Antiviral Activity of Engystol®: An *In Vitro* Analysis

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ABSTRACT

Objectives: To study the effects of the homeopathic preparation Engystol® (Biologische Heilmittel HEEL GmbH, Baden-Baden, Germany) on a panel of human pathogenic viruses *in vitro*.

Design: The effects of Engystol were studied using plaque-reduction assays and virus titration assays, and by quantification of newly synthesized viral proteins in virus-specific enzyme-linked immunoabsorbent assays (ELISAs).

Subjects: The DNA viruses Adeno 5 and herpes simplex type 1 (HSV-1), the RNA virus respiratory syncytial virus (RSV), and human rhinovirus (HRV)

Results: A 73% reduction of Adeno 5 specific proteins and an 80% reduction in HSV-1 specific proteins were observed in ELISAs of virus-infected cells treated with Engystol after infection. The effects appeared to be dose-dependent. With these viruses, similar results were observed in titration assays of viral offspring from cells treated with Engystol. Pretreatment of adenovirus with Engystol did not inhibit the infectivity of the virus suspension and no Engystol-induced stimulation of interferon-α could be observed. Plaque-reduction assays with the RNA viruses, RSV and HRV, showed reductions in infectivity by 37% (RSV) and 20% (HRV), respectively.

Conclusions: The results indicate antiviral activity of Engystol independent of the activation of the cellular interferon system.

INTRODUCTION

Many respiratory viruses, most commonly influenza virus, respiratory syncytial viruses (RSV) and rhinovirus, are capable of causing respiratory disease, either by their direct effects or by exacerbating underlying conditions. A number of substances are available for effective treatment of viral infections, such as amandatine,[1] neuraminidase inhibitors,[2] and nucleoside analogues.[3] However, many antiviral substances are associated with side-effects[4–6] and there is still a need for antiviral substances with good efficacy and tolerability, and low toxicity.

The homeopathic preparation Engystol® (Biologische Heilmittel HEEL GmbH, Baden-Baden, Germany) is fre-

quently used in complementary medicine for the prophylactic treatment of infectious diseases. Engystol is used in the form of tablets or in solution for injections and contains two main active ingredients, *Vincetoxicum hirundinaria* (swallowort), which has been associated with stimulation of host defense mechanisms, and sulfur, which has been used homeopathically for treating a range of conditions, including skin diseases, acute and chronic inflammations, and hepatic and gastrointestinal complaints. The remedy has been shown to be effective in various clinical settings for treating viral and respiratory infections.[7–9] Other studies have indicated Engystol treatment to be associated with a stimulation of the immune system in terms of phagocytic activity, granulocyte function, and improved humoral response.[10–13]

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However, it is currently unclear whether these effects are mediated by the immunostimulatory properties of Engystol or whether Engystol by itself has direct antiviral activity.

The current study investigated a possible antiviral activity of Engystol on a panel of human pathogenic viruses, including both RNA and DNA viruses: human rhinovirus (HRV); a nonenveloped, single-stranded RNA virus; two enveloped RNA viruses, influenza A virus and RSV; and two DNA-viruses, herpes simplex type 1 virus (HSV-1), which belongs to enveloped DNA-viruses, and adenovirus, a DNA virus lacking an outer membrane. Besides a direct effect of Engystol on virus-infected cells, the potential stimulation of cellular antiviral activity by induction of the interferon system by Engystol was investigated.

MATERIALS AND METHODS

Test substance

Commercially available formulations of Engystol® (lot 06959 11-2006; E1, and lot 09203 08-2008; E2) were supplied free of charge by the manufacturer as sterile ampoules (1.1 mL H_2O). The composition is outlined in Table 1. In all experiments, the test substances were diluted in a cell-culture medium before they were added to the cell cultures.

Reference drugs

Acyclovir (Zovirax,® Deutsche Wellcome GmbH, Burgwedel, Germany), ribavirin (Virazole,® ICN Pharmaceuticals, Frankfurt, Germany), and amantadine (amantadine hydrochloride, Ratiopharm, Ulm, Germany) were used as positive controls. Acyclovir was used in infections with HSV 1, ribavirin with RSV, and amantadine with influenza A virus.[1,3,14] These reagents were diluted according to their *in vitro* cytotoxicity.

Cells and viruses

Human rhinovirus 14 (HRV 14) was obtained from the Institute for Virology of the Friedrich-Schiller-University, Jena, Germany. RSV, (strain Long), influenza A/Chile 1/83 (H1N1) virus, HSV 1 (strain Thea), and adenovirus (Adeno 5) were obtained from the former Department of Medical

TABLE 1. COMPOSITION OF ENGYSTOL®a (PER 1.1 mL AMPOULE)

Ingredient	Potency	Quantity
Vincetoxicum hirundinaria	D6	6.6 mg
Vincetoxicum hirundinaria	D10	6.6 mg
Vincetoxicum hirundinaria	D30	6.6 mg
Sulfur	D4	3.3 mg
Sulfur	D10	3.3 mg

aBiologische Heilmittel HEEL GmbH, Baden-Baden, Germany.

Virology and Epidemiology of Virus Diseases of the Hygiene Institute of the University of Tübingen, Germany. All viruses were identified and characterized with a panel of monoclonal antibodies (BioWhittacker Products, Walkersville, MD). RSV, adenovirus, and HSV strains were propagated on human epithelial cells (HEP); HRV on HeLa cells in Hank's/Earle's minimal essential medium (MEM) containing 2% fetal calf serum, 25 mM $MgCl_2$, 2 mM of L-glutamine, 100 U/mL of penicillin, and 0.1 mg/mL of streptomycin. Influenza A was grown on Madin-Darby-Canine-Kidney (MDCK) cells with serum-free MEM containing 1 μg/mL of trypsin, 2 mM of L-glutamine, 100 U/mL of penicillin, and 0.1 mg/mL of streptomycin.

Virus assays

Enzyme immunoassays (Virion/Serion, Würzburg, Germany; Merlin Diagnostika GmbH, Bornheim-Hersel, Germany; Dako, Hamburg, Germany), plaque assays, and virus titrations were carried out using standard procedures. Plaque assays and virus titrations for the detection of infectious particles were performed with MDCK, HEP, and HeLa-cell cultures.

Cytotoxicity tests

Analyses of the *in vitro* cytotoxicity of the test substances was done with physiologically active cells by an enzymatic assay (MTT-assay) as described[15] and by microscopic examination of the cell cultures for altered cell morphology.

Assays for antiviral activity

Plaque-reduction assay. Determination of the antiviral activity of Engystol against influenza, RSV and HRV was performed with plaque-reduction assays. Cell monolayers were infected for 1 hour at 34°C with a multiplicity of infection (MOI) of 0.0002 (Influenza A, HRV) or 0.0004 (RSV) without, or in the presence of, different nontoxic dilutions of the test substance and the respective controls. The cell cultures were cultivated until lesions were visible in the cell monolayer (plaques) of the control group cultivated in medium alone, fixed with paraformaldehyde, and the remaining cell monolayers were stained with a crystal violet solution. Plaques were quantified with an optical evaluation system.

Determination of the infectivity of newly synthesized virus preparations (virus-titration assays). When infection doses were too high to allow the evaluation of virus titers by plaque-reduction assay, the amount of newly synthesized viruses was determined either by an enzyme-linked immunoabsorbant assay (ELISA) or by virus titrations for tissue culture infectious dose $(TCID)_{50}$ values. Virus-sensitive cell lines were infected with supernatants derived from substance-treated cultures and the respective 100-fold dilutions for 1 hour at

34°C. Subsequently, the medium was replaced by semisolid agarose and cells were cultivated for an additional 3–4 days until plaques were visible in the nontreated control groups.

Quantification of newly synthesized viral proteins in virus-specific ELISA. Newly synthesized viral proteins were quantified by a commercially available ELISA according to the manufacturers' specifications. The quantitative analysis of the virus protein–dependent extinction of the respective supernatants was performed with an ELISA reader (Tecan, Crailsheim, Germany) at an optical density (OD) of 450 nm.

Controls. A number of controls were included in the assays for the determination of the antiviral activity: uninfected cells (cell control); infected cells; or cells grown in medium without antiviral agent or test substance (virus control).

Calculation of antiviral activity. Mean values were calculated from four parallel probes and two independent tests. The results from the nontreated virus control groups were defined as 100% infection and *in vitro* effects of the substances were standardized as relative inhibitory effects.

Analysis of direct antiviral influence of Engystol on adenovirus. Engystol was added in a cell-free system in dilutions of 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:640, and 1:6400 to a high-titer adenovirus suspension (10^8 TCID$_{50}$/mL) for 120 minutes at 34°C and 5% CO_2. Subsequently, the viral protein content of the pretreated suspensions was analyzed by using twofold titrations in an adenovirus capsid-specific ELISA. The infectivity of the pretreated virus suspensions (MOI of 4) was determined by endpoint virus titrations on HEP cells starting with an MOI of 0.004, corresponding to a 1000-fold dilution of remaining Engystol in the pretreated virus suspension. In addition to a nontreated virus suspension, cetylpyridiniumchloride (CPC, 20 μg/mL) was included in the test system as a positive control.

Quantification of Type I interferon in cell culture supernatants of Engystol-treated cells. Engystol was added in dilutions of 1:2, 1:4, 1:8, 1:16, 1:32, and 1:320 to virus-sensitive cells. Costimulation-dependent interferon-inducing activity of Engystol was tested by adding Engystol together with a high-titer suspension of ultraviolet (UV)–inactivated (UV-GS-linker 254 nm, Stratagene, Heidelberg, Germany) HSV (1.25×10^7 plaque-forming units/mL prior to UV inactivation) to the respective cells. The virus suspension was tested for optimal interferon (IFN)–α–inducing capacity in time kinetics (days 1–6 after addition to the cells).

For the evaluation of the Engystol-induced interferon production, HEP and HFF cells were incubated with the test substance in different dilutions for 48 hours with and without a costimulus. The following controls were included: nonstimulated cells; cells stimulated by inactivated viral antigen alone; and human recombinant IFN-α. Subsequently, cell-culture supernatants were tested for their interferon content with an IFN-α–specific ELISA (R&D, Cologne, Germany). The absolute amount of IFN-α was determined by the use of an internal standard. All results were confirmed by a bioassay, in which the antiviral activity of IFN-containing supernatants were determined on IFN-α–sensitive cells.

RESULTS

In vitro *cytotoxicity of Engystol*

To determine the highest possible concentration of Engystol that could be used without affecting basic cell functions creating background artifacts, changes in OD of the dye (at 492 nm) relative to controls (medium only) were assessed on HEP, MDCK- Human mucosa fibroblasts (HFF), and HeLa cells after 5 days of exposure to the two batches of Engystol. The results of the cytotoxicity analyses are shown in Table 2. Cytotoxicity on HEP and MDCK cells

TABLE 2. DETERMINATION OF THE CYTOTOXICITY OF ENGYSTOL®a

Assay	HeLa	HEP	MDCK
ID90			
Engystol batch E1	Undiluted	Undiluted	Undiluted
Engystol batch E2	Undiluted	Undiluted	Undiluted
ID$_{50}$			
Engystol batch E1	Undiluted	Undiluted	1:5
Engystol batch E2	Undiluted	Undiluted	1:25
Lowest dilution used in virus assays			
Engystol batch E1	1:2	1:2	1:4
Engystol batch E2	1:2	1:2	1:4

aBiologische Heilmittel HEEL GmbH, Baden-Baden, Germany. The viability of cells (HeLa, human epithelial cells [HEP], Madin-Darby-Canine-Kidney [MDCK]) cultivated with different dilutions of two different batches of Engystol (E1 = lot 0659-11-2006; E2 = 09203-08-2008). Engystol was quantified with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT)-test. All data represent six replicates derived from two independent experiments. Standard deviations were less than 8%. The two batches of Engystol were evaluated for their cytotoxicity on HEP, MDCK, human mucosa fibroblasts (HFF), and HeLa cells.

was low. There was no cytotoxicity with Engystol, either in MTT-assays or in microscopic examinations (not shown) at a dilution of 1:2 for HEP cells and dilution of 1:4 for MDCK cells. The inhibitory concentration (IC)$_{50}$ of Engystol on MDCK cells calculated from several experiments was a dilution of 1:1.5. The cell morphology of HEP and MDCK cells was not affected by the treatment. HeLa cells treated with

undiluted test substances showed similar viability as non-treated controls. No toxic effects were found with the highest Engystol concentration in HFF cells (data not shown).

Based on these experiments, all batches of Engystol were used in the antiviral assays in dilutions of 1:2 to 1:64 on HeLa (HRV) and HEP cells (adeno and RSV) and of 1:4 to 1:128 on MDCK cells (Influenza A).
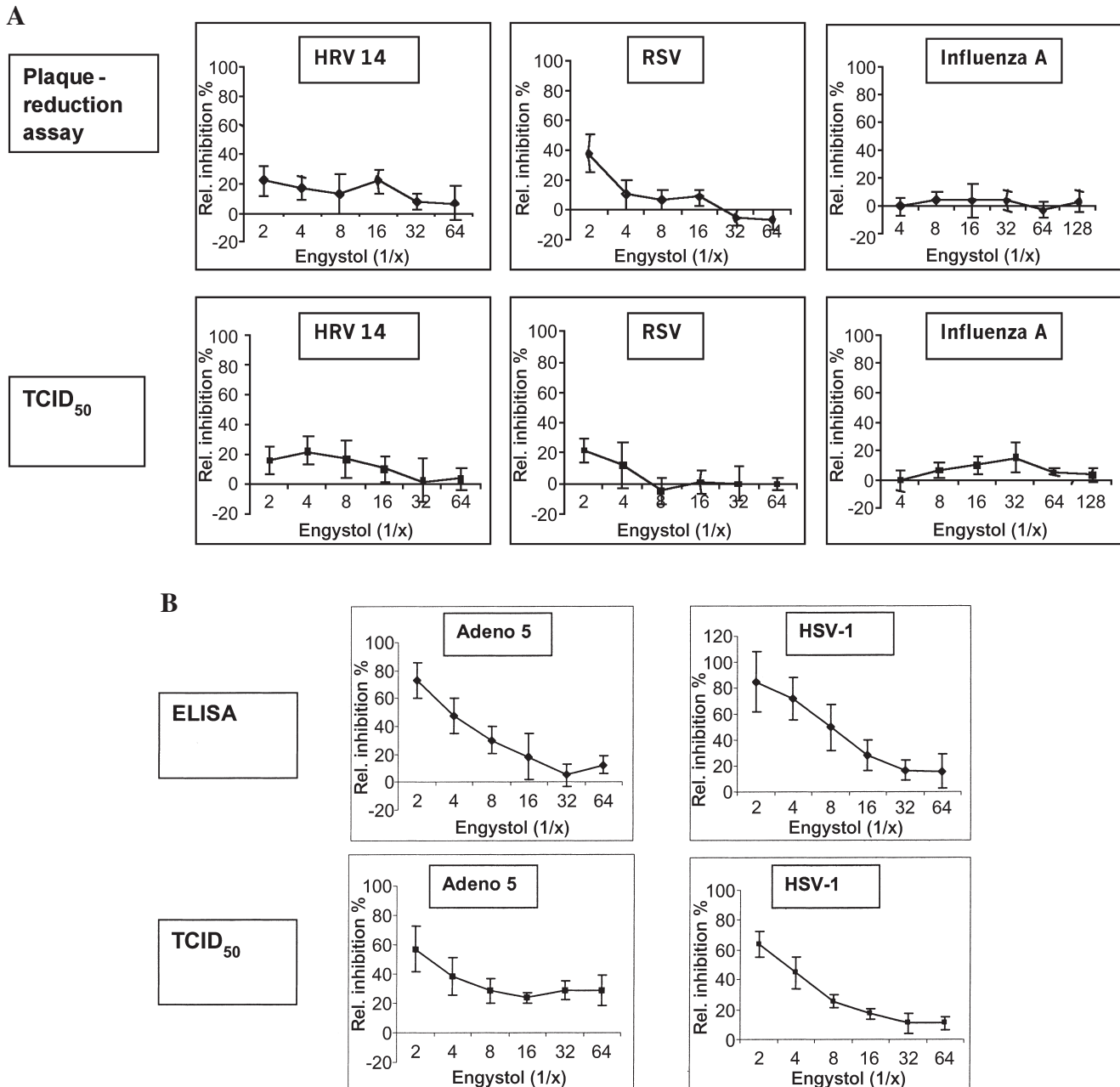
FIG. 1. Percentage inhibition of viral activity of different dilutions of Engystol® (Biologische Heilmittel HEEL GmbH, Baden-Baden, Germany) (E1) lot 0695911-2006 tested on a panel of RNA and DNA viruses is presented. Inhibition was based on (**A**) plaque-reduction assay (HRV 14, RSV, Influenza A), (**B**) viral protein-specific enzyme-linked immunoabsorbent assay (ELISA) (Adenoviruses 5 and HSV-1), and virus titrations on the permissive cell lines, resulting in TCID$_{50}$ values and was calculated as a percentage of the equivalent value from control cell cultures not exposed to Engystol. Mean of four values (two repetitions of two separate experiments) with standard deviations. HRV, human rhinovirus; RSV, respiratory syncytial virus; Adeno5, Adenovirus 5; HSV-1, herpes simplex virus type 1.

Antiviral activity of Engystol on Influenza A, RSV, HRV 14, Adeno 5, and HSV-1

The influence of Engystol (lot 0659-11-2006, E1) is shown on the RNA viruses HRV 14, RSV and Influenza A in Figure 1A, on the DNA viruses Adeno 5 and HSV-1 in Figure 1B. In plaque-reduction assays for HRV 14 (Fig. 1A, left) a slight reduction in plaques (22%) was observed with the lowest dilution of Engystol. A similar low effect was detectable in the virus-titration assays with Engystol-treated HRV 14-infected cell cultures (Fig. 1A, left). Comparable results were obtained with RSV-infected Engystol-treated cell cultures (Fig. 1A, middle). Only the highest concentration of Engystol showed an effect on RSV with an approximately 37% reduction in virus plaques. Endpoint titration of newly synthesized RSV showed a 20% reduction in viral infectivity (Fig. 1A, middle). No effect of Engystol was seen on influenza A virus, neither in the plaque-reduction assay (Fig. 1A, right) nor in the endpoint-titration assay (Fig. 1A, right).

In contrast, there was a marked effect of Engystol against adenovirus infection (Fig. 1B). With undiluted agent, a 73% inhibition of newly synthesized adenoviral proteins was determined in ELISA (Fig. 1B, left) and there was a 57% decrease in the infectivity of the offspring of adenovirus-infected cell cultures in endpoint titration experiments (Fig. 1B, TCID$_{50}$). Higher dilutions of Engystol, 1:4 and 1:8 showed an approximately 40% reduction in the ELISA as well as in the endpoint-titration assay. Engystol at a dilution of 1:16 was associated with a more than 20% reduction in virus antigen and infectivity.

Similar effects were observed on HSV-1–infected cell cultures (Fig. 1B, right). Engystol at a dilution of 1:2 was associated with a reduction of 80% in viral proteins assayed by HSV-1–specific ELISA. At a dilution of 1:16, a 28% re-

duction was observed. There appeared to be a dose-dependent effect for this antiviral activity (Fig. 1, third row, right). These data could be confirmed by the endpoint-titration experiments for infectivity of the offspring of Engystol-treated HSV-1–infected cell cultures (Fig. 1B, right, TCID$_{50}$). The lowest dilution of Engystol led to a 64% inhibition and a dilution of 1:16 led to a 20% reduction in HSV-1–specific infectivity. The efficacy of the reference substances was confirmed in the assays. For RSV a concentration of 6.2 μg/mL of ribavirin reduced infectivity by 80%. Five (5) μg/mL of amandatine hydrochloride reduced Influenza A virus infectivity by 60% and 10 μg/mL of acyclovir reduced the infectivity of HSV by 72% (data not shown).

Several alternative reasons for the observed effects were addressed in control experiments with the adenovirus. The possible heterogeneity of the Engystol preparations was assessed by assaying two independent lots of Engystol (lot 0659-11-2006, E1 and lot 09203-08-2008, E2). There was no difference in reactivity against the adenovirus between these preparations. The results were consistent using tenfold differences in virus titers (MOI 0.0004 and 0.004). Both preparations of Engystol showed very similar dose effects: 63% and 68% in ELISA for E1 and E2, respectively; and 42% and 48% reductions in TCID$_{50}$ endpoint-titration assays for E1 and E2, respectively. These results also indicated that the efficacy of Engystol was not a consequence of low doses of infectivity in the assays.

The possible direct interaction of Engystol with DNA-viruses was analyzed by treating a high-titer adenovirus-stock solution (TCID$_{50}$/mL 10^8) with different dilutions of Engystol. Engystol at dilutions down to 1:640 inhibited virus-specific capsid protein binding by 80% (Fig. 2A). These results were comparable to those with the internal control CPC. The infectious capacity of the Engystol-treated viruses was tested by titration experiments (Fig. 2B). The
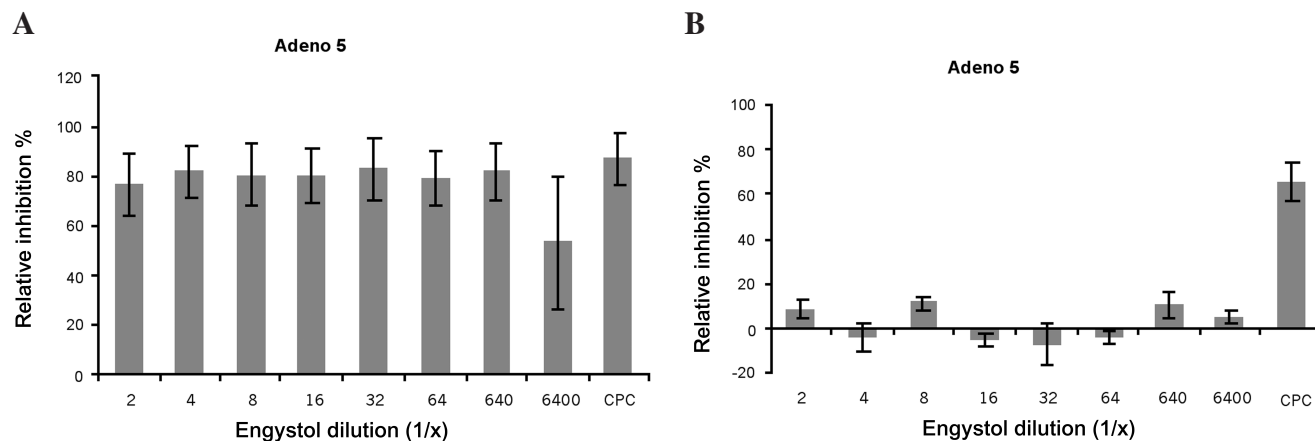
FIG. 2. Effect of Engystol® (Biologische Heilmittel HEEL GmbH, Baden-Baden, Germany) on suspensions of Adenovirus 5 (Adeno5). The interaction of Engystol with viral capsid proteins was evaluated in an adenovirus-specific ELISA (**A**). The effect on the infectivity of the virus suspension was assessed by titration of the infectious particles (**B**). Cethylpyridiniumchloride (CPC) served as an internal control. The mean of four values (two repetitions of two separate experiments) with standard deviations is shown.

internal control CPC demonstrated a clear antiviral activity but treatment with Engystol had no influence on the infectivity of the viral particles (Fig. 2B).

The possible IFN-α inducing or coinducing activity of Engystol was addressed by assessing the time to 50% induction of IFN-α in HEP cells and HFF cells after incubation with Engystol, alone or in combination with inactivated HSV-1 antigen. There was no enhancement of cellular IFN-α production with Engystol neither alone nor in combination with HSV-1 (Fig. 3). These data were confirmed by bioassays (data not shown).

DISCUSSION

The results of these *in vitro* experiments indicate a specific, dose-dependent antiviral activity of the herbal-based homeopathic preparation Engystol assessed in plaque-reduction assays and via analysis of the infectivity of newly synthesized viruses. The greatest effects were seen on enveloped (HSV-1) and nonenveloped (Adeno-5) DNA-

viruses. At high concentrations, there was also a marked effect from Engystol incubation on the RNA virus RSV in both assays. The degree of inhibition varied between 80% on adenovirus and HSV-1 and 20%–30% on RSV. Although the latter is a rather low degree of inhibition, the experiments were consistent and reproducible. The effects on HSV and Adeno 5 were similar to those of the control substance CPC.

Four different alternative explanations for the results were addressed: a possible heterogeneity between preparations (a risk with homeopathic and herbal preparations); a too low dose of virus in the assays; a direct interaction of Engystol with DNA-viruses; or an indirect antiviral effect of Engystol by induction of the cellular antiviral IFN system. The results were highly similar with two different lots of Engystol, indicating homogeneity between preparations. Different virus titers showed similar outcomes for endpoint-titration assays, and in ELISAs, indicating that the effect is not dependent on the virus dose. Preincubating adenovirus with Engystol produced capsid protein inhibition in ELISA similar to that of CPC but without reducing the infectivity
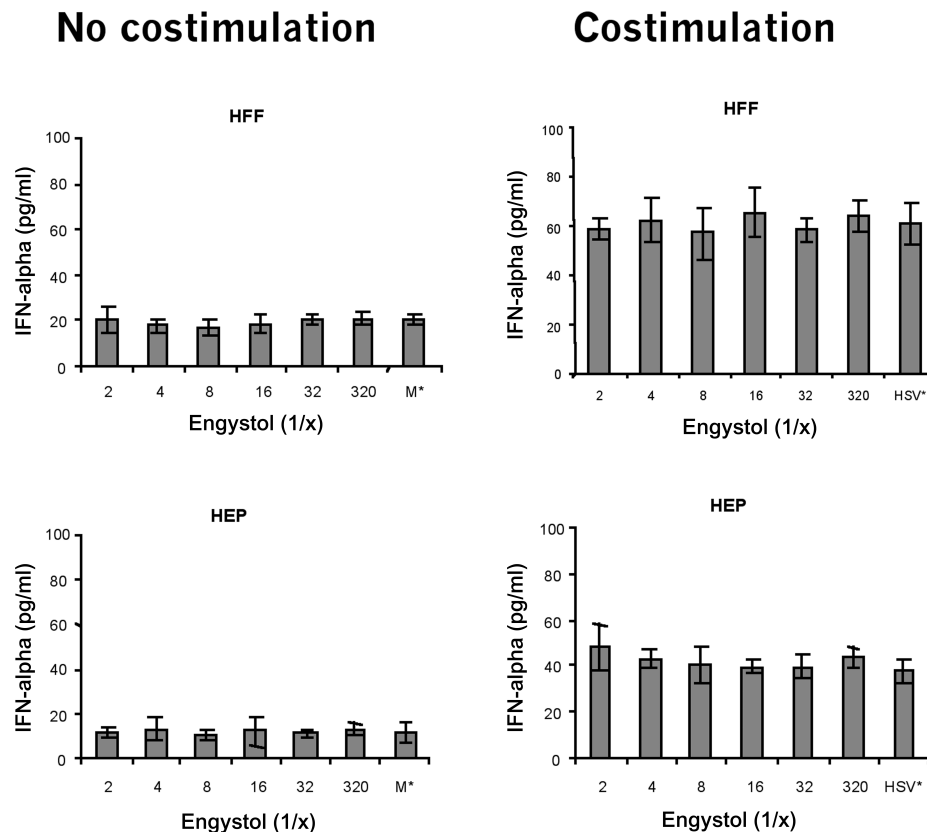
FIG. 3. Interferon (IFN)–stimulating activity of Engystol® (Biologische Heilmittel HEEL GmbH, Baden-Baden, Germany) was studied on human mucosa fibroblasts (HFF) cells (upper row) and on human epithelial (HEP) cells (lower row). The left-hand figures show IFN-α production by Engystol-treated cells without a costimulus. The right graph shows IFN-α synthesis by Engystol-treated cells stimulated with ultraviolet (UV)–inactivated HSV-1. IFN-α in the cell culture supernatants was quantified in an IFN-α–specific enzyme-linked immunoabsorbent assay. The mean of four values (two repetitions of two separate experiments) with standard deviations is shown. M*, medium as control; HSV*, stimulation with UV-inactivated HSV-1.

of the virus suspension. This indicates that Engystol interacts with the virus capsid, affecting the structure of capsid proteins to mask or alter the specific epitope of the virus but without affecting infectivity. A detailed analysis is needed to clarify the nature of the changes to the capsid by the homeopathic agent.

Finally, the alternative explanation of an Engystol-mediated induction of cellular IFN-α was tested in HEP and HFF cells with UV-inactivated virus suspensions. Engystol was unable to enhance IFN-α levels in this assay. Thus, it seems highly likely that the observed effects are the result of a real antiviral effect of Engystol.

Clinical studies have indicated beneficial effects of Engystol in a variety of settings[7–9,11,12] and the present study provides *in vitro* support for a direct effect of the homeopathic preparation on viral function. Engystol is reported to have immunomodulating effects in humans.[8,16,17] It is currently an open question to which degree the observed direct antiviral effects may be responsible for clinical benefits. The concentrations of the active components of Engystol in the current experiments are not directly comparable to those of the clinically used agent and, in the case of the effects of HSV, the concentrations used are unlikely to be achievable in clinical use. As for most homeopathic treatments, there are no pharmacodynamic data available. A similar question is whether the degree of inhibition observed is sufficient to account for clinical effects on viral infection. However, both discussions are beyond the scope of the current work, which focused on possible interaction of Engystol with virus-infected cells and demonstrated antiviral activity *in vitro*. It would be interesting in a clinical setting to address the degree to which these different activities of Engystol contribute to the reported beneficial effects in patients.

The molecular mechanisms of the antiviral activity of Engystol are unknown. We could exclude a surface active antiviral reactivity as the dominant mechanism. Viral capsid proteins may be affected by direct treatment with the preparation, as indicated by the ELISA data from the directly treated adenovirus suspension, but there was no effect on the infectivity of directly treated viral particles. Based on the present data, it seems that Engystol has an influence on virus-specific components necessary for viral replication. It is quite possible that several mechanisms with possible additive antiviral effects are involved.

CONCLUSIONS

The scarcity of antiviral drugs without problematic side-effects may be one reason why complementary and alternative medicine is enjoying a rising popularity in both the United States and Europe. If it can be shown conclusively that Engystol, a plant-derived substance at quite a high dilution, has antiviral effects, this would be a welcome addition to the treatment options for viral infections. Homeo-

pathic agents in general have a well-established record of tolerability and there are no adverse effects indicated for Engystol.[18]

In summary, this work supports a direct effect from the homeopathic agent Engystol on a variety of human viruses, assessed in specific assays. Further work is needed to establish an explanation for the effects at the molecular level and to assess the relevance of the *in vitro* results in our assays to benefits observed with Engystol in clinical practice.

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