INCREASED EFFECTS OF TOPICALLY APPLIED INTERFERON ON HERPES SIMPLEX VIRUS-INDUCED LESIONS BY CAFFEINE

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Summary. – Caffeine (Cf, 0.15 – 0.6 mg/ml) and human leukocyte interferon (IFN, >5 x 10^2 IU/ml) partially inhibited the replication of herpes simplex virus type 1 (HSV-1) in human diploid LEP cells and HaCaT cells. When the two drugs were applied simultaneously the inhibitory effects exceeded those achieved with either substance alone. In the subsequent clinical trial, which was conducted as a one-center, randomised, and placebo-controlled study, the same substances included in either oinment or solution were used for topical treatment of recurrent herpetic lesions. Of 115 patients taken into the study 20 were treated with Cf only (50 mg/g of oinment or solution), 25 with IFN only (5 x 10^4 IU/g), 25 with mixture of Cf and IFN (same concentrations) and 45 with placebo. Both the healing time (HT, period between the prodromes and reepithelization) and the spreading time (ST,period between the prodromes and the appearance of the last new lesion) were recorded in each patient. While the placebo effects were negligible, the other treatments tended to abort the lesions. HT was shortened by at least 4 days in 75% of patients treated with Cf alone, in 88% of those treated with IFN alone, and in all patients treated with their mixture. The effects on ST were less marked; in the case of Cf alone they were negligible. A shortening of ST by at least 2 days was recorded in 40% and 60% of patients treated with IFN alone and the drug mixture, respectively. Statistical analysis confirmed that both in terms of HT and ST the disease was more significantly alleviated by the mixture of Cf and IFN than by either drug alone.

Key words: herpes simplex virus type 1; lesions; interferon; caffeine

Introduction

Recurrent HSV lesions continue to represent a major health problem. In the past both peroral and topical treatments including a variety of substances have been utilized with varying success. Topical application of solutions or oinments seems to be increasingly appealing because it allows the use of high local concentration of such substances which cannot be achieved by systemic administration. The state of the art has been reviewed by Spruance and Freeman (1990).

Abbreviations: Cf = caffeine; HSV-1 = herpes simplex virus type 1; HT = healing time; IFN = interferon; MOI = multiplicity of infection; p.i. = post infection; ST = spreading time

In the present study we utilized observations made when studying the *in vitro* systems of HSV latency established in human embryo lung cells by treatment with (E)-5-(2'-bromovinyl)-2'-deoxyuridine and human leukocyte IFN (Wighdahl *et al.*, 1982). In these experiments, we have recognized a number of factors which have either facilitated or blocked the reactivation of the virus (Vonka *et al.*, 1985). In an extension of these experiments we subsequently observed that the reactivation was efficiently blocked by Cf at a concentration that was non-inhibitory to HSV replication (Vonka V., unpublished data). These findings have stimulated further investigation to determine the capacity of Cf to potentiate the action of various substances with antiviral effect. In this paper we report on the ability of Cf to enhance the inhibitory effects of human IFN on HSV replica-

tion both *in vitro* and *in vivo*. The latter study was conducted as a single-center, randomised, double-blind, placebo-controlled trial with a topical application of oinments or solutions containing IFN and/or Cf.

Materials and Methods

Tissue cultures. Human diploid embryo lung (LEP) cells were cultivated and maintained as described previously (Kutinová *et al.*, 1973). Vero cells were obtained from Dr. J. Rajčáni, Institute of Virology, Bratislava, and the spontaneously immortalized aneuploid human keratinocyte HaCaT cells (Bonkamp *et al.*, 1988) were kindly provided by Dr. N.E. Fusenig, Heidelberg. In our laboratory, they were cultivated in the same medium as LEP cells.

Virus. HSV-1 strain KOS was the same as in previous experiments (Holý *et al.*, 1973). Virus stocks were prepared in Vero cells.

Drugs. Human leukocyte IFN was prepared at the Institute of Virology, Bratislava, as described by Cantell and Hirvonen (1977). The activity of the semipurified IFN preparation was assayed in antiviral tests by the CPE inhibition method using primary human lung fibroblasts and vesicular stomatitis virus. In the present study, the IFN batches B-84-10 and CH-88-1,2,3 with the specific activity of 10^6 IU/mg of protein were used. The lyophilized preparation was kept at 4 °C. For tissue culture experiments, it was dissolved in water, diluted 1:10 in tissue culture media, aliquoted and kept at -70 °C until use. Cf (SPOFA, Czechoslovakia) was dissolved in PBS. The stock solution (30 mg/ml) was sterilized by filtration, aliquoted and stored at -20 °C. After thawing it was heated before use to ensure complete dissolution of the drug.

Growth experiments. The effects of both Cf and IFN on HSV growth were tested in LEP and HaCaT cells. In various experiments cultures were infected at multiplicity of infection (MOI) ranging from 5.0 to 0.00001 PFU per cell, incubated for 1 hr at room temperature and washed. Drug-free medium or medium containing varying concentrations of one or both drugs was then added. In the first series of experiments the drug(s) was(were) present only post infection (p.i.), in the second series cultures were, moreover, pretreated with drug(s) for 22 – 24 hrs before infection. At intervals samples were withdrawn and placed at -70 °C. After all samples had been collected, their virus titer (log PFU/ml) was determined by titration in Vero cells.

Patients. A total of 115 patients were enrolled in the study. All of them had been sufferring for 2 to 12 years from frequent herpetic lesions (3 to 12 recrudescences per year) and had been followed for this disease for prolonged periods of time. All had given informed consent to be included in the study and were confirmed to be otherwise in good health at the enrollment. Forty-five were males, 70 females; age range was 15 – 55 years; average age was 35 years. In 85 patients the lesions were localized above the waist (AW-lesions), nearly entirely in the facial area; in 30, below the waist (BW-lesions), predominantly in the genital area. No attempts were made to isolate and type the associated viruses. However, a recent virological investigation done within the frame of a study on HSV vaccine in a group of 42 patients originating from the same population had revealed that all but one of the AW lesions were associated with HSV-1 and all BW lesions with HSV-2 (Kutinová *et al.*, 1988). It is reasonable to assume that the situation in the present patients was similar.

Oinments and solutions. For the preparation of oinments, the hydrophilic oinment base Ambiderman (SPOFA, Czechoslovakia) was used. It was mixed with reconstituted IFN solution to give the final concentration of 5×10^4 IU/g. The final concentration of Cf was 50 mg/g. Four oinments were prepared: oinment A did not contain any drug and served as placebo, oinment B contained Cf only, oinment C contained IFN only, and oinment D contained both drugs. Analogous solutions of Cf and/or IFN in PBS were prepared and initially used in a portion of the patients with genital lesions. The drug concentrations were the same as in the oinments. Both the oinments and the solutions were kept at +4 °C and dark. They were used for up to 4 months after preparation.

Design of the study. The patients were divided into four groups: A, B, C, D. Group A (control) consisted of 45 subjects: 33 patients with AW lesions and 12 with BW lesions. They were treated with placebo (oinment or solution A). Group B included 20 patients: 15 with AW and 5 with BW lesions. These patients were treated with Cf only (oinment or solution B). Group C, 25 patients, 20 with AW and 5 with BW lesions, were treated with IFN only (oinment or solution C). Group D, 25 patients, 17 with AW and 8 with BW lesions, were treated with a mixture of Cf and IFN (oinment or solution D). The patients had been allocated randomly and they were not aware that some of them were treated with placebo.

The oinments (or, in a few patients, the solutions) were applied topically within 24 hrs of the onset of first prodromal symptoms or, exceptionally, next day when the papules or vesicles were already present. The patients were instructed to apply their respective preparations 5 to 8 times a day until complete healing. They were instructed how to follow and record the course of their disease on a special questionnaire on a daily basis. The patients were seen by the physician on the day of the first signs of the recurrence and thereafter at least twice a week. On these occasions they were interviewed, the size and stage of the lesions were examined and the patients' records were checked. In principle, two parameters of the reccurent disease were followed and recorded: HT, i.e. the interval (in days) between onset of prodromes and epithelization of all lesions (thus this parameter combines the phase of lesion development and lesion resolution) and, separately, ST, i.e. the interval (in days) between onset of prodroms and appearance of the last new lesions. The patients were not being given any concomitant antiviral therapy.

Statistical methods. The HT and ST, respectively, reported by each subject before and on the treatment were analyzed to determine the significance of the effects observed. The data from the period prior to treatment (control period) served as control for evaluating the treatment effects. Since in the control period the statistical distribution of both the variables followed showed positive assymetry towards longer times, the lognormal statistical density model seemed appropriate and was used for the analysis. In the presentation of results the logarithmic means and their confidence intervals were transformed to the original metrics, thus forming the respective geometric means and their confidence intervals. The results of analyses of data from the control periods showed

no significant differences between the groups (A-D) for both variables; the assumption of homogeneity of the individual patient groups complied with the results of statistical tests (F = 1.000 for the first variable and F = 2.046 for the second variable, both with 3 and 111 degrees of freedom). For the evaluation of treatment effects the data collected on treatment were related to those of the control period. In accordance with the statistical model, the differences in transformed variables between the treatment and control periods were used; this amounted to using the logarithms of the ratios between treatment and control variables. These new variables analyzed by simple one-way analysis of variance in addition to which the multiple Scheffé's range test (Scheffé, 1959) was adopted for pairwise comparison of means. The results (in Tables 4 and 5) present the relative shortenings of HT and ST, respectively, in terms of geometric means and their 95% confidence limits.

Results

In vitro study

Cf exhibited a slight inhibitory effect on HSV replication at doses equal to or exceeding 0.15 mg/ml. This inhibition manifested itself by a delay of cytopathic changes, reduced plaque size and reduced infectious virus production. As shown in Table 1, these effects were dependent on the Cf concentration. At concentrations exceeding 1 mg/ml the toxicity of the drug for LEP cells was apparent. For this reason, most *in vitro* tests were carried out with Cf doses not exceeding 0.6 mg/ml. The effect of IFN varied, depending on its concentration and the MOI, but it also varied from experiment to experiment, presumably being influenced by the physiological state of the cells and possibly some other experimental variables. Results of a typical experiment are shown in Table 2. On the basis of this and similar tests it was decided that IFN would be used at concentrations of $10^3 - 10^4$ IU/ml in combination experiments.

Table 1. HSV-1 replication in LEP cells in the presence of Cf

Cf (mg/ml)	log TCD_{50}/ml at intervals (hrs) p.i. 0	24	48	72
0	2.0	4.3	6.6	7.0
0.15		3.6	6.6	6.8
0.3		3.7	6.0	6.0
0.6		3.3	5.3	6.0
1.2		2.0	3.0	2.0

MOI 0.05 $TCID_{50}$/cell, drug present p.i.
Cf = caffeine.

Table 2. HSV-1 replication in LEP cells in the presence of IFN

IFN (IU)	log TCD_{50} at intervals (hrs) p.i. 0	24	48	72
0	2.0	4.6	7.3	7.6
5×10^2		3.3	5.9	6.6
10^3		2.9	5.5	5.8
2.5×10^3		2.3	5.3	5.3

MOI 0.005 $TCID_{50}$/cell, drug present p.i.
IFN = interferon.

Table 3. Combined effects of IFN and Cf on the growth of HSV-1 in LEP cells

Exp. No.	MOI ($TCID_{50}$/cell)	IFN (IU/ml)	Cf (mg/ml)	Time of harvest (hrs p.i.)	Control virus titer (log TCD_{50}/ml)	Reduction of virus titer in the presence of Cf	IFN	Cf+IFN
1[a]	0.0001	1.2×10^3	0.3	48	6.5	1.0	1.4	2.4
	0.1			20	7.1	0.6	0.8	1.2
2	0.03	1.0×10^4	0.3	48	7.7	1.7	2.0	3.2
3	0.005	5.0×10^3	0.3	48	6.5	0.7	1.2	3.2
4	0.0001	2.5×10^3	0.15	48	7.5	0.2	1.2	2.6
			0.6			2.4		4.3

[a]In Exp. No. 1 the drugs were added only after virus infection, in other experiments they were present both before and after infection.
Cf = caffeine; IFN = interferon.

In examining the combined effects of IFN and Cf, different experimental approaches were used. Initially, the substances, whether alone or in mixture, were added only after infection. In subsequent series of experiments, cultures were pretreated for 24 hrs with either IFN or Cf or both, and then infected; after the infection the same concentrations of the drugs as used for the pretreatment were incorporated into the maintenance medium. It was this last modification which gave the most consistent data in repeated tests. In all experiments, cultures untreated with either drug were used as controls. Results of several experiments each of which is a representative of a larger series of experiments are summarized in Table 3. In all of them the combined effects of the two substances exceeded those of either substance alone; however, these effects varied markedly from test to test. In some of them the combined effects corresponded to the sum of the two effects (experiments No. 1 and 2); in others (experiments No. 3 and 4) the combined effects were more pronounced suggesting synergistic action. Extensive efforts to clarify this variation have failed thus far.

Experiments in which LEP cells were substituted by HaCaT cells yielded similar results (data not shown).

Clinical study

In spite of the unclarified nature of Cf-IFN-HSV-cell interactions, the observation that the combined effect of the two drugs *in vitro* exceeded the effect of either of them,

when applied alone and the innocuity of the two substances stimulated a clinical study.

After it had been checked that oinments or solutions containing 20 – 100 mg/g of Cf did not irritate human skin, four oinments and solutions containing per gram either 50 mg of Cf, or 5 x 10^4 IU of IFN, or both, or placebo were prepared and applied in patients suffering from recurrent herpetic lesions (see Materials and Methods). The results are summarized in Tables 4 and 5. While placebo produced only negligible effects on either HT (Table 4) or ST (Table 5), treatments with the other preparations favourably influenced one or both of the disease parameters followed. In general, the effects on HT were more pronounced than those on ST, which is in line with earlier observations indicating that antiviral drug treatment influenced lesion resolution more markedly than lesion development (Spruance *et al.*, 1990). At the concentrations used, IFN was more efficient than Cf. However, the most marked was the combined effect of both the substances. Either treatment provided highly significant effects on HT ($p<0.001$); the effects of the combination significantly exceeded those produced by Cf ($p<0.01$) or by IFN alone ($p<0.05$). It is

Table 4. Effect of treatment with Cf, IFN and their combination on healing of herpetic lesions

Group	Treatment	No. of patients	Geometric mean		Ratio of geometric means (A/B) (95% confidence interval)	No. of patients affected (%)[a]	Significance
			before treatment (A)	on treatment (B)			
A	Placebo	45	13.17 (7-9 days)	12.61 (7-18 days)	0.96 (0.87–1.05)	2 (4.4)	NS
B	Cf	20	11.42 (7-16 days)	6.25 (3-10 days)	0.55 (0.47–0.63)	15 (75.0)	$p<0.01$
C	IFN	25	12.44 (10-13 days)	4.78 (3-8 days)	0.38 (0.33–0.44)	22 (88.0)	$p<0.001$
D	Cf+IFN	25	12.54 (7-16 days)	3.18 (1-5 days)	0.25 (0.22–0.29)	25 (100.0)	$p<0.001$

[a]Shortening of HT by at least 4 days.
Cf = caffeine; IFN = interferon; NS = non-significant.

Table 5. Influence of treatment with Cf, IFN and their combination on spreading of herpetic lesions

Group	Treatment	No. of patients	Geometric mean		Ratio of geometric means (A/B) (95% confidence interval)	No. of patients affected (%)[a]	Significance
			before treatment (A)	on treatment (B)			
A	Placebo	45	2.86 (1-6 days)	2.53 (1-5 days)	0.88 (0.78–1.01)	6 (13.3)	NS
B	Cf	20	2.70 (2-4 days)	2.13 (1-3 days)	0.79 (0.65–0.96)	3 (15.0)	NS
C	IFN	25	2.79 (2-4 days)	1.64 (1-3 days)	0.59 (0.50–0.70)	10 (40.0)	$p<0.01$
D	Cf+IFN	25	3.20 (2-6 days)	1.38 (1-3 days)	0.43 (0.36–0.51)	15 (60.0)	$p<0.01$

[a]Shortening of ST by at least 2 days.
Cf = caffeine; INF = interferon; NS = non-significant.

remarkable that by combined treatment all patients were favourably affected.

In the case of ST, Cf alone was without effect, and IFN alone or in combination with Cf reduced this parameter significantly ($p<0.01$ and $p<0.001$, respectively). Again, Cf potentiated the effect of IFN ($p<0.05$). While a favourable effect of IFN alone could be observed in about 40% of patients, 60% of them responded to treatment with both drugs.

Variations associated with drug application form, lesion site or the age or sex of the patients were not observed. No untoward reactions were reported.

Discussion

The present data indicate that Cf, which is a rather weak inhibitor of HSV replication (Yamasaki and Tagaya, 1980; Shiraki and Rapp, 1988) enhanced the inhibitory effect of IFN when added simultaneously with it to virus-infected cultures. From the results obtained, it is not possible to decide whether this combined effect was due to the summation of two independent effects or to modulation of the IFN effect by Cf or *vice versa*. Because of the complexity of the biological effects of both Cf (Timson, 1977; Sivak *et al.*, 1980) and IFN (Sen 1984; Borecký, 1989; Taylor and Grossberg, 1990) it is difficult to propose a reasonable explanation of the co-operative actions. While HSV has often been found as moderately sensitive to IFN (Stewart, 1975), it should be recalled that different mechanisms of the inhibitory effect of IFN on this virus replication have been considered (Mittnacht *et al.*, 1988; Chatterjee *et al.*, 1985).

The mechanisms operative *in vivo* are probably even more complex, because of the possible involvement of systemic anti-HSV immune reactions curtailing the infection, local immunomodulatory effects of IFN and drug-metabolizing enzymes. Irrespective of the nature of the various effects it appears that the drugs used favourably influenced the course of the disease in patients suffering from recurrent herpes simplex lesions. Although the topical application of either Cf or IFN alone did reduce the severity of herpetic lesions, the effects were more marked when the two substances were applied simultaneously. The treatment was well tolerated, and no side-effects were observed. The present data thus confirm the beneficial effects of topical application of IFN on herpes simplex lesions reported by Sacks *et al.* (1991) for IFN and by Movshovitz *et al.* (1986) and Glezerman *et al.* (1988) for IFN ß, and indicate, in addition, that this favourable effect can be further enhanced by the simultaneous application of Cf, a cheap and innocuous drug.

In a recent study with subunit HSV vaccine in patients suffering from recurrent herpetic lesions we observed a marked placebo effect (Kutinová *et al.*, 1988). This was virtually absent in the present study. The difference may be due to the fact that the use of a vaccine represented a very substantial departure from the previous regimen of treatment based on the use of various oinments, solutions or peroral drugs. It seems that psychoneuroendocrine pathways which may influence many physiological functions and eventually influence the course of herpetic disease, were considerably less activated by the present treatment.

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