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Induction of gene mutations by lectins of different origin and cytokine EMAPII in somatic mammalian cells *in vitro*

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Exogenous carbohydrate binding proteins, namely, three lectins of different origin and cytokine EMAPII, have revealed the ability to induce mutations in hprt locus in Chinese hamster cells in vitro. In the investigations of their biological action at the wide range of doses (from 0.002 to 2000 μg/ml) direct dependence of mutagenic effect on the concentration of exogenous protein in cellular system has been manifested. Mathematical modelling has shown that resulting curve consists of three processes: the first one proceeds with saturation, the second one – with linear dependence on the dose, and the third one is described by non-linear equation of the second degree. Mutagenic activity of lectins is realized according to the latter equation and characterized by gradual increasing of mutation frequency with the increase in the protein dose.

Keywords: mammalian cells, cytokine, lectin, resistance, mutations, hprt locus, apoptosis, mathematical modelling.

Introduction. Exogenous carbohydrate binding proteins under investigation are specific for various biological functions and capable of influencing intramolecular processes, namely, adhesion, migration, apoptosis, proliferation, and differentiation. Their influence is realized via numerous cell signalling pathways [1–3]. One of the least studied issues is the possible role of exogenous allogenic proteins in genetic processes, *i.e.* in mutagenic processes and processes of reparation of recipient cell DNA. Earlier we have shown the capability of one of *Sumbucus nigra* lectins to induce both primary DNA damages and transgenations [4–7]. EMAP II cytokine (endothelial and monocyte activating polypeptide II) is multifunctional mediator protein, specific for both cytokine- and RNA-binding functions. EMAP II is capable of initiating apoptosis, it is also the factor of antiangiogenesis in the development of tumour vessel system. It also performs several important functions in embryogenesis [8, 9].

The goal of current work was to investigate the regularities of effect of exogenous carbohydrate binding proteins, different in origin and carbohydrate specificity of lectins and EMAP II cytokine, on spontaneous mutagenic process in mammalian cells *in vitro*.

Materials and Methods. Chinese hamster cells of Blld-ii-FAF28Cl237-8Glu⁻tsIII cell line, sensitive to the effect of purine bases analogues, including 6-mercaptopurine (6-MP), were used in all experiments. Therefore, test-system applied allows studying induction of direct recessive mutations on hypoxanthine-guanine phosphoribosyl transferase locus (*hprt*).

Commercially available plant lectin preparations of *Sumbucus nigra* bark (SNL), *Lens culinaris* seeds (LCL) and those of animal origin – *Persa fluviatilis* spawn (PFL), produced by *Lectinotest*, Ukraine, were used as biological factors. EMAP II cytokine was expressed in *Escherichia coli* cells and purified to homogenous condition (not less than 95%) using the method of metal-chelating chromatography on Ni-NTA agarose, according to the description presented in [10]. Biological activity of proteins was investigated in the wide range of concentrations (from 0.002 to 2 000 g/mol). The cells were treated with each of the investigated proteins for 3–4 hours in MEM medium, free from bovine fetal serum (BFS). Alkylating agent N-methyl-N -nitro-N-nitrosoguanidine (MNNG), concentration of 500 ng/ml, was selected to be the positive control; the cells were treated for 1 hour in serumless medium [15]. The method of the experiments on induction of gene mutations on *hprt* locus is presented in the previous work [14]. Expression time was 3–4 days depending on the experiments. Selection of mutants was performed with 6-MP addition, 60 g/ml, to the cultural medium. The frequency of 6-MP-resistant clones was determined taking into account cloning efficiency.

To investigate inheritance and nature of 6-MP resistance, cell clones of independent origin, cultivated in different selective media, were gathered using sterile Cloning Disks (*Scienceware*, USA), transferred into the standard growth medium with 10% BFS, and cultivated for 20–25 passages. Next, cloning efficiency of cells of different investigated clones on different selective media, *i.e.* with 6-MP, 6-thioguanine (6-TG), 8-azaguanine (8-AG), and aminopterine in the concentrations of 60, 10, 30, and 0.003 g/ml, respectively, was analysed [16]. The results obtained were compared with data on cloning efficiency in growth medium and ATG medium (*PanEco*,Russia), which is suitable for cells with active or partially active enzyme hypoxanthine-guanine-phosphoribosyltransferase (HGPRT). The cells with direct mutations on *hprt* locus, resulting in HGPRT loss of its activity, are not capable of multiplying on ATG medium, yet they form colonies on other selective media.

Statistical processing of data obtained was performed according to Fisher's criterion and mathematical modelling of mutational process, induced by biological factors, was conducted.

Results and Discussion. The results of two experiments on biological activity of EMAP II cytokine in the range of 2–200 000 ng/ml were revealed to be very similar and thus were averaged. In all of these experimental concentrations, protein caused statistically reliable increase in the frequency of 6-MP resistance mutations.

The effect of protein in *C* concentration on mutagenesis in population of Chinese hamster cells on the example of direct mutations on *hprt* locus may be presented as a common effect of three biological processes with their own mechanisms:

$$F \quad \sum_{n\ 1}^{3} F_n \quad F_1 \quad F_2 \quad F_3 , \qquad (1)$$

where F – frequency of mutants in the experiment, F_1, F_2, F_3 – frequencies of mutants, obtained due to the effect of each separate process.

These three processes can be either dependent or independent from each other. The number of processes reviewed is sufficiently enough to get a certain idea on the interaction of cells with protein molecules, taking into account 10% measurement error.

The first process F_1 may result in mutagenic effect with the frequency saturation of F_{01} = 205 at the concentrations of C 2 g/ml:

$$F_1 \quad F_{01} \ (1 \quad e \quad), \qquad (2)$$

F_{01} – maximal possible value of mutation frequency at the given condition; = $ln(C)$ – function of protein concentration; *i.e.* according to this mechanism mutagenic effect is revealed in the concentration {C_1,

$C_2\}$, where $C_1 = 0.002$ g/ml and $C_2 = 2$ g/ml. For $C>C_2$ increase in concentration does not influence the degree of effect.

The second process results in mutagenic effect with linear dependence on in the whole range of investigated concentrations. Minus (–) indicates the decrease of effect at the increase in concentration. $F_{02} = 30$ indicates initial conditions; $f_{02} = 12$ – the value of spontaneous mutagenesis:

$$F_2 \quad (F_{02} \quad f_{02}). \qquad (3)$$

The third process is described by non-linear equation of the second order and in our case can be presented as quadratic dependence with parameter F_{03} = 2.5 and spontaneous mutagenesis $f_{03} = 12$:

$$F_3 \quad F_{03} \quad ^2 \quad f_{03}. \qquad (4)$$

F_{0x} values, where x = 1, 2, 3, may testify in favour of initial characteristics of the investigated cells and proteins. Comparative analysis of these parameters with the similar ones for other objects may supply new data on protein-cell interactions.

F mutant measurement error was accepted to be ±10% and unchangeable in all measured ranges.

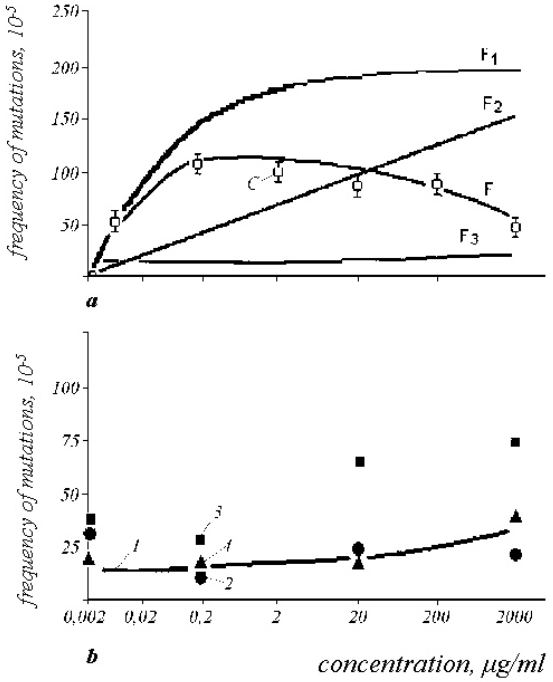
Combined formula (1) for all three processes is as follows:

$$F \quad F_{\exp} \quad F_{01} \ (1 \quad e^{\ \ln C}) \quad F_{02} \ \ln C \quad F_{03} \ (\ln C)^2. \quad (5)$$

In Figure, a, F_1, F_2, F_3 – indicate theoretically calculated separate dependence curves $F_n = f(C)$; F – the resulting curve.

Experimental data for cytokine are marked with special markers in Figure as well. Noteworthy, according to the model applied, processes start contributing to the common mutagenic effect at the lowest concentrations, *i.e.* from the initial values of the experiment, which testifies about independent activity of each of the processes at low concentrations.

However, negative effect in the case of F_2 testifies that there are the same cell targets for all three processes, which are the constituents of resulting manifestation of mutagenic effect and objects of competition for macromolecules.



Dependence of frequency of mutations, induced by EMAP II (*a*: theoretical calculations F_1–F_3 – mechanisms 1–3, F – resulting, experimental cytokine C) and by lectins (*b*: 1 – theoretically calculated curve for mechanism 3; *2 – S. nigra* lectins; *3 – L. culinaris* seeds; *4 – P. fluviatilis* spawn) in Chinese hamster cells, on protein concentration.

Further development of these ideas will allow determining molecular mechanisms of main biological processes. In Figure, *b*, the data on lectins of *S. nigra*, *L. culinaris*, and *P. fluviatilis* are presented for comparative analysis with effect of cytokine. The effect of lectins is notably lower than that of cytokine. F_3 may be considered the process of revealed mutagenesis for the investigated range of concentrations, hence, the obtaining of more or less significant effect requires higher concentrations than in the case of cytokine (Table 1).

All three lectins were shown to increase the frequency of 6-MP resistant clones in populations of Chinese hamster cells. Fig. indicates gradual increase in mutagenic effect depending on the concentration, while the character of this dependence was similar to that of the process, described by non-linear second-degree equation (process 3, according to the model). At

Table 1
Value of experimental and theoretical data on frequency of mutants, induced by EMAPII cytokine

C, ng/ml	ln*C*	F_{exp}	Modeled processes			
			F_1	F_2	F_3	$F \sum_{n1}^{3} F_n$
0	0,00	0,00	00,0	00,0	0,0	00,0
2	0,30	52,8	52,0	9,0	0,0	43,0
20	1,30	107,6	145,5	39,0	0,8	107,4
200	2,30	100,8	180,0	69,0	2,6	113,6
2000	3,30	87,9	192,6	99,0	5,4	99,0
20000	3,30	92,5	197,3	129,0	9,2	77,5
200000	5,30	50,9	199,0	159,0	14,1	54,0

the lowest investigated concentration of 0.002 g/ml the frequency of induced mutations did not differ much from that of control. Statistically reliable increase in the frequencies of mutants has been noted in all three lectin cases at higher concentrations. Mutagenesis, induced by lectins, can be compared to the mutagenic effect of chemical super-mutagen MNNG. Induced apoptosis has been observed at lectin concentrations, resulting in statistically reliable mutagenic effect [17]. Possibly there is more than one way of protein influencing genetic stability of mammalian cells in the mentioned model and at current conditions [18]. Further development of these ideas may allow revealing molecular mechanisms of genetic processes, induced by proteins.

The results obtained were in good correlation with rare literature data on capacity of some growth factors and cytokines to induce genetic damages in mammalian cells [19]. We have studied the dependence of protein mutagenic effect on their concentration and performed mathematical modelling of mutagenic process.

In the course of investigation we have selected and analysed 11 6-MP-resistant cell clones (Table 2). Control sample of Chinese hamster cell clone was expectedly shown to be sensitive to all analogues of purine bases and multiplied on selective ATG medium at the same time, which is a common behaviour pattern for cells with active HGPRT enzyme. The cells of all investigated 6-MP-resistant clones preserved this feature after long time cultivation in the growth medium, *i.e.* all of them were mutants. All analysed mutant clones were capable of multiplying not only in 6-MP-containing medium but also demonstrated cross-resistance to other purine bases analogues. All but MP31 and MP34 did not multiply on ATG and aminopterine media, which demonstrated the absence of enzymatic activity of HGPRT (direct *hprt* mutations).

The behaviour of MP31 and MP34 was rather abnormal, *i.e.* they not only demonstrated cross-resistance to purine base analogues but also formed the colonies in aminopterine medium and ATG selective medium. The authors suppose that the reason is mutation of resistance to aminopterine in the cells of these two clones.

Thus, all obtained and analysed 6-MP resistant clone cells inherited this feature and revealed cross resistance to different purine base analogues. This fact testifies that resistance of clone cells to 6-MP is conditioned by mutation. In the majority of mutant clones this mutation resulted in complete loss of HGPRT enzymatic activity. Clarifying the nature of the aforementioned mutations requires sequencing of complete nucleotide sequence of *hprt* encoding part.

Therefore, the dependence of mutagenic effect on the concentration at EMAPII cytokine influence is of a more complicated character, compared to lectins. Biological effect of the latter is characterised by gradual increase in the frequency of mutants depending on in-

Table 2
Cloning efficiency analysis of control and 6-MP resistant clone cells of Chinese hamster in growth and selective media

Clone No.	Agent, concentration, g/ml	Cloning efficiency of control and selective media, %					
		Growth medium	6-MP, 60 g/ml	6-TG, 10 g/ml	8-AG 30 g/ml	Aminopterine 0.003 g/ml	ATG
MP30	–	66,00	63,45	65,40	75,00	0,00	00,0
MP37	–	39,50	34,15	42,35	28,65	0,00	00,0
MP31	LCL, 20	30,80	33,45	23,65	30,80	1,60	1,40
MP32	LCL, 20	44,50	38,52	31,25	43,45	0,00	0,00
MP34	SNL, 0.2	63,50	60,02	68,50	65,80	0,15	0,80
MP39	PFL, 200	62,01	58,32	60,53	59,20	0,00	0,00
MP40	PFL, 20	65,60	63,00	53,70	70,40	0,00	0,00
MP41	EMAPII	47,40	54,30	49,65	52,80	0,00	0,00
MP42	EMAPII	66,10	51,20	53,21	57,00	0,01	0,06
MP44	EMAPII	51,60	41,60	52,30	56,20	Data is absent	Data is absent
MP36	MNNG, 0.5	47,35	42,15	47,80	44,55	0,00	0,00
Output cell line	–	68,32	0,00	0,00	0,00	0,00	57,14

crease in dose. This dependence is described by non-linear equation of the second degree. Mutagenic activity of lectins is correlated with their capability to induce apoptosis in Chinese hamster cells.

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Индукция генных мутаций лектинами разного происхождения и цитокином EMAP II в соматических клетках млекопитающих *in vitro*

Резюме

Экзогенные углеводсвязывающие белки – три лектина разного происхождения и цитокин EMAP II проявляют способность индуцировать мутации по локусу hprt в клетках китайского хомячка in vitro. При исследовании их биологического действия в широком диапазоне доз (от 0,002 до 2000 мкг/мл) выявлена прямая зависимость мутагенного эффекта от концентрации чужеродного белка в клеточной системе. Как показало математическое моделирование, результирующая кривая зависимости частоты мутантов от концентрации для EMAP II является составляющей трех процессов: первый протекает з насыщением, второй – с линейной зависимостью от дозы, а третий описывается нелинейным уравнением второй степени. Мутагенная активность лектинов реализуется по последнему уравнению и характеризуется постепенным повышением частоты мутантов с увеличением дозы белка.

Ключевые слова: клетки млекопитающих, цитокин, лектин, резистентность к 6-меркаптопурину, мутации, локус hprt, апоптоз, математическое моделирование.

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