ELSEVIER

International Immunopharmacology 1 (2001) 1841–1848

International Immunopharmacology

www.elsevier.com/locate/intimp

Enhanced resistance to Sendai virus infection in DBA/2J mice with a botanical drug combination (Sinupret®)

M. Schmolz [a], D. Ottendorfer [b,*], R.W. März [c], C. Sieder [c]

[a] *EDI GmbH, Reutlingen, Germany*
[b] *Battelle Institut, Frankfurt, Germany*
[c] *Bionorica Arzneimittel GmbH, Neumarkt / Opf., Germany*

Received 29 March 2001; received in revised form 30 May 2001; accepted 31 May 2001

Abstract

It was investigated whether the botanical drug combination Sinupret is able to modulate the resistance of mice to a respiratory tract infection with Sendai virus (*Parainfluenza viridae*) if given prophylactically to the animals. Three doses of Sinupret drops (SD) and Sinupret tablets (ST, p.o.), and two active controls, the chemical secretolytic ambroxol (p.o.) and the immunomodulator Muramyldipeptide (MDP, i.v.) were used. Test and reference substances were applied at days −3 and −1 before infection, except MDP, which was given once on day −1 before infection. CD4+ and CD8+ lymphocyte subpopulations were measured after infection as indicators of immunological treatment response. Groups of 20 mice each were infected by intranasal application of Sendai virus under anaesthesia. We found that the 1× and 5× human doses of Sinupret drops significantly prolonged the survival times ($p < 0.05$) compared to placebo. Additionally, ambroxol and MDP were comparably less effective. In all groups, changes in CD4+ and CD8+ T-lymphocyte subpopulations of the peripheral blood were observed, but no clear relationship to the treatment results was seen.

It was concluded that Sinupret increases the resistance to an experimentally induced respiratory tract infection in mice. Moreover, the effect of Sinupret was superior to that of an immunostimulant (MDP) and of a synthetic secretagogue (ambroxolhydrochloride).

Keywords: Sendai virus; Plant medicine; Respiratory tract infection; T-lymphocytes; Muramyldipeptide; Ambroxol

1. Introduction

The herbal preparation used in our investigations (Sinupret®, manufacturer Bionorica Arzneimittel, Germany), is one of the most popular herbal medicines in Germany for the treatment of sinusitis and infections of the respiratory tract. It has been formulated in 1934 as a combination of five medicinal plants (see below) and remained unchanged since that time. Several controlled clinical studies support the effectiveness of this combination [1,2]. As main pharmacological activities, secretolytic, anti-inflammatory, immunomodulatory, and recently, antiviral activities of the combination and its ingredients were reported [3,4]. Based on the clinical, pharmacological and toxicological data, the preparation was approved by the German regulatory authorities (BfArM) in 1997. From the clinical data, and from pharmacological investigations, a protective ef-

* Corresponding author. Battelle Institut, Regerstr. 6, 55127 Mainz, Germany, Tel.: +49-6131-78384; fax: +49-6131-993848.
E-mail address: ottendorfer@mainz.netsurf.de (D. Ottendorfer).

1567-5769/01/$ - see front matter
PII: S1567-5769(01)00108-4

fect of Sinupret against airway infections was postulated. A pilot study conducted at the school of veterinary medicine in Hannover supported this hypothesis, showing a 40% reduction of mortality in a bacterial infection model. Therefore, a project comprising two studies investigating prophylactic and therapeutic activities, respectively, were designed and realised at Battelle Institute, Frankfurt, using a murine infection model with Parainfluenza virus, strain Sendai (Paramyxoviridae). Here, the study of the activities of a prophylactic treatment protocol is reported. The second study was conducted using a therapeutic treatment regimen. In both experimental studies, the efficacy of different formulations and dosages of the herbal preparation were analysed with respect to mortality rate and survival function, by comparison with a placebo treatment. As active controls, a drug with known secretagogue activities (ambroxol) and a drug with documented immunostimulating activities (*N*-acetyl-muramyl-L-alanyl-D-*iso*-glutamine, MDP), were included. Repeated measurements of peripheral CD4 + and CD8 + T-lymphocyte counts served to monitor the response of the immune system upon the different treatments.

2. Methods

2.1. Animals and animal keeping

The whole study was carried out under Good Laboratory Practice (GLP) conditions. For the infection with Parainfluenza virus, strain Sendai, female mice, 8–12 weeks old (25 g) of the DBA/2J strain were used. Animals were purchased specific pathogen free (SPF) from Charles River, Wiga. All animals were left in quarantine over a period of up to 1 week, followed by an examination by a veterinary physician to monitor the housing conditions. The animals were housed in groups of six in Makrolon cages within a quarantine station. Water and standard nutrition were fed ad libitum.

2.2. Dose range-finding tests for Parainfluenza virus, strain Sendai

The viral agent selected for the study was the Enders strain of Parainfluenza virus 1, strain Sendai. To increase the infectivity and the viral titer of the purchased virus, the stock virus was passaged on chick embryos. For this purpose 12-day embryonated hen's eggs were inoculated into the allantois using different virus stock dilutions (serial 10-fold dilutions); 72 h later, the allantoic fluid was collected by aspiration, cleared by ultracentrifugation and infectivity titers were determined with a group of 50 DBA/2J mice at least. Concentrations between 10^{0} and 10^{-3} were able to kill reproducibly 70% to 90% of the animals within 14 days. Thus, a pool of these three virus concentrations was used to infect the animals to ensure equal conditions in the infection model and to avoid loss of infectivity due to longer storage.

2.3. Dosage of test substances, application and experimental groups

The Sinupret doses administered to the animals are multiples of the human doses (per kilogram): for the drops (SD) the 1-fold (1 ×), 5-fold (5 ×) and 50-fold (50 ×) were chosen; for the tablets (ST) the 1 ×, 5 × and 20 × doses were used. Additionally, a group of animals received ambroxol to assess the contribution of a secretagogue compound (1 × human dose). The bacterial immune response modifier Muramyldipeptide (MDP) was used at a dose of 0.1 mg/animal (i.v.). All other treatments were applied intragastrically (the ground Sinupret sugar-coated tablets were suspended in pyrogen-free 0.9% saline). Daily freshly prepared test and reference solutions/suspensions were used. Control animals received 0.2 ml saline (0.9%). The different treatment groups for the prophylactic as well as for the therapeutic application protocol for the second study comprised eight treatment groups and one control group each with 20 animals. Main outcome criteria were mortality rate or survival time, rsp., and CD4 + and CD8 + lymphocyte counts as the immunological response variable.

2.3.1. Test substances: Sinupret, ambroxol and N-acetyl-muramyl-L-alanyl-D-iso-glutamine (MDP)

Sinupret drops (100 g) contain 29 g of an aqueous–alcoholic extract (extraction agent: ethanol 59% v/v) of 0.2 g *Gentianae radix* (gentian root), 0.6 g *Primulae flos* (primrose flowers), 0.6 g *Rumicis*

herba (sour dock herbs), 0.6 g *Sambuci flos* (elder flowers) and 0.6 g *Verbenae herba* (shop vervain wort herbs). One human dose is 3 ml of the final product. The composition of Sinupret sugar-coated tablets is identical regarding the medicinal plants. One dragee contains 6 mg of *G. radix* (gentian root), 18 mg of *P. flos* (primrose flowers), 18 mg *R. herba* (sour dock herbs), 18 mg *S. flos* (elder flowers) and 18 mg *V. herba* (shop vervain wort herbs). One human dose is two dragees. The preparation is given three times a day. The quality of the herbal ingredients is defined by strong selection criteria reaching beyond the specifications of the German Pharmacopoeia DAB 10 (1996), the standard registrations and the DAC 1986 and 1991 (German pharmaceutical codex). The preparation is produced according to the guidelines of Good Manufacturing Practices (GMP). Specifications and standardised production processes guarantee high batch-to-batch comparability. Sinupret drops and sugar-coated tablets were supplied by the manufacturer. The latter were ground as a whole and applied as suspension. Ambroxol-hydrochloride (commercial preparation) was supplied by Ratiopharm; *N*-acetyl-muramyl-L-alanyl-D-*iso*-glutamine (MDP) was purchased from Sigma.

2.4. Experimental groups in the Sendai virus infection model for the prophylactic application

The different Sinupret formulations, ambroxol and 0.9% saline (placebo) were given on days −3 and −1. MDP (0.1 mg/animal in 0.2 ml 0.9% saline) was administered i.v. on day −1 only, due to previous experiences with murine infection models.

2.5. Infection of the animals with Parainfluenza virus strain Sendai

The animals were infected by the natural, i.e. intranasal, route. Before, they were anesthesized with a mixture of Ketanest® (ketaminhydrochloride solution Parke Davies) and Rompun® 2% solution (Bayer). Then, the virus solution was administered i.n. with a hamilton syringe and the day of the infection is called day 0. After infection, the animals were observed for the following 14 days; mortality was recorded daily.

2.6. Determination of CD4+ and CD8+ T-cells (FACS analysis with peripheral blood)

CD4-positive (T-helper) lymphocytes and CD8-positive lymphocytes (cytotoxic T-cells) were determined by flow cytometry (FACS) as immune response endpoints. As FACS analyses were repeated at least six times, only small blood volumes per animal could be obtained to avoid additional health problems due to blood loss. Thus, the samples had to be pooled from seven, seven and six animals, respectively, to *enable* three independent determinations for each time point in each group. Blood was taken from the retro-orbital plexus by means of a heparinised microcapillary tube and directly transferred into Eppendorf tubes. Staining of the cells was performed with FITC-conjugated antibodies (Becton Dickinson, anti-L3T4 and anti-Ly2). The negative control was a nonspecific mouse IgG isotype. Biotest Institute, Frankfurt, Germany, kindly performed the FACS analyses. The determination of lymphocyte subpopulations in the prophylactic treatment was performed at days 0, +1, +4, +8, +11 and +14.

2.7. Statistics

Survival time was the primary outcome variable. Mice that were still alive at the end of the observation time (14 days) were treated as censored observations. Survival times were compared using the Cox Proportional Hazards Regression Model. Treatment was the only covariate used in the model. This model implies, that the hazard of death (h) of each animal depends on a basic risk, the treatment and the observation time (t). The basic risk ($=h$) is represented by the placebo treatment, whereas the risk in the verum group is expressed by the term $h_{(i)}$. Thus, the following equation results:

$$h_{(i)}(t) = c_{(i)} h_{\text{placebo}}(t).$$

$c_{(i)}$ is the risk ratio (= RR) or the relative hazard of death under verum$_{(i)}$ compared to placebo. If RR = 1, the hazard of death under verum is the same com-

Table 1
Cox regression analysis of maximum likelihood estimates, conditional risk ratio and 95% confidence limits

Variable	DF	Parameter estimate	Standard error	Wald chi-square	Pr > chi-square	Risk ratio	Lower	Upper
MDP	1	−0.479640	0.44145	1.18053	0.2772	0.619	0.261	1.470
Ambroxol	1	−0.426338	0.44115	0.93396	0.3338	0.653	0.275	1.550
ST1 ×	1	−0.137052	0.40846	0.11259	0.7372	0.872	0.392	1.942
ST5 ×	1	−0.178810	0.40852	0.19158	0.6616	0.836	0.376	1.862
ST20 ×	1	0.290877	0.39367	0.54596	0.4600	1.338	0.618	2.893
SD1 ×	1	−1.633811	0.64606	6.39534	0.0114	0.195	0.055	0.692
SD5 ×	1	−1.207638	0.53288	5.13595	0.0234	0.299	0.105	0.849
SD50 ×	1	0.113700	0.39359	0.08345	0.7727	1.120	0.518	2.423

Table 1 summarises the risk ratios for the different treatment groups and shows, for example, that the lower concentrations of the Sinupret drops (SD 5 × and 1 × human dose) were clearly more effective in decreasing the risk ratio compared to the highest doses of both Sinupret formulations (SD 20 × and ST 50 × human dose).

pared to placebo. RR < 1 indicates that the hazard of death under verum is lower relative to placebo. All calculations were done using the statistical software SAS PROC PHREG. The survivor function estimates were not calculated by the Kaplan–Meier-Method, but are generated from the regression results.

To have sufficient amounts of volume, blood samples for the FACS-analyses had to be pooled. An unknown bias is introduced with increasing mortality rates. Thus, we restrict interpretation of lymphocyte counts to the first two measurements, where only little, if any, mortality was recorded. The original data were adjusted to form a common baseline; all evaluations are seen on an exploratory level.

3. Results

Table 1 and Figs. 1–3 summarise the results of the Cox regression analyses for the prophylactic

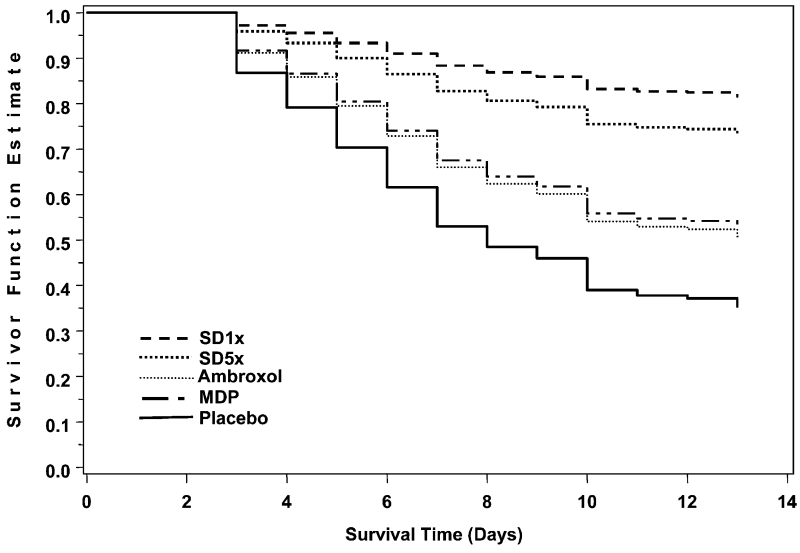

Fig. 1. Compares the survival time (days) for the treatment groups "Sinupret drops" (SD1 × , SD5 ×) and the two active controls ambroxol and muramyldipeptide vs. placebo, given prophylactically to the animals. It can be seen that the prophylactic treatment of the animals with 1 × and 5 × human dose of the Sinupret drops significantly enhanced the survival time of the animals ($p < 0.05$).

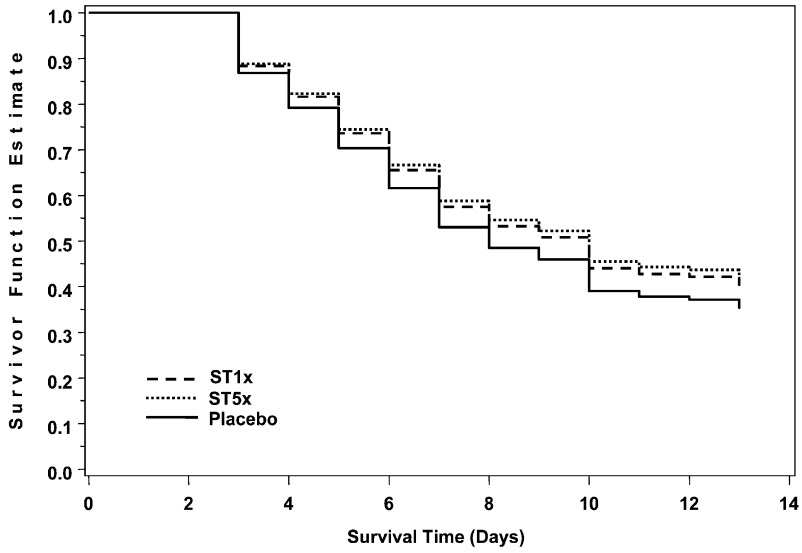

Fig. 2. Illustrates the suvival time (days) of animals for the prophylactic treatment with the Sinupret tablet formulation (ST1 × and ST5 ×) vs. placebo. Compared to the Sinupret drops, the Sinupret tablets were weakly effective.

treatment. Fig. 1 compares the survival time of the animals treated with the lower doses of the Sinupret drops and the two active controls vs. placebo. It can be seen that the prophylactic treatment of animals with 1 × and 5 × human dose of the Sinupret drops (SD1 × , SD5 ×) decreases the hazard to 19% (SD1 ×) resp. 30% (SD5 ×), compared to placebo; see Fig. 1 and Table 1). Both results are statistically significant (Wald chi-square, $p < 0.05$). Remarkably, the two active controls, ambroxol and muramyldipeptide, were less effective than the Sinupret drops (Fig. 1). In contrast, the Sinupret tablet formu-

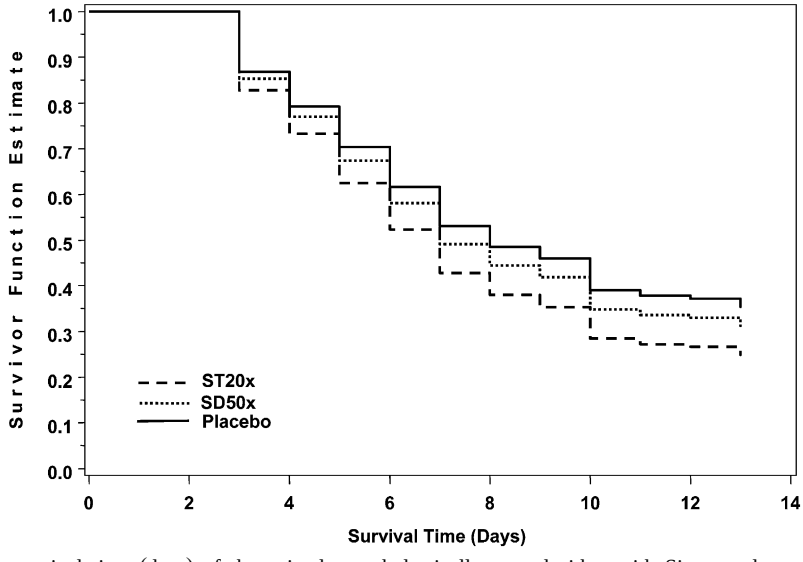

Fig. 3. summarizes the survival time (days) of the animals prophylactically treated either with Sinupret drops (50 × human dose) or Sinupret tablets (20 × human dose) vs. placebo treatment.

lation (ST) given prophylactically proved to be hardly effective in protection of the animals compared to the drops (see Fig. 2); the hazard for ST5× was 83%, for ST1× 87% of the placebo hazard ($p > 0.5$). The highest doses of both Sinupret formulations (ST20×, SD50×, Fig. 3) did not result in a better protection of the animals. The hazard of death for the animals given ST20× was 134%, for SD50× it was 112% of the placebo hazard (compare Fig. 3, not significant).

3.1. Results of the FACS analyses for T-helper and T-suppressor / cytotoxic T-cells

As mortality increases with time, blood volumes obtainable for lymphocyte staining were reduced for FACS analyses. The data from the first two observations (day 0, and day 1 under prophylactic conditions, day 0 and day 4 under therapeutic conditions) are valid and unbiased by mortality. No consistent pattern of correlation between survival time and the CD4+/CD8+ ratios can be detected between the groups. However, smaller, therapeutic doses (1×, 5×) of SD increased this ratio and mean survival time under prophylactic conditions as did smaller doses (1×, 5×) of ST under therapeutic conditions. Further investigations into the mechanisms are recommended.

4. Discussion

The infection of mice with Parainfluenza virus type 1 (Paramyxovirus), strain Sendai, was used to investigate effects of a well established herbal combination for the treatment of airway diseases on the course of disease. Our results show a marked protection from this virus-induced mortality in the prophylactic treatment schedule, demonstrating that Sinupret was generally more effective than both active controls, MDP and ambroxol. The intra-nasal infection with Sendai virus is characterized by an acute bronchiolitis and an interstitial pneumonia [5]. Normally, the disease is completely resolved after 10–14 days (depending upon the dose of infection) predominantly by cell mediated antiviral immunity. In our study, the virus load was such that up to 90% of the animals died after this time. Also speculative at the moment, the protection of animals after peroral application of Sinupret may be a hint for the involvement of the mucosal immune system. Nevertheless, Sendai virus infections are strictly limited to the respiratory tract, where a successful clearance of virus depends on the immunological site mainly but not completely upon virus-specific CD8-positive T-lymphocytes [6,7]. Mucociliary clearance as the physiologic mechanisms is the basic underlying mechanism.

Generally, immunopotentiating drugs, acting through the antigen-specific stimulation of the immune response, accompanied later by cytokine-mediated (e.g. non-antigen specific) enhancement of effector functions of immune cells, participate in successful virus clearance. However, a prophylactic treatment of animals before viral challenge, as in our study, stimulates mainly nonspecific functions of the immune system, such as phagocytosis, release of type 1 interferons or synthesis of T-and B-cell activating cytokines like TNF-alpha, Interleukin-1β or Interleukin-2. Nevertheless, the latter factors can help to potentiate *specific* antiviral immunity later on during the course of infection by further activation/recruitment of the antigen-activated precursor T-cells of the CD8+ and/or CD4+ phenotype.

Concerning our results of the FACS analysis, it has been reported [8] that the antigen-specific activation of precursor T-cells in the regional lymph nodes takes place between days 0 and +4 to +5 after infection. Cells recovered from the blood during this period consequently represent a mixture of T-helper and cytotoxic T-cell precursors (Th_p, CTL_p). As the flow-cytometric differentiation of lymphocytes into CD4- and CD8-positive T-cells can only identify cells phenotypically but not functionally, the application of Sinupret may at least partially be related to changes in the numbers of antigen-specific CD4+ and CD8+ T-cells in the peripheral blood, due to the mainly mucosal and local activation of T-cells [8].

With regard to the results of MDP, showing a slightly better protection compared to placebo (Fig. 1), this result was not unexpected, but those of ambroxol under prophylactic conditions. MDP was shown in numerous reports to be an efficient immunostimulant, capable of enhancing nonspecific immunity [9]. Among multiple mechanisms, secre-

tion of cytokines and activation of monocytes/macropahges, NK-cells and B-lymphocytes were described [9–14].

Concerning the mechanisms of action involved in the positive outcomes of this study, the prophylactic design with two applications of the herbal drugs and ambroxol on days −3 and −1 and one application of MDP on day −1 is a clear hint on immunomodulating effects of responder groups, as a secretolytic activity surely is lasting not more than a few hours. Of interest, ambroxol was shown recently not only to be a potent secretolytic drug. It inhibits the secretion of several pro-inflammatory cytokines and oxygen derived mediators in-vitro [15,16], which may contribute to the amelioration of the virus-induced local cell damage, possibly explaining the unexpected effects of ambroxol in prophylaxis. Since a broad spectrum of immunopharmacological activities has been identified for the herbal combination and its ingredients [17], the results described here confirm the relevance of these in-vitro data. Especially, some nonspecific immune mechanisms like phagocytosis and cytokine production by human leukocytes were dose-dependently influenced by the single components [17], that partially evoked also contradictory effects, which is interesting in the light of the seemingly inhibitory effects of higher concentrations of Sinupret formulations on mortality rates. For example, high concentrations of *R. herba* reduced the zymosan-stimulated oxidative burst in human granulocytes, whereas *G. radix* or *P. flos* were inactive [17].

The combination Sinupret stimulated the granulocytes, but to a lesser extent than the single components *V. herba* and *S. flos*. Similarly, a monocytic cell line, MonoMac 6, showed augmented phagocytosis after pretreatment with SD and *P. flos* as well as SD increased the synthesis of Interleukin-1β in MonoMac 6 cells [17]. The latter cytokine plays a key role in autocrine-mediated T-cell and B-cell differentiation [18].

Concerning the marked enhancement of survival rates for the Sinupret drops at lower concentrations, the cytokine modulating capacities of Sinupret and some of its components seem to be important for the observed effects. The lower protection rates and somewhat contrasting results with high-dose Sinupret formulations may be otherwise *sub-effectively* concentrated substances with anti-inflammatory (inhibitory) characteristics. A prophylactic treatment with immunomodulating agents usually triggers primarily nonspecific defense mechanisms, simply because they do not contain specific antigens (except for vaccinations and some bacterial lysates). In summary, the present results indicate that the herbal combination remedy Sinupret exhibits activities that reach far beyond secretolysis, and may contribute to explain its excellent therapeutic efficacy.

Acknowledgements

The infection model with Sendai virus was conducted at the Battelle Institute Frankfurt, Germany in 1992, sponsored by Bionorica Arzneimittel. The determinations of the lymphocyte subpopulations were kindly performed by Dr. Ernst, Fa. Biotest, Frankfurt, Germany.

References

[1] Neubauer N, März RW. Placebo-controlled, randomized double blind clinical trial with Sinupret sugar-coated tablets on the basis of a treatment with antibiotics and decongestant nasal drops in acute sinusitis. Phytomedicine 1994;1(3): 177–81.

[2] März RW, Ismail C, Popp MA. Profile and effectiveness of a phytogenic combination preparation for treatment of sinusitis. Wien Med Wochenschr (Austria) 1999;149(8–10):202–8.

[3] Schwartner C, Wagner H, Christoffel V. Chemical and pharmacological investigations on *Rumex acetosa*. Oral presentation at the 2nd and International Congress on Phytotherapy, September 11–14, Munich, Germany; 1996. Abstract SL 117.

[4] Chibanguza G, März RW, Sterner W. On the secretolytic and toxic effects of a physiomedical secretolytic drug combination and its componets. Arzneim-Forsch/Drug Res 1984; 34(I):32–6.

[5] Parker JC, Whiteman MD, Richter CB. Susceptibility of inbred and outbred mouse strains to sendai virus and prevalence of infection in laboratory rodents. Am Soc Microbiol 1978;19:123–30.

[6] Doherty PC. Anatomical environment as a determinant in viral immunity. J Immunol 1995;155:1023–7.

[7] Doherty PC, Hamilton-Easton AM, Topham DJ, Riberdy J, Brooks JW, Cardin RD. Consequences of viral infections for lymphocyte compartmentalization and homeostasis. Immunology 1997;9:365–73.

[8] Doherty PC. Cell mediated immunity in virus infections. Biosci Rep 1997;17:367–87.

[9] Masihi KN, Lange W, editors. Immunomodulators and non-specific host defence mechanisms against microbial infections. Advances in the biosciences, vol. 68, Section 1. Oxford: Pergamon; 1997. p. 3–29.

[10] Yamaguchi F, Akasaki M, Tsukada W. Induction of Colony stimulating factor and stimulation of stem cell proliferation by injection of muroctasin. Arzneim-Forsch/Drug Res 1988; 38(II, 7A):980–3.

[11] Chedid L, Parant M, Lefrancher P, Choay J, Lederer E. Enhancement of non-specific immunity to *Klebsiella pneumoniae* infection by a synthetic immunoadjuvant (*N*-acetylmuramyl-L-alanyl-D-isoglutamine) and several analogs. Proc Natl Acad Sci 1977;74(5):2089–93.

[12] Suzu S, Yokota H, Yamada M, et al. Enhancing effect of human monocytic colony stimulating factor on monocyte tumoricidal activity. Cancer Res 1989;49:5913–7.

[13] Moore RN, Larsen HS, Horohov DW, Rouse BT. Endogenous regulation of macrophage proliferative expansion by colony-stimulating-factor induced interferon. Science 1984; 223:178–81.

[14] Tamura M, Yoo YC, Yoshimatsu K, et al. Effect of muramyl dipeptide derivatives as adjuvants on the induction of antibody response to recombinant hepatitis B surface antigen. Vaccine 1995;3:77–82.

[15] Suzuki M, Teramoto S, Matsuse T, Ohga E, Katayama H, Fukuchi Y, et al. Inhibitory effect of ambroxol on superoxide anion production and generation by murine lung alveolar macrophages. J Asthma 1998;35:267–72.

[16] Pfeifer S, Zissel G, Kienast K, Muller-Quernheim J. Reduction of cytokine release of blood and bronchoalveolar mononuclear cells by ambroxol. Eur J Med Res 1997; 24:129–32.

[17] Schmolz M. Immunological features of extracts from *Radix gentianae*, *Flores primulae*, *Flores sambuci*, *Herba verbenae* and *Herba rumicis* as well as from a combination thereof (Sinupret®). Oral presentation at the 4th and International Congress on Phytotherapy, September 10–13, Munich, Germany; 1992. Abstract SL 36.

[18] Kirckpatrick CH. Biological response modifiers: interferons, interleukins, and transfer factor. Ann Allergy 1989;62:170–5.