Antiviral Mechanisms of the drug 'Gepon': Modulation of Cytokine Gene Transcription in a J-96 Human Cell Line.

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The immuno-modulator 'Gepon' has been successfully used in the treatment of acute and chronic infectious-inflammatory process of different etiology and localisation. We have noticed a high efficacy of 'Gepon' in the treatment of recurring candidiasis of mucous membranes[1], chronic pharyngitis[2], recurring respiratory infection[3], and recurrent herpes-virus infection[4]. In the treatment of serious laryngo-tracheal bronchitis in children, a very effective anti-inflammatory action was shown with visible disappearance of swelling of the throat (in patients who have had Croup Syndrome[5]). In addition, the rapid and visible anti-inflammatory action of 'Gepon' was shown when it was locally used in treatment of inflamed mucous membranes of urogenital tract. The current research was undertaken for a better understanding of antiviral and anti-inflammatory mechanisms of Gepon. This paper demonstrates how Gepon can affect the stability, immunity and resistance of human cells *in vitro* to the cytotoxic affect of Encephalomyocarditis Virus (EMC Virus). The results indicate that there is a association between the antiviral action of Gepon and modulation of gene transcription of regulatory cytokines in human cells.

Materials and Methods

Cells.

Fibroblast cell culture J-96 was derived from human blood cells of a patient suffering from sub-acute monocytic leukemia. The cells were cultivated in Medium 199, with addition of 2 mM L-glutamine, 10% inactivated Fetal Calf Serum, 100 units/ml penicillin and anti-PPLO.

Virus.

Encephalomiocarditis Virus (EMC Virus) from mouse strain Columbia SK-Col-SK at an initial concentration 10^5 $TCID_{50}$ (Tissue Culture Infectious Dose that causes death of 50% cells) in 1 ml.

Titration of EMC Virus

24 hours before the experiment, J-96 cells were cultured in 96-well plastic microtitre plates to a monolayer then infected by EMC Virus in 10-serial dilutions from 10^{-1} to

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[1] *Russian publication*: New approach and therapy for the treatment of urogenital candidiasis, Gynecology, 2001 Vol3 No6 210-212

[2] *Russian publication*: New approach and therapy for chronic disease of the throat, Prescribing Physician, 2002 No4 64-65

[3] *Russian publication*: First experiment using intranasal application of Gepon in children with respiratory disease, Pediatry, 2002 No2 86-88

[4] *Russian publication*: Immunomodulator Gepon for local therapy of herpes virus infection, Russian National Congress, Man and Medicine, Moscow 2002, thesis report 55

Russian publication: Treatment of recurrent genital herpes with immuno-modulator Gepon, Russian National Congress, Man and Medicine, Moscow 2002, thesis report 56

[5] *Russian Publication*: Effective treatment for croup syndrome with Immunomodulator Gepon, Russian Medical Journal 2002 Vol 10, No3, 138-141

10^{-8}. The cell cultures were incubated at 37 ^{o}C in an atmosphere of 5% CO_2. After 24 hours the CytoPathogenic Effect (CPE) of the virus was assessed using an inverted Leitz microscope. For investigating the effect of Gepon and during analysis of synthesis of cytokine mRNA, the infectious dose of virus used in culture was 100x $TCID_{50}$ viral dose in 1 ml.

Drugs

The Gepon used was in the form of lyophilized sterile drug in 2ml vials produced by OOO Immapharma (Moscow). "Ridostin" a well known inducer of interferon used for protection from virus infection (NPO Vector, Novosibirsk) was the control drug for comparison.

The antiviral effect of Gepon

Cell line J-96, at a concentration of 200 000 cells per 1 ml were inoculated in 96-well microtitre plates and incubated in Medium 199 with 10 % fetal Calf Serum, 300mcg/ml glutamine and 100 units/ml penicillin for 24 hours. Gepon was added to the wells with J-96 cells to a concentration of 1mg/ml and then titrated by two fold serial dilution in 24 wells (3 wells for every concentration of the drug). The experiments were repeated 3 times. The antiviral effect was evaluated as the minimum effective concentration (maximum effective dilution) of drug which protected 50% of the cells from the CytoPathogenic Effect (CPE) of 100x $TCID_{50}$ EMC Virus in 1 ml.

Investigation of the influence of the timing of exposure of the cells to Gepon and EMC Virus on CPE.

Gepon was used in 3 concentrations: 10 mcg/ml,1,0 mcg/ml, 0,1 mcg/ml (9 wells for every concentration). The cultures were incubated for 24 hours before EMC Virus was added to three wells of every concentration of drug. Three types of Gepon treatment protocol were used: 1) drug was added 24 hours before cells were infected by the virus (without washing drug away), 2) drug was added 24 hours before infection but just before infection, the cells were washed of the drug 3) drug was added to the cell culture then after only 10 minutes, the cells were washed of drug, incubated for 24 hours, and then infected by virus. The level of Gepon's antiviral effect was based on its effect on CPE of EMC virus. J-96 cells where grown to a monolayer in 96 well micro-titre culture plates and for the determination of CPE, an inverted microscope at a magnification of 100x was used. The intensity of CPE was recorded as percentage of cell death of each infected culture. A CPE as a result of EMC virus which resulted in death of all of the cells was recorded as 100% cell death. Partial killing was scored as either 75% cell death, 50% cell death or 25% cell death. Absence of cell death in culture infected by virus was marked as 0%

Determination of cytokine mRNA.

J-96 cell culture was inoculated into 250ml culture flasks and on day 3 of cultivation, growth medium was changed to supporting Medium 199 supplemented with 2 % Fetal Calf Serum with no added antibiotics. Gepon was added to a concentration of 1mcg/ml and cells cultured for 24 hours. Treated and untreated cell cultures were infected EMC Virus for 4 hours before RNA precipitation. The mRNA was precipitated from four types of J96 cell culture: 1) Untreated and non-infected control cells; 2) Cells that were incubated for 24 hours in the presence of Gepon; 3) Cells incubated in the absence of drug for 20 hours and then virally infected and incubated for 4 hours more, before RNA precipitation; 4) cells incubated in presence of Gepon during 20 hours, and then virally infected and incubated for 4 hours more, before RNA precipitation. The quantity of 11 cytokine mRNAs were determined by reverse transcription and polymerase chain reaction (RT-PCR). RNA precipitation and

Reverse transcription and PCR- amplification was made according published methods. Two primers were used for the following cytokines: IFN-a, IL-6, IL-8, IL-1b, IL-2, IL-4, IL-10 TNF-a, IFN-y, IL18, IL12. For a positive control primers for b-actin were used. Samples without mRNA were used as a negative control. Analysis of the PCR incubations was made by electrophoresis in 2.5% agar gel visualised with ethidium bromide. Electrophoresis markers produced by Promega were used for the identification of nucleotide sequences (G1758).

Results

Gepon protects J-96 cells *in vitro* from the cytopathogenic effect of EMC Virus.
In control cell cultures of J-96, infection by EMC Virus led to a CPE which resulted in the death of 100% cells. In some experiments Gepon totally suppressed the growth of virus, reducing CPE induced by EMC Virus from 100% to 0%. Previous incubation of cells J-96 in the presence of Gepon significantly increased their resistance to EMC Virus infection.

Table 1
Protective Effect of Gepon in human J-96 cell culture better than Ridostin

Drug			**Protective Effect**			
Initial drug concentration		**Initial culture**	**Expt-1**	**Expt-2**	**Expt-3**	**M± m**
Gepon 1mg/ml	Culture dilution: Culture Conc in mcg/ml:	1/ 2 500	1/ 128 7.8	1/1024 0.98	1/4096 0.24	3 ± 2.4
Ridostin 1mg/ml	Culture dilution: Culture Conc in mcg/ml:	1/ 2 500	1/ 512 2	1/ 128 8	1/ 128 8	6± 2

Culture dilution is the maximum dilution in an experiment that the protective effect was observed (CPE_{50}/ml)
Conc in mcg/ml is the minimum drug concentration in an experiment that the protective effect was observed (CPE_{50}/ml)

The data present in Table 1 shows the minimum effective concentration and maximum effective culture dilution, in which Gepon displays its protective activity. The minimum effective concentration of the drug, which provides the protective effect, was a concentration of 3.0± 2.4 mcg/ml. The effect of Gepon was at least comparable with the protective effect of Ridostin, a well-known interferon inducer. Between separate observations, a variation in the minimum effective concentration of Gepon was observed. This variation was related to the natural variation in culture conditions, the times of the experiments with different culture sera and other additional factors necessary to maintain cell culture.

Time of exposure of cells to Gepon sufficient for the manifestation of antiviral effect of the drug
The data presented in Table 2 shows Gepon's antiviral activity in concentrations 10mcg/ ml, 1 mcg/ml, 0.1mcg/ml with different times of exposure of cells to Gepon. The results prove that incubation of cells in the presence of Gepon for 24 hours induces a high level resistance to cytopathogenic effect of the virus. For example, a 24 hour pre-incubation of J-96 cells with 1.0 mcg/ml Gepon on average protected 92% of the cells from the cytopathogenic effect of EMC Virus, reducing cell death from 100% in the control to 8% in the Gepon group. Reduction of the exposure time of the

cells to Gepon to 10 minutes caused a significant decrease of Gepon's protective antiviral effect. It appears than 10 minutes exposure of the cells to Gepon was insufficient time for important cellular changes necessary for the protection from the virus. The data proves the need for the prolonged contact between the cells and Gepon to induce signals that allow the transformation of the cells into a state of high resistance to viral infection.

Table 2.

Cytopathic efficiency of EMC Virus (100 $TCID_{50}$ /ml) in J-96 cells dependent on the time of incubation cells in presence of Gepon

Cytopathogenic effect (CPE) of EMC Virus measured as percent cell death													
Drug	Gepon Conc. mcg/ml	24 hour incubation of J-96 cells with Gepon, then EMC virus added				24 hour incubation of J-96 cells with Gepon, wash out drug, then EMC Virus added				10 min. incubation of J-96 cells with Gepon, wash out drug, incubate for a further 24 hours, then EMC Virus added			
		I	II	III	**M**	I	II	III	**M**	I	II	III	**M**
Gepon	10	0	25	25	**17**	25	25	0	**17**	25	25	100	**50**
Gepon	1.0	0	0	25	**8**	25	0	0	**8**	50	25	100	**58**
Gepon	0.1	0	25	0	**8**	0	0	0	**0**	75	25	100	**67**
Control	0	100	100	100	**100**	100	100	100	**100**	100	100	100	**100**

Control: EMC Virus (100 $TICD_{50}$ /1ml), no Gepon

I II III are three parallel experiments with different concentrations of Gepon and M is the average result

Gepon changes the spectrum of cytokine mRNAs produced in the cells

The increase of cell resistance to viral infection often depends on the synthesis of interferons and some of the other cytokines. In this experiment the production of 11 different cytokine mRNAs in J-96 cells before and after a 24 hour incubation with Gepon, in the presence and absence of virus were studied (Table 3).

Table 3.

Influence of Gepon and EMC Virus on the activity of cytokine mRNA in J-96 cells

	J96 Cells incubated for 24 hours			
	In absence of Gepon		**In presence of Gepon for 24hours**	
	No Virus	**EMC Virus added**	**No Virus**	**EMC Virus added**
INF alpha mRNA	No	**Yes**	**Yes**	No
INF gamma mRNA	**Yes**	No	No	No
IL-1b mRNA	No	**Yes**	**Yes**	No
IL-2 mRNA	**Yes**	**Yes**	**Yes**	**Yes**
IL-4 mRNA	**Yes**	No	No	No
IL-6 mRNA	No	No	**Yes**	**Yes**
IL-8 mRNA	No	No	No	No
IL-10 mRNA	**Yes**	No	**Yes**	**Yes**
IL-12 mRNA	No	No	No	**Yes**
IL-18 mRNA	**Yes**	**Yes**	**Yes**	**Yes**
TNFa mRNA	**Yes**	No	**Yes**	**Yes**

EMC Virus added to the culture after 20 hours of incubation with Gepon, then incubated for a further 4 hours.

Yes: presence of cytokine mRNA

No: absence of cytokine mRNA

The effect of Gepon on the synthesis of cytokine mRNA in uninfected cells

The spectrum of cytokine mRNA synthesised in control cells and in cells incubated in the presence of Gepon was significantly different and this suggests that Gepon had a regulatory effect on cytokine gene expression in J-96 cells. In control cultures, in the absence of drug and virus, J-96 cells synthesised mRNAs for IFN-gamma, IL-2, IL-4, IL10, IL-18 and TNF-a. In J-96 cells, which had been incubated for 24 hours in the presence of Gepon, the spectrum of cytokine mRNAs included the additional synthesis of mRNA for IFN-a, IL-1B and IL-6, but production of mRNA for IFN-gamma and IL-4 was stopped. The effect of Gepon was to change the cells regulatory program of gene transcription that was coincident with the transition of the cells into a state of high resistance to EMC Virus infection.

The effect of Gepon on the synthesis of cytokine mRNA in virus infected cells

Under the influence Gepon, there was a transformation of the cytokine response of cells to viral infection. In response to EMC Virus infection, J-96 Cells started the transcription of mRNA IFN-alpha and IL-1b while the synthesis IFN-gamma, IL-4, IL-10 and TNF-alpha mRNA was stopped. Transcription of mRNAs for IL-2, IL-6, IL-8, IL12 and IL18 did not change during EMC Virus infection. Therefor in EMC Virus infected cells there were significant changes in the program of cytokine mRNA synthesis and this regulatory change was coincident with cells failure to oppose the virus infection.

When J-96 Cells were incubated for 24 hours in presence of Gepon and then infected with EMC Virus, the spectrum of transcribed cytokine mRNAs was different to that found in cells infected without previous incubation with drug. In this case where the cells had been previously treated by Gepon, and then infected by virus, it was found that the synthesis of mRNAs IL-2, IL-6, IL-10, IL-12, IL-18, TNF-alpha had been activated. The result of culturing with Gepon, was that neither did the cells respond on virus infection by synthesising INF-alpha and IL-1beta mRNAs nor did they cease to synthesise INF-gamma, IL-10 and TNF-alpha mRNAs.

Discussion

Cytokines are strong regulators of the condition of different cells and tissues. In some cases cytokines regulate the state of cells that produce them (an autocrine mechanisms). In some other cases with the help of cytokines, cells can affect their nearest neighbours in a tissue (a paracrine mechanism) or affect cells from other organs and tissues (an endocrine mechanism). The mutual effects between cells, mediated through cytokines can be quite complicated and in a system of cytokines, every cytokine can seriously affect the synthesis of many other cytokines and also affect its own synthesis. In the case where the synthesis program of cytokines is changing under the influence of Gepon, we suggest that the protection of J-96 cells from EMC Virus may happen as a result of both autocrine or paracrine cytokine effects. In first case, cells are changing the internal spectrum of cytokine synthesis under the influence of Gepon and changing themselves to become more resistant to the cytopathogenic effect of virus. In other case, through the modification of mRNA synthesis, the new cytokine 'cocktail' produced may affect other cells and bring them to state of high resistance to virus infection.

For instance in initial phase of viral infection, the synthesis IFN-alpha is stimulated which then induces increased synthesis of IL-12. IFN-alpha and IL-12 act synergistically to switch on IL2 and IFN-gamma synthesis. In the immune system, IL-2 activates T-cells and IFN-gamma activates monocyte/macrophages. In both cases, this increases mechanisms of protection from intracellular infections. This paper shows that Gepon regulates the synthesis of IL-6, IL-10, TNF-alpha, IFN-alpha and IL-1b. The reaction of J-96 cells to Gepon was very different to their reaction to EMC Virus. The fundamental difference between the Gepon effect and the virus effect was related to transcription of mRNAs for IL-6, IL-10 and TNF-alpha. In contrast, Gepon's effect and the virus effect on the synthesis of mRNA for IFN-alpha and IL-1b were similar. Therefor the increase of the resistance of the J-96 cells to virus infection was based on the modification of the synthesis mRNAs for IL-6, IL-10 and TNF-alpha but not INF-alpha and IL-1 beta. It is possible that this type of regulatory effect on cells in immune system increases the effectiveness of the antiviral protection by the immune system.

Generally the results of this work show only part of the mechanism behind the antiviral effect of Gepon. The modulation of the spectrum of synthesised cytokines induced by Gepon, can protect cells either by other direct influences of Gepon on the cell or by increasing infection resistance of other cells that been affected by the modified cytokine cocktail. Both effects could be taking place during Gepon's influence on J-96 cells *in vitro*. Under clinical conditions with the intake of Gepon into human body via mucus membranes, in addition to direct contact with Gepon leading to the increase of antiviral resistance in the mucus membrane, Gepon may systemically activate immune protection from infection by modulating the spectrum cytokine production in different organs and tissues. The influence of Gepon on the systemic spectrum of synthesised cytokines could result in the observed activation macrophages, NK-cells, cytolitic T-cells observed in clinical studies and which are probably responsible for enhancing the immunity that protects the human body and help it fight the infections.