in rats with the Morris tumor, neuraminidase treatment failed to decrease the tumor size.

Immune system cells are subject to apoptosis, like many other cell systems (7-9). However, the literature concerning the influence of neuraminidase on effector cells of the immune system is rather meager. On the basis of available articles (7, 10, 11), it was difficult to explicitly foresee changes in peripheral blood lymphocytes of a cancer host under the influence of neuraminidase. Neuraminidase, a part of viruses, has the ability to induce apoptosis in cultures of mammal cells (4, 12). Some authors state that neuraminidase of the influenza virus may evoke apoptosis mainly through activating TGF- $\beta$  and directly through the internal apoptosis track (13). The anti-neuraminidase compound - Zanamivir (4-guanidino-2,3-dihydro-N-acetylneuraminic acid) – may slow down the host's cell apoptosis caused by the influenza virus. This fact indicates the participation of neuraminidase in stimulating the apoptosis of immune system cells of the organism attacked by the influenza virus (5, 13, 14).

The above data may indicate that neuraminidase causes immunosuppression. However, the results of the current study clearly indicate a slowdown influence of neuraminidase on apoptosis in peripheral blood lymphocytes. The study shows that neuraminidase decreased the level of apoptosis in blood lymphocytes and increased their vitality. The slowing of apoptosis after neuraminidase was supported by a simultaneous decrease in lymphocytic caspase-3 activity. Thus, neuraminidase may counteract the immunosuppressive influence of the Morris tumor cells on the immune system of a cancer host. Interestingly, the effect of neuraminidase seems to depend on its dose. This is difficult to discuss with the literature data, as we could not find any previous information concerning the dose dependence of neuraminidase effects in regard to peripheral blood lymphocytes.

In the current study, the proportion of blood lymphocytes that entered the process of apoptosis was evidently enhanced in rats with an implanted Morris tumor, which is in accord with the notion that the number of T and B lymphocytes decreases in the cancer host's body. Suzuki et al (15) showed that apoptosis of cancer cells originating from T lymphocytes was inhibited by adding neuraminidase. It is possible that the characteristics of neuraminidase effects depend on the type of cell exposed to its action and the influence of some ill-defined at the present state of knowledge cofactors; the elements that cannot be easily controlled for in experimental studies. In this context it should be stressed that lymphocytes originating from the rat's blood are more sensitive to apoptosis than human lymphocytes in an *in vitro* culture (2).

Caspase-3 is a key mediator of apoptosis and its activity is required for morphological changes typical for this process in all types of cells tested so far, which has made it a good marker of apoptosis (16-18). The current study demonstrates a substantial increase in caspase-3 activity in rats with an implanted Morris tumor which remained free of neuraminidase treatment. The reasonable assumption is that the process of apoptosis must have been increased in blood lymphocytes in this group of animals. On the other hand, animals exposed to a bigger dose of neuraminidase showed a marked decrease in caspase-3 activity, which also reflected a decreased number of apoptotic cells. It thus seems reasonable to conclude that neuraminidase had anti-apoptotic activity in our study.

In the present study neuraminidase, although effective in inhibiting apoptosis of blood lymphocytes, failed to influence the tumor size. Thus, neuraminidase treatment did not play a role in the local cancer development. This result is somehow at variance with those of Alley and Snodgrass (19) and Watkins et al (20) who found that neuraminidase applied directly into the tumor caused a local slowdown of the cancer development. Alley and Snodgrass (19) indicated that in some cases a full recovery was observed among rats with a lung malignant neoplasm after applying neuraminidase directly to the tumor. It should be noted that in the current study neuraminidase was administered intravenously, and not directly into the tumor, which may be a reason of its different effect on the tumor size.

In conclusion, the study demonstrates that neuraminidase administered to rats with the Morris tumor caused an appreciable decline in the proportion of apoptotic lymphocytes in the blood, which was accompanied by a decline in lymphocytic caspase-3 activity, a biochemical marker of apoptosis. The anti-apoptotic effect of neuraminidase was dose-dependent. Although neuraminidase failed to influence the local cancer development in terms of tumor size, its anti-apoptotic effect toward the cells of the immune system of a cancer host is of research interest as it may potentially offer a way to strengthen the host's immune response. Further research is required to investigate the exact determinants of neuraminidase action in a cancer host.

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Author's address: U. Grata-Borkowska, Department of Family Medicine, Wrocław Medical University, Wrocław, Poland; e-mail: ulagrata@op.pl