A COMBINED EFFECT OF PSEUDORABIES VIRUS GROWTH FACTOR /PRGF/ AND VARIOUS CYTOSTATICS ON TUMOUR /Hep - 2/ CELLS IN VITRO

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Abstract

Kocáková P., J. Leško, K. Horáková, F. Golais: *A Combined Effect of Pseudorabies Virus Growth Factor (PRGF) and Various Cytostatics on Tumour (Hep-2) Cells in vitro.* Acta vet. Brno 1997, 66:159-169.

One of the activities of pseudorabies virus /PRV/ growth factor /PRGF/ is its ability to change the transformed phenotype of cultured tumour cells to the normal one.This activity of PRGF was studied in combination with antiproliferative activity of various cytostatics. An undesirable outcome was observed, when PRGF was combined with 11 out of 12 cytostatics. These cytostatics suppressed the ability of PRGF to change the transformed phenotype of Hep-2 cells, and on the other hand, PRGF diminished the effect of these cytostatics on growth and metabolism (DNA and protein content, glucose consumption, lactate and ammonia production) compared to non-treated control cells. This effect was not observed when the cells were cultured in the presence of PRGF and methotrexate (MTX). In this case both suppression of transformed phenotype and inhibition of cell growth were observed. Taking these results into account, PRGF combined with MTX is discussed as a possible method for cancer studies.

Pseudorabies virus growth factor, cytostatics, transformed phenotype, cell proliferation

Pseudorabies virus growth factor (PRGF) was detected in our laboratory in some virus infected or transformed cells. This factor has been shown to have two effects on cultured cells *in vitro*. Normal, non-transformed cells, when cultured in the presence of PRGF acquired the appearance of transformed cells (criss-cross morphology and anchorage independent growth). However, the phenotype of transformed cells cultured with PRGF became converted towards the normal one (Golais et al. 1990; Golais et al. 1992; Dušinská et al. 1994). In this paper, the effect of PRGF on morphology, growth and metabolism of human carcinoma (Hep-2) cells was studied along with 12 cytostatics in attempts to find an appropriate combination for a possible cancer treatment.

Materials and Methods

Preparation of PRGF

Non resolved PRGF (Gašperík et al. 1994) was used. Monkey kidney (MK-2) cells grown in Eagle's basal medium (BEM) with 5 % heated bovine serum, were infected with TOP strain of PRV at input multiplicity of 1 PFU/cell. To remove the virus, the medium from infected cultures was harvested 3 days p.i. acidified to pH 3, kept for 3 days at 4 °C and then raised again to neutrality (pH 7.2). Five ml aliquots of the medium were then lyophilized, resuspended in 0.5 ml phosphate buffer (pH 7.2) and applied to a column (3,5 x 40 cm) of Sephadex G 15. The column was washed with deionized water at a constant flow rate 1 ml /min. The samples from the column collected in 5 ml aliquots were tested in MK-2 cells for transforming and in Hep-2 cells for transformed phenotype repressing activity (for details see Golais et al.1990). Positive samples were lyophilized, resuspended in 0.2 ml deionized water and applied onto column of Sephadex G 75.

The column (3 x 30 cm) was washed with deionized water at a constant flow rate 0.2 ml /min. Collected samples in amounts of 1 ml were tested again in MK-2 and Hep-2 cells. Positive samles were pooled, lyophilized, resuspended in 0.5 ml deionized water and concentrated on the Biogel P 20 column. The separation pattern on

Sephadex G 15 and Biogel P 20 columns have been given in G o l a i s et al. (1992). Biogel P 20 concentrated PRGF samples served as stocks for other studies.

Cultivation of cells in the presence of PRGF and cytostatics

Hep-2 cells were seeded into 25 cm²/ 50 ml plastic NUCLON bottles (2.5 - 3x10⁵ cells per bottle) and cultivated for 24 h in BEM containing 5% heated bovine serum, glucose (1g/L) and glutamine (300 mg/L). Afterwards, the medium was removed and replaced with the same medium containing cytostatics in chosen concentrations (see Table 1) and 10⁴ PRGF units /ml (for definition of one PRGF unit see G o l a i s et al. 1992). A part of cultures received medium containing cytostatics without PRGF, or medium with PRGF without cytostatics. Control cells received intact medium without cytostatics and PRGF. 6-thioquanine (6-TG) as recommended (T i d d et al. 1972) was removed following 24 h incubation and replaced with normal medium.

Table 1
Cytostatics used in combination with PRGF

Drug	Producer	Optimal concentration
		(μM)
Carboplatin	Lachema	200
Doxorubicin	Lemery	90
6-Thioquannine (6-TG)	Sigma	2
Cytosar (Cytarabine)	Upjohn	50
Fluoro-uracil	Roche	70
Ifosfamid	Asta	380
Ethyl-isothiocyanatobutanoate (E-4 IB)*	STU	1,5
Uromitexan	Asta	300
Vincristine	Lemery	6
Taxol	Bristol Mayers Squibb	5
Etoposide	Pierre Fabre	5
Methotrexate	Ebewe	5,5

*E-4 IB was synthetized by F l o c h and K o v á č (1975), and an antiproliferative activity of this compound was demonstrated by H o r á k o v á et al. (1993).

Cells cultured for 24 h in the presence of PRGF and 6-TG received only PRGF following 6-TG removal. To maintain equal initial conditions (pH, serum and glucose content etc.) in all cultures, the media of other samples were changed along with those containing 6-TG, however, the cytostatics were added again. All cultures were then cultivated for 3 days at 37 °C before tested for DNA and protein content as well as for glucose consumption and production of ammonia and lactate.

To test the anchorage independent growth of cells cultured in the presence of PRGF and cytostatics, 0.5 ml aliquots of 0.7% agar (DIFCO) in Earle's medium with 5% heated fetal bovine serum were dispensed into 2 cm² wells of flat-bottom plates (NUCLON) and overlayed with 0.5 ml aliquots of 0.35 % agar in the same medium containing 2 x 10⁴ Hep-2 cells. Both agar layers contained either PRGF itself (1x10⁴ unit /ml), or combined with cytostatics in concentrations given in Table 1. Control wells contained cells cultivated in agar without PRGF and cytostatics. The cells cultivated at 37 °C were controlled after 18 days for formation of colonies.

Growth curves

The cells cultivated in 25 cm² /50 ml NUCLON bottles were trypsinized at 24 hrs intervals using „dry" trypsinization method (H a y f l i c k and M o o r h e a d 1961), resuspended in equal amounts of serum containing BEM (1 ml per bottle), counted in a haemocytometer and their number was expressed in cells/ml. Viable cell counts were determined by the trypan blue exclusion method. The number of cells from samples kept in cultures till the last interval was expressed as saturation density (cell /cm²) and was further used for estimation of doubling time of culture population (P a t t e r s o n 1979).

Staining technique
The cell grown in 60 mm FALCON Petri dishes for 48 hrs were rinsed with phosphate buffer (pH 7.2), fixed with methanol and stained with Giemsa according to the method described by Freshney (1987).

Cell metabolism and cytotoxicity studies
The cells cultivated in the presence of PRGF and cytostatics as described in this section were trypsinized and the number of cells was determined. One portion of cell suspensions was used for estimation of protein content by Lowry method (Freshney 1987). Another part of suspension was then frozen and thawed three times at 70 °C to disrupt cells and the nucleic acid were extracted using procedure described by Munro and Fleck (1966). The DNA content of the extract was estimated by a shortened diphenylamine method (Gendimenico et al. 1988). The media of all samples were clarified by low-speed centrifugation (15 min at 180 r.p.m) and tested for glucose uptake (Dahlquist 1964), as well as for lactate (Horost et al. 1974) and ammonia (Bolleter et al. 1961) production. The probable percentage of conversion of glucose into lactate was calculated according to the formula:

$$\frac{\text{lactate production}}{\text{glucose consumption x 2}} \text{ x 100.}$$

Results

The effect of PRGF and cytostatics on transformed phenotype of Hep-2 cells

PRGF itself, when present in the medium of cultured Hep-2 cells supressed the appearance of transformed phenotype („criss-cross" pattern of growth) confirming previously obtained data (Golais et al. 1990; Golais et al. 1992), see Plate XI., Fig.1a,b.

An interesting phenomenon was observed, when PRGF was combined with 11 out of 12 cytostatics. These cytostatics interfered with the transformed phenotype repressing activity of PRGF, so that the cells retained the appearance of transformed control cells (Plate XI., Fig. 1c, Plate XII., Fig. 1d).This undesirable interference was not observed, when MTX was used in combination with PRGF (Plate XII., Fig.1e).

Similarly, when PRGF and each of 11 cytostatics were present in soft agar, the colonies of transformed cells appeared having the size resembling that of the control cells. On the other hand, a supression of the formation of colonies was observed, when this agar contained either PRGF alone or combined with MTX. The effect of cytostatics without PRGF on the formation of colonies was not studied.

Growth characteristics of cells treated with PRGF and cytostatics

All cytostatics, when present in the medium decreased the saturation density of Hep-2 cells to about 55 - 80% and brought about a 15 - 40% prolongation of their doubling time as compared to the control cells cultured in intact medium. Both these effect were diminished when 11 cytostatics (with the exception of MTX) were added to the cultures simultaneously with PRGF. In this case, the decrease of the saturation density, as well as the doubling time prolongation were slightly reduced (see Table 2).

Even though MTX showed most marked effect on the cell growth, its effect was not significantly influenced by PRGF. PRGF, present without cytostatics slightly stimulated the growth of Hep-2 cells. Subsequently, 6-TG, E- 4IB and MTX were chosen for more detailed studies. As follows from the growth curves depicted in Fig. 2, PRGF in spite of this transformed phenotype repressing activity enhanced the growth of Hep-2 cells and the same did in cells cultivated in the presence of 6-TG and E-4 IB (Fig. 2 a,b), however, in this case, as already mentioned (see Fig.1), its ability to repress transformation was lost. Different from other cytostatics, the inhibition of cell growth by MTX was not significantly influenced by PRGF (Fig. 2).

Table 2

Saturation densities and doubling times of Hep – 2 cells cultivated in the presence of PRGF and cytostatics

Cytostatics	Saturation density cells x $10^3/cm^2$	Doubling time hrs
Control	52±1.5	57
PRGF	64±1.7	49
6-TG	36±1.9	75
6-TG + PRGF	40±1.0	69
E-4 IB	34±1.7	80
E-4 IB + PRGF	38±1.8	72
MTX	28±1.7	98
MTX + PRGF	29±1.6	94
Carboplatin	37±1.2	74
Carboplatin + PRGF	41±1.6	67
Doxorubicin	35±1.1	78
Doxorubicin + PRGF	42±1.3	66
Ifosfamid	38±1.4	72
Ifosfamid + PRGF	42±1.1	66
Uromitexan	37±1.7	74
Uromitexan + PRGF	42±0.8	66
Taxol	32±1.8	85
Taxol + PRGF	35±1.3	78
Fluorouracil	36±1.8	75
Fluorouracil + PRGF	40±1.1	69
Cytarabine	37±1.5	74
Cytarabine + PRGF	40±1.1	69
Vincristine	31±1.2	87
Vincristine + PRGF	34±1.5	80
Etoposide	36±1.8	75
Etoposide + PRGF	40±0.9	69

Cells were trypsinized and counted after entire 120 hrs cultivation. Mean values taken from 5 repeated experiments.

Estimation of metabolic variables

To characterize the cytostatic and cytotoxic effect of 6-TG, E-4 IB and MTX combined with PRGF in greater detail, the changes in the amount of DNA and proteins, as well as those in the glucose and glutamine metabolism, resulting from exposure of the cells to these drugs were measured.

The treatment of cells with 6-TG and E-4 IB was accompanied with the increase in the cellular DNA content and PRGF interfered with this effect. On the other hand, MTX-induced decrease of DNA content. PRGF alone, reduced the amount of DNA in Hep-2 cells to about 60% (Fig. 3a).

A considerable increase in protein content was demonstrated after treatment of cells with E-4 IB and MTX. PRGF was able to abolish this effect when acted along with E-4 IB however, it was not able to influence the protein increase in MTX-treated cells. Less marked
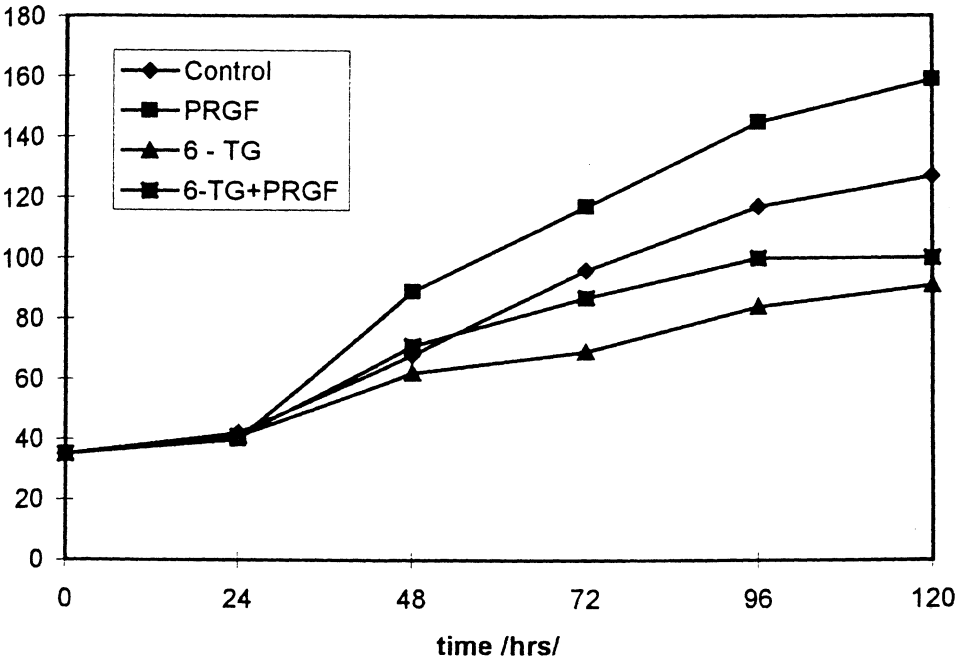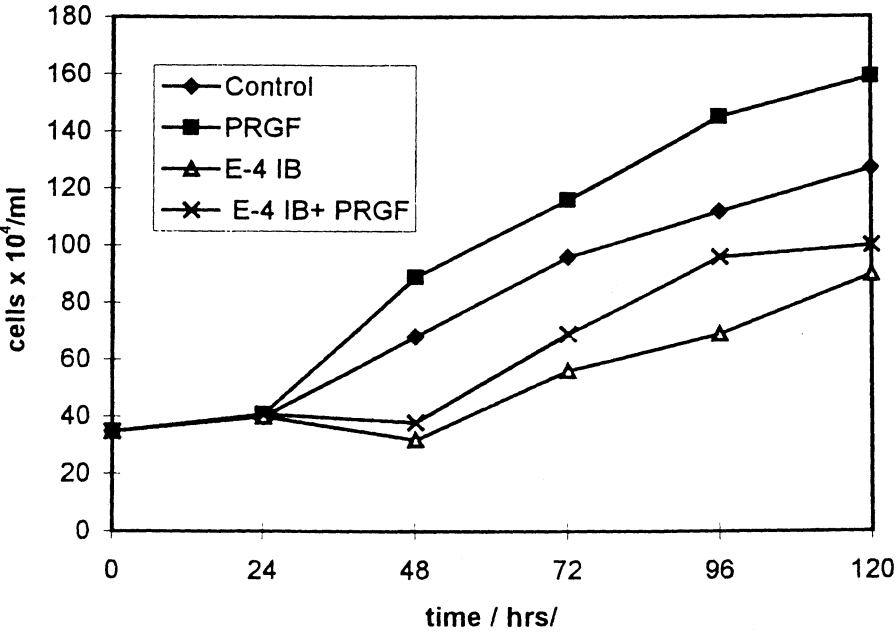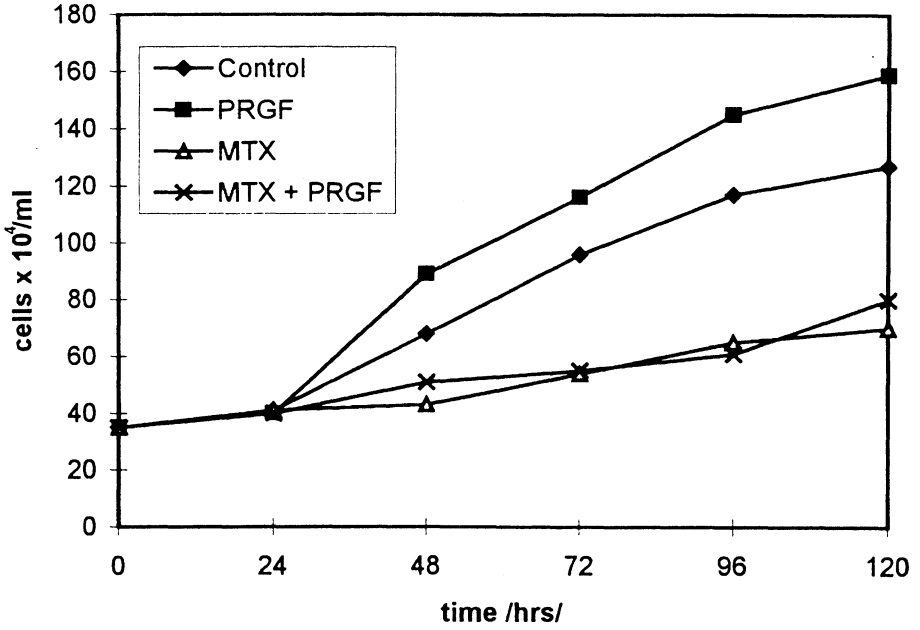
Fig. 2a

Fig. 2b

Fig. 2c

increase in protein content, which could be similarly abolished by PRGF was demonstrated in 6-TG treated cells. PRGF, present in medium without cytostatics had no effect on protein synthesis (Fig. 3b).
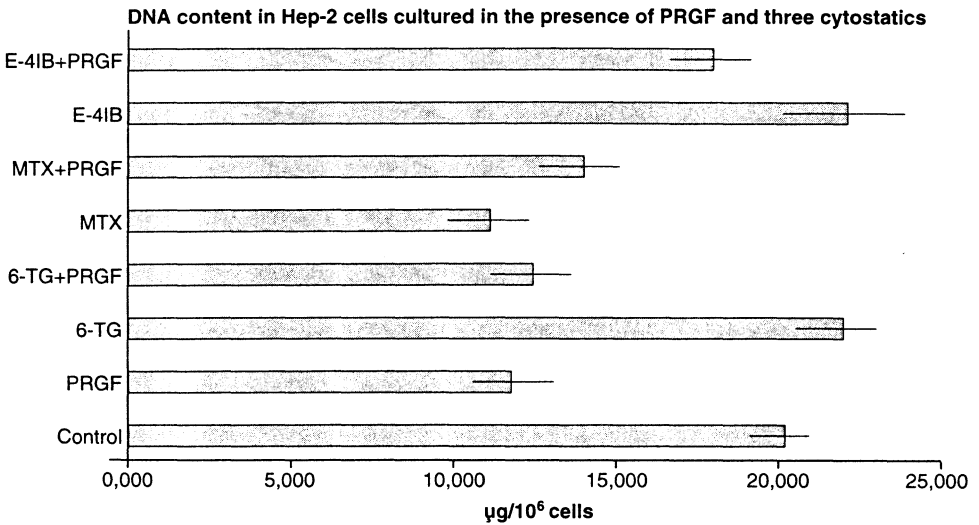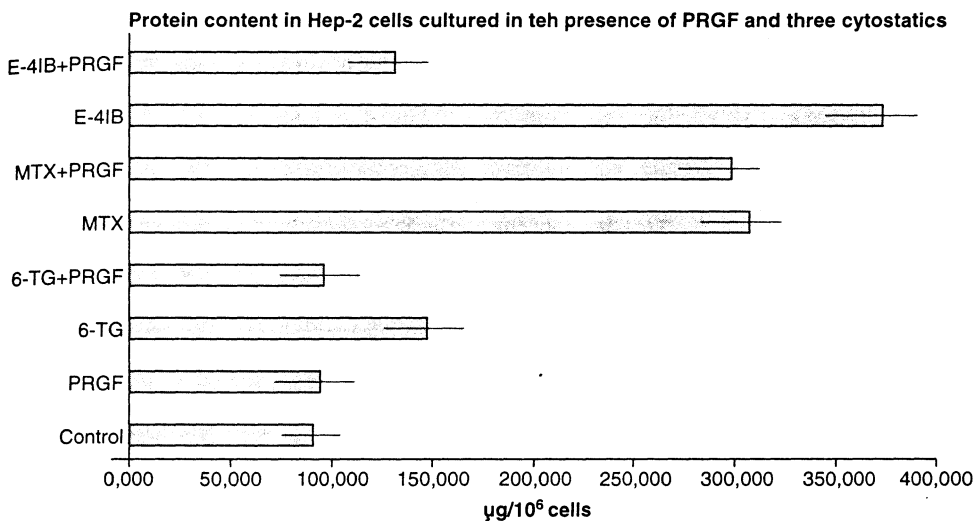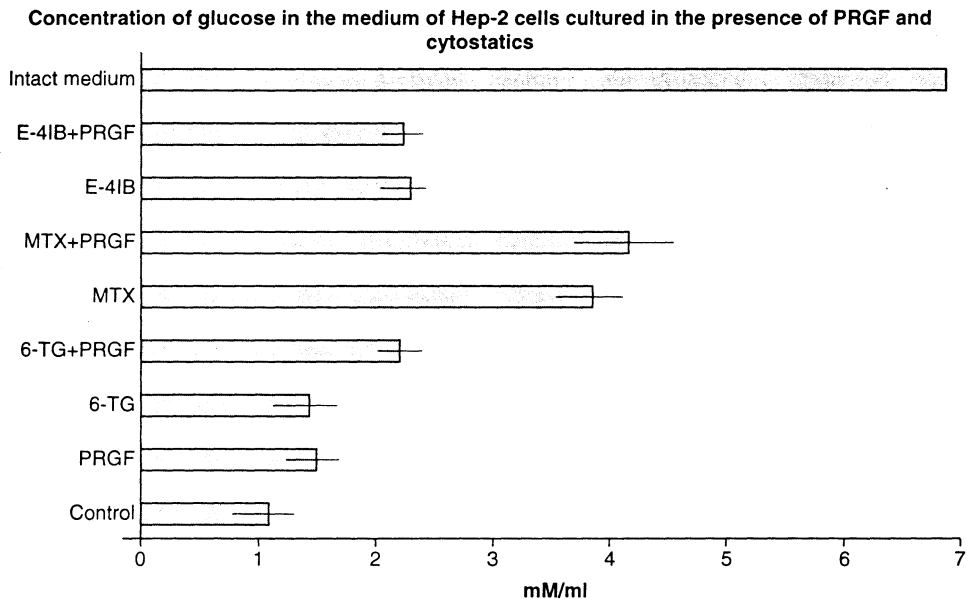
Fig. 3a

DNA content in Hep-2 cells cultured in the presence of PRGF and three cytostatics

Fig. 3b

Protein content in Hep-2 cells cultured in teh presence of PRGF and three cytostatics

Cells treated by MTX used about 50% less glucose from the culturing medium during the whole incubation period than control cells, whereas glucose uptake in E-4 IB treated cells was reduced only to about 80% or was not significantly changed in 6-TG treated cells. PRGF alone, or combined with these three cytostatics did not substantially affect the glucose consumption (Fig. 4). Lactate production, as well as conversion of glucose into lactate are

Fig. 4

Concentration of glucose in the medium of Hep-2 cells cultured in the presence of PRGF and cytostatics

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summarized in Table 3. As follows from Fig. 5 where the lactate concentration was expressed in mM/ 10^6 cells, MTX enhanced markedly the lactate production and a similar but less marked enhancement was observed in 6-TG and E-4 IB treated cells. PRGF retained this effect, however not influencing the lactate production in cells without cytostatics. Similar results were obtained when ammonia production was measured (see Fig. 6).
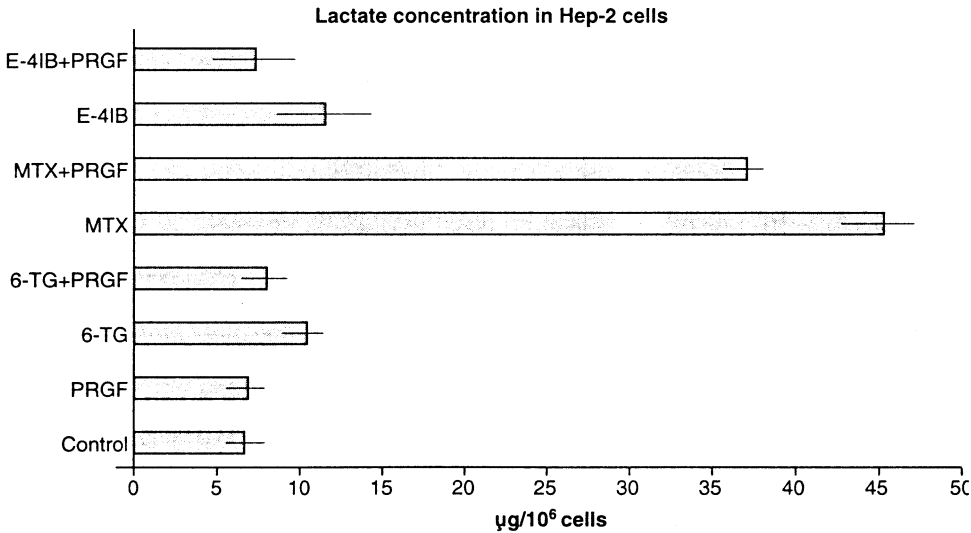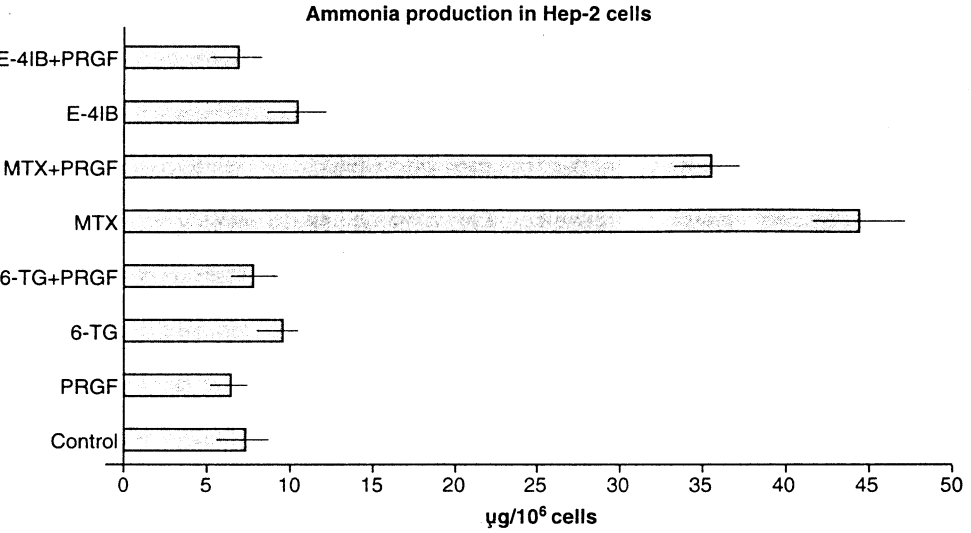
Fig. 5

Lactate concentration in Hep-2 cells

μg/10^6 cells

Fig. 6

Ammonia production in Hep-2 cells

μg/10^6 cells

Table 3

**Glucose metabolism of Hep – 2 cells cultivated in the presence
of PRGF and three cytostatics**

	Glucose consumption	Lactate production	Conversion of glucose into lactate
	mM/ml	mM/ml	%
Control	5.8 ± 0.3	8.6 ± 0.3	74.0
PRGF	5.5 ± 0.2	10.9 ± 0.2	100.5
6 – TG	5.5 ± 0.4	9.5 ± 0.3	87.1
6 – TG + PRGF	4.7 ± 0.2	8.0 ± 0.2	85.2
MTX	3.1 ± 0.3	16.6 ± 0.2	267.0
MTX + PRGF	2.7 ± 0.1	16.4 ± 0.3	292.0
E – 4 IB	4.6 ± 0.2	7.3 ± 0.2	79.1
E – 4 IB + PRGF	4.7 ± 0.3	5.7 ± 0.4	61.4

Discusssion

An undesirable mutual interaction was observed, when PRGF was combined with 11 out of 12 cytostatics and added to tumour Hep-2 cells. These cytostatics reversed the ability of PRGF to repress the transformed phenotype of cells, and on the other hand, PRGF decreased their cytostatic and cytotoxic effect.

An increase in DNA and protein synthesis is often observed after treatment of cells with some cytostatic drugs especialy with those which interfere with the synthesis of nucleic acids and proteins as a counsequence of unbalanced cell growth (C o h e n and S t u d z i n s k i 1967; H o r á k o v á et al. 1974; R o s s 1983; S a v w i c k a et al. 1986; H o r á k o v á et al. 1988). Both 6-TG and E-4 IB increased the synthesis of DNA and proteins in Hep-2 cells confirming previously obtained data (T i d d et al. 1972; H o r á k o v á et al. 1978; H o r á k o v á et al. 1993) and PRGF repaired these changes. Similarly, the increase in lactate and ammonia production brought about by 6-TG and E-4 IB treatment was supressed by PRGF. At present we have no explanation for these fimdings. This mutual relationship between PRGF and 11 cytostatics has to be studied at molecular level.

PRGF, present in the medium of cultured Hep-2 cells without cytostatics did not significantly influence the lactate and ammonia production, or the protein synthesis. However, it decreased the DNA content as compared to that of control cells cultivated without PRGF and cytostatics, in spite of its growth stimulating activity (Fig. 2). About 40% decrease in DNA content (see Fig. 3) might be due to the loss of the transformed phenotype of Hep-2 cells mediated by PRGF.

The results obtained with MTX were markedly different from those obtained with 6-TG, E-4 IB and other cytostatics. MTX was shown not to interfere with the transformed phenotype repressing activity of PRGF and on the other hand, its cytostatic effect in Hep-2 cells was not impaired by PRGF. As different from 6-TG and E-4 IB, DNA content in MTX treated cells was lower as compared to controls,which may be due to purineless and thymidineless state in cells owing to the depletion of tetrahydrofolates by inhibition of dihydrofolate reductase (F r i d l a n d 1974; J o h n s t o n et al. 1995). This effect was slightly repaired by PRGF. Elevated ammonia production in MTX treated cells might be

due to the reduced glucose uptake seen in Fig. 4, which might lead to increased glutamine utilization (B u t l e r and S p i e r 1984; L a n k s 1987). In spite of the reduced glucose consumption, the conversion of glucose to lactate was considerably enhanced (up to 260%). Thus, in the presence of MTX some other carbon compounds probably lipids (G e y e r, 1967), or amino acids (Z i e l k e et al. 1978; H o r á k o v á et al. 1989) might be converted into lactate. The values of ammonia and lactate cocentrations were lower, although still considerably remote from the control levels in non-treated Hep-2 cells, when MTX was combined with PRGF (see Fig. 5 and 6).

As follows from the results presented in this paper, MTX along with PRGF might represent an appropriate combination for the treatment of cancerogenic cells. As our in vitro studies showed, the cytostatic effect of MTX may be deapened by the loss of the transformed phenotype caused by PRGF, which may moreover slightly reduce the cytotoxic changes brought about by MTX. However, there is one problem concerning PRGF, which might hinder its successfull application to cancer. In addition to the transformed phenotype repressing activity, PRGF transforms normal, non-transformed cells (G o l a i s et al. 1990; D u š i n s k á et al. 1994). Although the oncogenicity of PRGF in organism was not demonstrated as yet, PRGF was only shown to facilitate the growth and postnatal development of newborn mice and rats, when administered subcutaneously without inducing tumours or pathological changes (C s a b a y o v á et al. 1995), it would be still riskfull to use PRGF for the treatment of tumours. It appears to be a general feature of most (if not all) herpesviruses to code under certain culture conditions for growth factors as shown in our last study (G a š p e r í k et al. 1996). Some of them could be divided into two, some other into three active components. Especialy the „middle" B component of the three-component factors might be of value for our purposes. These components are devoid of the transforming activity possessing only an ability to repress the transformed phenotype (G a š p e r í k et al. 1996). Thus these components might become appropriate candidates for a successfull application to cancer e.g. combined with MTX. This will be an aim of our future studies.

Vplyv rastového faktora vírusu pseudorabies (PRGF) a rôznych cytostatík na nádorové Hep-2 bunky in vitro

Jednou z aktivít rastového faktora vírusu pseudorabies (PRGF) je schopnosť meniť transformačný fenotyp pestovaných nádorových buniek na normálne. Táto aktivita PRGF sa študovala v kombinácii s antiproliferatívnou aktivitou rôznych cytostatík. K nežiadúcim výsledkom sme dospeli u 11 z 12 cytostatík. Tieto potlačovali schopnosť PRGF meniť transformačný fenotyp Hep-2 buniek, znižovali rast buniek a mali vplyv aj na ich metabolizmus (obsah DNA a bielkovín, spotrebu glukózy a tvorbu kyseliny mliečnej a amoniaku) v porovnaní s neovplyvnenou kontrolnou skupinou. Tento vplyv nebol pozorovaný pri kultivácii buniek Hep-2 v prítomnosti PRGF a Metotrexatu. V tomto prípade bola pozorovaná aj schopnosť transformovať nádorové bunky ako aj inhibícia rastu buniek. Diskutovaná je možnosť použitia kombinácie PRGF a Metotrexatu pri štúdiu nádorov.

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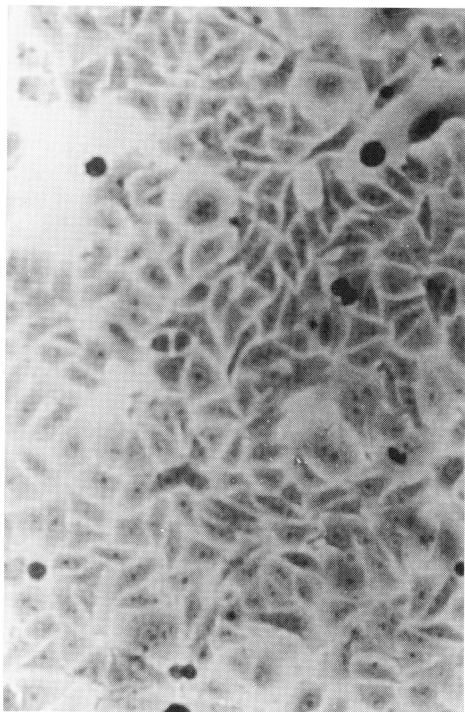842 15 Bratislava
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Plate XI.
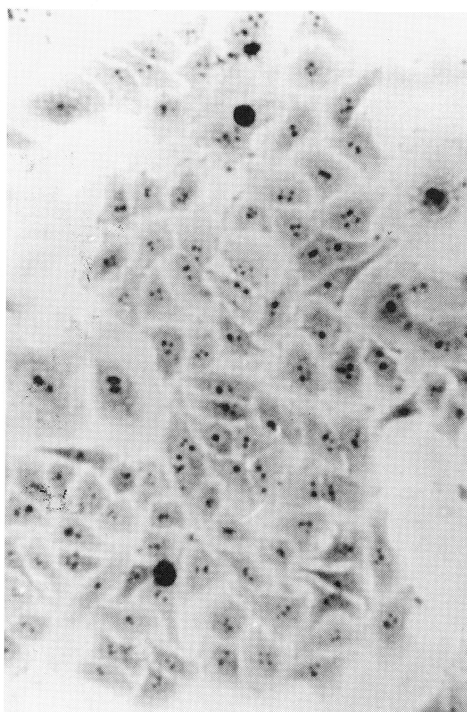Kocáková P. et al.: A combined... pp. 159-169

Fig.1a: Control Hep-2 cells (× 400)

Fig.1b: Hep-2 cells cultivated in the presence of PRGF (× 400)
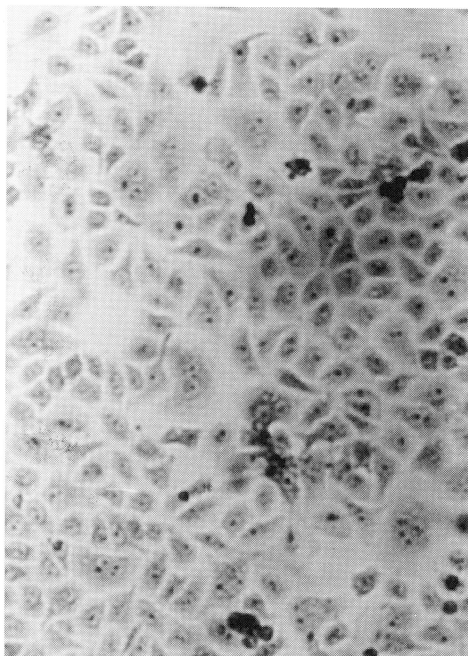
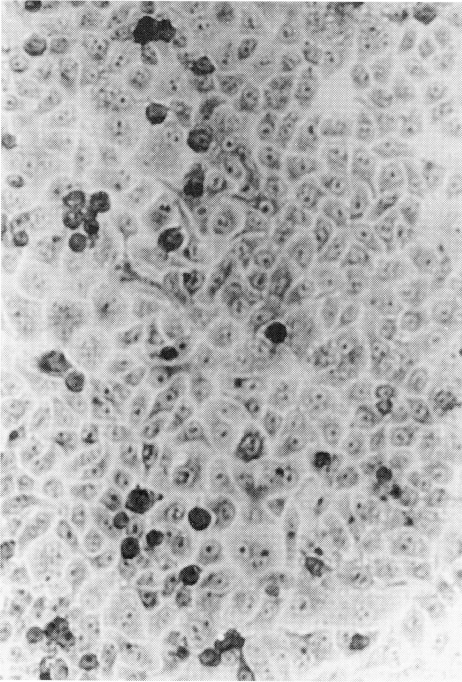Fig.1c: Hep-2 cells cultivated in the presence of E-4 IB (× 400)

Plate XII.

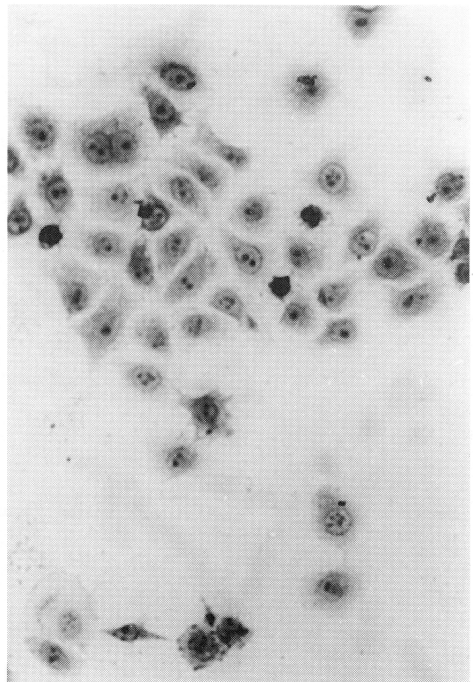Fig.1d: Hep-2 cells cultivated in the presence of E-4IB and PRGF (× 400)

Fig.1e: Hep-2 cells cultivated in the presence of MTX and PRGF (× 450)