ELSEVIER

Antiviral Research 26 (1995) 161–171

Antiviral Research

Inhibition of herpes simplex virus type 1, respiratory syncytial virus and echovirus type 11 by peroxidase-generated hypothiocyanite

Hannamari Mikola [a], Matti Waris [b], Jorma Tenovuo [a,*]

[a] *Department of Cariology, Institute of Dentistry, University of Turku, Lemminkäisenkatu 2, FIN-20520 Turku, Finland*

[b] *Department of Virology, Faculty of Medicine, University of Turku, Kiinamyllynkatu 13, FIN-20520 Turku, Finland*

Received 13 June 1994; accepted 24 October 1994

Abstract

The human mouth is an important route of viral transmission and evidence exists that human saliva can neutralize some viruses, e.g. herpes simplex type 1 (HSV-1) and human immunodeficiency virus (HIV) in vitro. However, little is known of the actual antiviral agents in saliva. We have analyzed how hypothiocyanite (HOSCN/$^-$OSCN) ions, present in human saliva and generated by salivary peroxidase systems, affect the viability of three different types of viruses: HSV-1 (capable of inducing oral lesions), respiratory syncytial virus (RSV, respiratory infections), and echovirus 11 (EV 11, enteric diseases). Viral suspensions were pretreated (30 min) with HOSCN/$^-$OSCN concentrations up to 180 μM both at pH 6.0 and 7.1 and inoculated into human gingival fibroblasts. The cultures were incubated at 37°C for 18–48 h, fixed and the infected cells were counted after immunoperoxidase staining. HSV-1 was most sensitive to HOSCN/$^-$OSCN with an IC_{50} of 8.5 μM at pH 6.0 and an IC_{50} of 20 μM at pH 7.1, respectively. RSV was inhibited by HOSCN/$^-$OSCN only at pH 6.0 with an IC_{50} of 8.0 μM. EV 11 was also resistant at neutral pH, but sensitive at pH 6.0 with an IC_{50} of 68 μM. In contrast to HSV-1 and RSV, the inhibition of EV 11 was not dependent on the concentration of HOSCN/$^-$OSCN. The inhibition was in all cases stronger at pH 6.0 than at neutral pH. Our

* Corresponding author. Fax: +358 (21) 6338300.

SSDI 0166-3542(94)00073-5

results suggest that hypothiocyanite, a normal component of human whole saliva, in physiological concentrations effectively inhibits HSV-1 and RSV at acidic pH, whereas EV 11 is more resistant in vitro.

Keywords: HSV-1; RSV; Echovirus; Peroxidase; Hypothiocyanite; Saliva

1. Introduction

Human saliva contains a number of antimicrobial agents which control the multiplication and metabolism of various oral bacteria and fungi (Mandel, 1987). These agents include both innate, non-immunoglobulin (lysozyme, lactoferrin, peroxidases, agglutinins, histidine-rich peptides) proteins as well as acquired factors, such as IgA, IgG and IgM antibodies (Brandtzaeg, 1989; Tenovuo, 1989). A large number of studies on their effects on bacteria and fungi are available, but much less is known of their possible effects against viruses. We considered this an important issue, since the human mouth is a major port of entry for viruses into the human body and saliva is the first body fluid to come into contact with orally transmitted viruses.

Although some studies have shown that human saliva can inhibit viruses, such as herpes simplex type 1 (HSV-1) (Gyselink et al., 1978) and human immunodeficiency virus type 1 (HIV) (Fox et al., 1988; Archibald and Cole, 1990; Fox, 1992; Archibald et al., 1993; Robinovitch et al., 1993), very little is known of the actual antiviral agents present in saliva. Archibald and Cole (1990) identified anti-HIV activity in submandibular/sublingual saliva, but not in parotid saliva. The anti-HIV activity seemed to be associated with the presence of salivary mucins. In contrast to HIV, HSV-1 neutralizing activity also exists to some extent in human parotid saliva (Bergey et al., 1993), but the main part of inhibition against HSV-1 seems to be due to serum-derived IgG antibodies present in whole saliva (Gyselink et al., 1978).

Interestingly, peroxidase-mediated antimicrobial systems present in human saliva effectively inhibit both HIV (Pourtois et al., 1990, 1991; Yamaguchi et al., 1993), HSV-1 (Courtois et al., 1990), and polioviruses (Belding et al., 1970) in vitro. In the present study, we have analyzed the inhibitory activity of the antibacterial/antifungal hypothiocyanite ($HOSCN/^{-}OSCN$) ions, produced by the oral peroxidase/SCN^{-}/H_2O_2 system, against three different types of viruses. The viruses chosen for this study were HSV-1 (an oral pathogen), respiratory syncytial virus (RSV, a respiratory virus) and echovirus 11 (EV 11, an enteropathogen). These three different viruses were chosen to represent the assumed differences in their ability to escape saliva-mediated virucidal systems, such as $HOSCN/^{-}OSCN$.

2. Materials and methods

2.1. Cells and viruses

All experiments were done with human gingival fibroblasts (HGFs), which were isolated from clinically healthy gingiva of a 27-year-old woman. These cells, previously

characterized by Kähäri et al. (1991), were used between passages 6 and 12 for the experiments. The viruses used were HSV type 1 (HSV-1, strain F), RSV (RSV; strain Randall) and echovirus 11 (EV 11; strain Gregory). HSV-1 and EV 11 were obtained from the American Type Culture Collection (ATCC, Rockville, MD). EV 11 was plaque-purified and shown to be neutralized with specific antiserum (ATCC) (Auvinen and Hyypiä, 1990). RSV originated from Dr. Taylor-Robinson (Harvard Hospital, Salisbury, UK) and it was plaque-purified before the current studies. Working stocks were produced in HGFs and stored frozen at −80°C. The titers of the stocks were 2.0×10^9, 2.7×10^5 and 6.7×10^7 plaque-forming units (PFU)/ml for HSV, RSV and EV 11, respectively.

2.2. Preparation of cell cultures

Approximately 80,000 HGF cells in 1.0 ml of Dulbecco's modification of Eagle's medium (Gibco BRL, Paisley, UK), containing 10% fetal calf serum (FCS; PAA Labor- und Forschungsges. M.B.H., Linz, Austria) and antibiotics, were seeded in 24-well tissue culture clusters (Costar, Badhoevedorp, The Netherlands) and incubated at 37°C until confluency. Prior to the addition of the studied agent, growth medium was replaced by 800 μl of maintenance medium supplemented with 2% of FCS and antibiotics.

2.3. Preparation of the HOSCN/$^-$OSCN-virus mixtures

Lactoperoxidase (LP), prepared from bovine milk, was a product of Sigma Chemical Co. (St. Louis, MO) and had a purity index (A_{412}/A_{280}) of 0.81. Human salivary peroxidase has several characteristics in common with LP (Månsson-Rahemtulla et al., 1988), so that in spite of some differences in many physical and kinetic properties, LP is frequently used as a model of salivary peroxidase in microbiological studies (Tenovuo, 1991). $(Nbs)_2$ [5,5′-dithiobis(2-nitrobenzoic acid)] was purchased by Aldrich Chemical Co. (Milwaukee, WI). Before use it was reduced to Nbs (5-thio-2-nitrobenzoic acid) with 2-mercaptoethanol as described previously (Pruitt et al., 1983). Hydrogen peroxide was obtained as a 30% solution (E. Merck AG, Darmstadt, Germany) and stored at 4°C. Potassium thiocyanate (KSCN) was a product of E. Merck AG. The VMG buffer, containing (in mmol/l) 74 NaCl, 6 KCl, 4 $Na_2HPO_4 \times 7\ H_2O$, 7 KH_2PO_4, 2 $CaCl_2$, 46 sodium β-glycerophosphate and 0.5 $MgCl_2$, used in all experiments, was prepared according to Möller (1966). The pH was adjusted by the addition of 1.0 M HCl and the buffer was sterilized before use.

For all experiments, the solutions were freshly prepared in sterilized tubes prior to the addition of the virus. KSCN and LP were dissolved in VMG buffer (pH 7.1 or 6.0) and the HOSCN/$^-$OSCN was generated by adding dropwise 2.5–50 μl of H_2O_2 into the solution during 1 min. The final concentration was 0.5 mM for KSCN and 10 μg/ml for LP. The solution was combined with equal volume of appropriate virus suspension freshly prepared from the stock in VMG (HSV 2.0×10^6, RSV 1.35×10^4 and EV 11 6.7×10^5 PFU/ml), vortexed and incubated at 37°C for 30 min. No notable pH change (< 0.12 pH units) was observed during the 30-min incubation. The control cultures,

incubated in the same way as the test cultures, comprised: (1) VMG buffer alone; (2) culture supplemented with the highest H_2O_2 concentration used (250 μM); (3) culture with LP and KSCN (but with no H_2O_2); and (4) culture supplemented with the whole LP system (LP/KSCN/H_2O_2) but with no viruses. The HOSCN/$^-$OSCN concentration was determined by reaction with the colored anionic monomer of $(Nbs)_2$ as described by Aune and Thomas (1977) and modified by Pruitt et al. (1986).

The possible cytotoxicity of HOSCN/$^-$OSCN against HGFs was assessed by light microscopy after 30 min incubation at 37°C of HGFs exposed to HOSCN/$^-$OSCN (175 μmol/l) without any viruses. Previous studies indicated no cytotoxicity of HOSCN/$^-$OSCN against HGFs (Tenovuo and Larjava, 1984).

Ten-fold dilutions of the treated or control virus suspensions were made in the maintenance medium and inoculated into duplicate cultures (200 μl/well). The plates were centrifuged at 25°C in a Sorvall Technospin R centrifuge at 740 *g* for 45 min. The plates were then immediately placed in 5% CO_2-atmosphere at 37°C and incubated to the exponential phase, i.e. for 16–18 h (HSV-1 and EV 11) or 46–48 h (RSV).

2.3. Immunoperoxidase staining of the virus-infected cells

Viruses were detected by using direct immunoperoxidase-staining (IPS) with monoclonal antibodies (Mabs) for HSV-1 and RSV and indirect IPS with polyclonal antibodies (Pabs) for EV 11. Specific Mabs against HSV-1 (2/7/30) (Ziegler et al., 1985) and RSV (101A) (Waris et al., 1990) have been described earlier. The antibodies were labeled with horseradish peroxidase (HRP, type 6; Sigma Chemical Co.) for IPS (Wilson and Nakane, 1978). EV 11-specific Pabs were produced in rabbit by using heat-inactivated virus. Peroxidase-conjugated swine immunoglobulins (Igs) to rabbit Igs (anti-rabbit) were obtained from Dako-immunoglobulins a/s (Copenhagen, Denmark). After appropriate incubation times the maintenance medium was aspirated and the cells were rinsed twice with phosphate-buffered saline (PBS). The cells were fixed at room temperature for 15 min with methanol (HSV-1, EV 11) or cold 75% acetone in PBS (RSV). The fixative was removed and cells were rinsed twice with PBS containing 0.1% Tween 20 (PBS-T). HRP-labeled Mabs were diluted 1 : 500 (HSV-1), 1 : 2000 (RSV) and Pabs 1 : 1000 (EV 11) in PBS supplemented with 5% fat-free dry milk (Valio, Helsinki, Finland) and 0.01% antifoaming agent (Cuplaton, Orion Co., Espoo, Finland) (PBS-M). An amount of 300 μl of appropriate antibody dilution was added to each well and incubated for 1 h at 37°C. In the EV 11 assay, the wells were washed twice with PBS-T and once with PBS and the incubation was repeated with anti-rabbit Igs diluted 1 : 200 in PBS-M. Before staining, the wells were washed twice with PBS-T and once with PBS.

The stain contained 2.0 mg of 3-amino-9-ethylcarbazole (Sigma Chemical Co.), dissolved in 0.5 ml of dimethylformamide (BDH Ltd, Poole, UK) and brought up to final volume by adding 9.5 ml of acetate buffer (pH 5.0). Just before plate staining, 10 μl of 30% H_2O_2 was added and 300 μl of the substrate solution was pipetted into each well. The plates were left at room temperature until the plaques were clearly distinguishable for reading the results by light microscopy (approximately 30 min).

3. Results

All experiments were done at pH 7.1 and 6.0. Although for different viruses variable titers were used, it was observed that almost identical HOSCN/$^-$OSCN-mediated inhibition curves were found despite the differences in the final titers (Figs. 1–3). In order to obtain a wide range of observations, IPS plaque titration was performed in three 10-fold dilutions. When present, the inhibition was similar in all dilutions. Because of the pronounced decrease in RSV-titers at low pH, pH 6.0 was chosen to represent the acidic conditions in the experiments. Compared to pH 7.1, the control RSV-titers decreased by 27 and 47% at pH 6.0 and 5.0, respectively.

Hypothiocyanite displayed variable inhibitory effects on viruses depending on the HOSCN/$^-$OSCN concentration, pH and the virus studied (Figs. 1–3). No significant inhibition of any virus and no detectable HOSCN/$^-$OSCN generation were observed if any of the components (enzyme-SCN^-, H_2O_2) of the lactoperoxidase system was omitted, neither did H_2O_2 alone have any inhibitory effect. The complete peroxidase

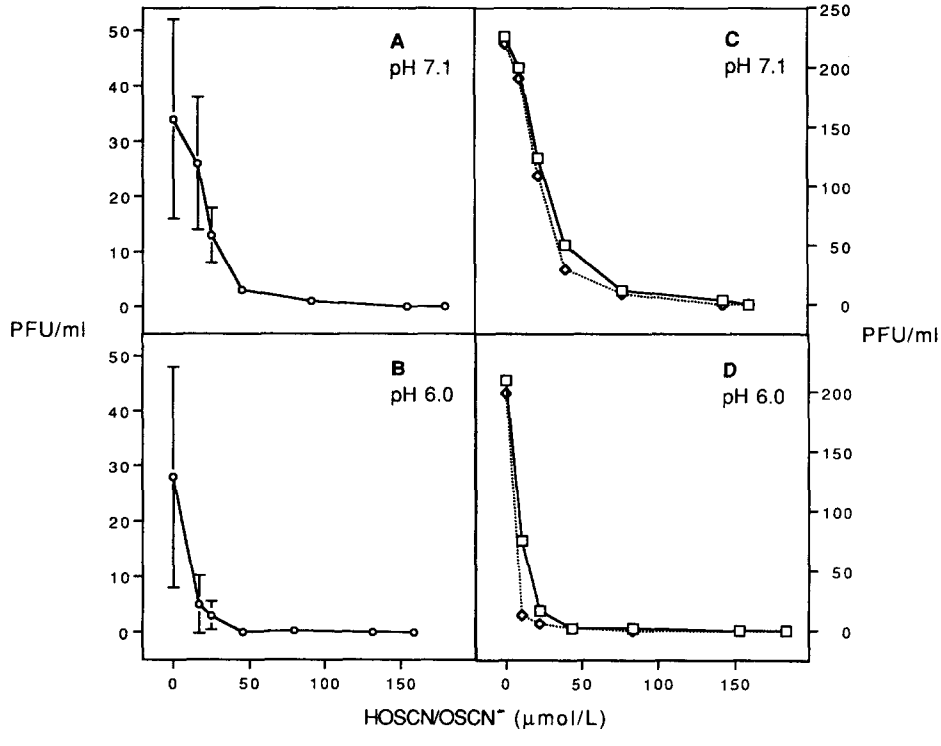

Fig. 1. Inhibition of HSV-1 (PFU/ml) by peroxidase-generated HOSCN/$^-$OSCN at pH 7.1 and 6.0. (A, B) The values indicate mean ± S.D. of 3 experiments, in which the incubation titer was 2.0×10^6 PFU/ml, followed by a 1 : 300 dilution to get the final PFU/ml. (C, D) Results of experiments in which the incubation titer was either 3.5×10^5 (□) or 3.5×10^4 (◇) PFU/ml. The dilutions made after the incubation giving the final PFUs/ml were 1 : 600 (□) and 1 : 60 (◇), respectively.

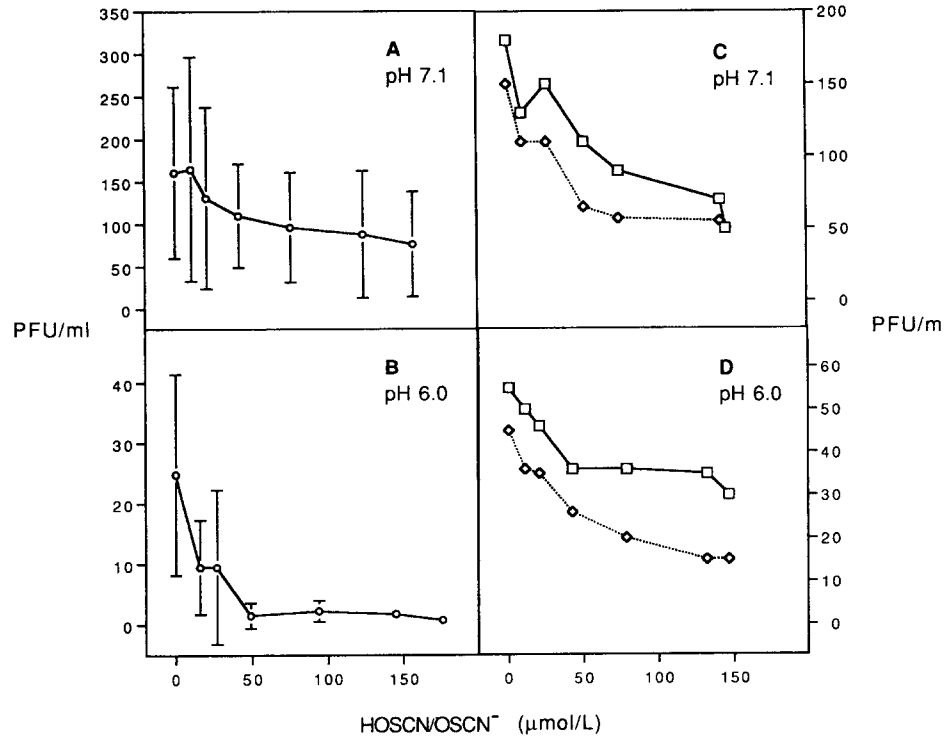

Fig. 2. Inhibition of RSV (PFU/ml) by peroxidase-generated HOSCN/$^-$OSCN at pH 7.1 and 6.0. (A, B) The values indicate mean ± S.D. of 3 experiments, in which the incubation titer was 1.35×10^4 PFU/ml, followed by a 1 : 60 dilution to get the final PFU/ml. (C, D) Results of experiments in which the incubation titer was either 2.5×10^5 (□) or 2.5×10^4 (◇) PFU/ml. The dilutions made after the incubation to get the final PFUs/ml were 1 : 600 (□) and 1 : 60 (◇), respectively.

system without any viruses did not affect HGFs, as observed by light microscopy, suggesting that HOSCN/$^-$OSCN, as such, had no cytotoxic effect. All these findings indicate that HOSCN/$^-$OSCN was indeed the antiviral agent in this system.

No significant difference existed between pH 6.0 and 7.1 in the HSV-1 control titer (Fig. 1). HSV-1 was effectively inhibited by HOSCN/$^-$OSCN and the virus was slightly more sensitive at pH 6.0 ($IC_{50} = 8.5$ μM) than at pH 7.1 ($IC_{50} = 20$ μM) (Fig. 1).

The RSV control titer was remarkably lower at acidic pH than at pH 7.1 (Fig. 2). The inhibition of RSV by HOSCN/$^-$OSCN was strongly pH-dependent: the IC_{50} was as low as 8 μM at pH 6.0, while at pH 7.1 the IC_{50} value could not even be calculated due to the almost non-existing inhibition, even with the highest HOSCN/$^-$OSCN concentration studied (157 μM) (Fig. 2).

EV 11 reacted to HOSCN/$^-$OSCN in a very different way than HSV-1 and RSV. EV 11 tolerated the acidic pH well and the original titer was even higher at pH 6.0 than at neutral pH (Fig. 3). At pH 7.1, the HOSCN/$^-$OSCN seemed to be totally ineffective

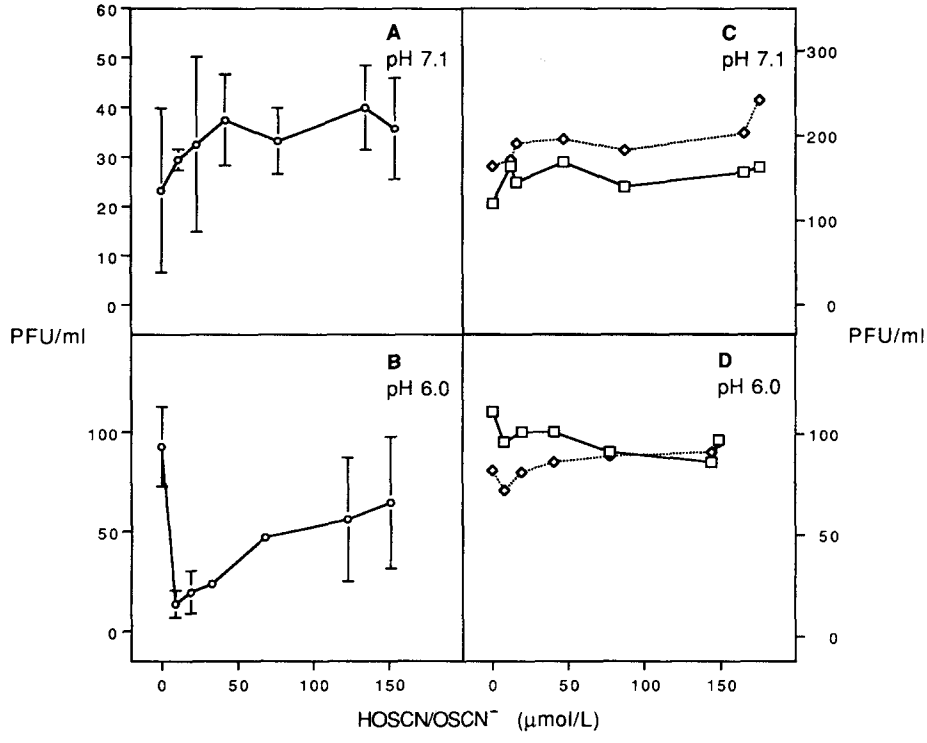

Fig. 3. Inhibition of EV 11 (PFU/ml) by peroxidase-generated HOSCN/$^-$OSCN at pH 7.1 and 6.0. (A, B) The values indicate mean ± S.D. of 3 experiments, in which the incubation titer was 6.7×10^5 PFU/ml, followed by a 1 : 60 dilution to get the final PFU/ml. (C, D) Results of experiments in which the incubation titer was 1.1×10^5 (□) and 1.1×10^4 (◇) PFU/ml. The dilutions made after the incubation to get the final PFUs/ml were 1 : 600 (□) and 1 : 60 (◇), respectively.

against EV 11, even at high concentrations, but at lower pH (6.0) as low concentrations of HOSCN/$^-$OSCN as 10–100 μM effectively inhibited the virus ($IC_{50} = 68$ μM). The lower the HOSCN/$^-$OSCN concentration was, the more EV 11 was inhibited, but with no effect at neutral pH (Fig. 3). Surprisingly, HOSCN/$^-$OSCN concentrations $\geqslant 100$ μM had no statistically significant inhibitory effect on EV 11, not even at low pH.

4. Discussion

The three different viruses selected for this study may theoretically differ in their susceptibility to salivary neutralizing agents. HSV-1 represents a virus, which is frequently transmitted by saliva, which may cause intraoral lesions (Scully and Bagg, 1992) and which, according to serum HSV-1 antibody analyses, has a prevalence of

approximately 70–80% (Nahmias and Roizman, 1973). Intraoral shedding of HSV-1 has been documented in approximately 3–7% of the population (Kameyama et al., 1988).

The role of salivary antibodies in neutralizing HSV-1 is obvious. Antibodies, mainly IgG and IgM isotypes but to a lesser degree also IgA, mediate viral neutralization and are directed either against virus-infected cell proteins exposed on the surface of infected cells or directly binding to extracellular viruses (Norrild, 1985). A suppressed immune response, such as in cancer patients receiving therapeutic irradiation to the head and neck regions, easily leads to HSV-1 reactivation and recurrent infections (Hedner, 1993). Cancer treatment also impairs the function of the salivary peroxidase system (Månsson-Rahemtulla et al., 1992), which according to the present results, may influence the intraoral activity of HSV-1.

According to our study, HSV-1 is sensitive to HOSCN/$^{-}$OSCN in physiological salivary concentrations (Tenovuo et al., 1982), in particular at pH 6.0. The sensitivity of HSV-1 to the peroxidase system has been previously reported by Courtois et al. (1990), who, however, did their experiments only with $^{-}$OSCN concentration of 100 μM and at pH 7.4. It is likely that neutralization of HSV-1 in saliva occurs both by serum-derived antibodies (mainly IgG and IgM isotypes) as well as by peroxidase-generated HOSCN/$^{-}$OSCN.

We observed that the virucidal activity of the peroxidase system was more effective at acidic pH (6.0), as also observed with bacterial species (Thomas et al., 1983; Tenovuo et al., 1988). Belding et al. (1970) found that the virucidal effect of the peroxidase system against poliovirus was strongest at pH 4.5, but some virucidal activity could also be detected at neutral pH. In HIV-HOSCN/$^{-}$OSCN studies, the pH has been 7.4 (Pourtois et al., 1990) or has not been given (Yamaguchi et al., 1993). Since the HOSCN/$^{-}$OSCN-mediated virucidal activity seems to be both time- (Courtois et al., 1990; Yamaguchi et al., 1993) and dose-dependent, the above studies indicate that the pH range should also be analyzed in more detail. The pH of human whole saliva may range between 5 and 8, depending on, for example, the diet.

Interestingly, activation of the salivary peroxidase system has been reported to reduce the incidence of oral aphthous ulcers by 70–80% (Hoogendoorn, 1985). Whether this has anything to do with viral neutralization is as yet unknown, but it is interesting to note that DNA from another herpes virus, varicella zoster, is frequently detected in oral recurrent aphthous ulcers (Pedersen et al., 1993). Thus, a possibility exists that if herpes viruses are pathogenic in oral ulcers, HOSCN/$^{-}$OSCN could act as a non-immune defense against these lesions.

RSV is a respiratory virus which is not known to induce any oral symptoms. It turned out to also be relatively sensitive to neutralization by HOSCN/$^{-}$OSCN, but only at acidic pH. The studied enteropathogen, EV 11, can be assumed to pass fast through the oral cavity, and at neutral pH, no inhibition at all of EV 11 was detected. However, an unexplained inhibition by low (10–100 μM) concentrations of HOSCN/$^{-}$OSCN was constantly detected at pH 6.0. Although some inhibition of EV 11 by HOSCN/$^{-}$OSCN could be detected in vitro, this virus was, as expected, clearly most resistant to peroxidase-mediated inhibition. The virucidal mechanism of HOSCN/$^{-}$OSCN is as yet unknown. We showed the inactivation of free viruses, but recently Yamaguchi et al. (1993) also demonstrated peroxidase-mediated inhibition of cytopathic effects in already

HIV-1 infected cells. Thus, it may be concluded that HOSCN/$^-$OSCN has potential virucidal activity, not only against free, but also against cell-associated viruses.

In summary, although the studied viruses were susceptible to HOSCN/$^-$OSCN in vitro, this inhibition, as such, does not explain differences in their infectivity in vivo. First, due to many practical reasons, we did our studies with HGFs, although gingival and mucosal keratinocytes are more likely to be the first cells to come into contact with orally transmitted viruses in vivo. Secondly, although HSV-1 was very susceptible to HOSCN/$^-$OSCN, this virus can still cause oral lesions in spite of the presence of saliva. EV 11, which has to pass the oral defense systems, is least sensitive to HOSCN/$^-$OSCN. Our results support the assumption that oral peroxidase systems are not only antibacterial (Pruitt and Reiter, 1985) and antifungal (Lenander-Lumikari et al., 1992), but also, to some extent, antiviral. This is of particular interest since HOSCN/$^-$OSCN-generating (up to 250 μM) dentifrices, mouthrinses and moisturizing gels have recently come onto the market in the USA and in many European countries (Lenander-Lumikari et al., 1993). The generation of HOSCN/$^-$OSCN in saliva has resulted in HIV-1 inactivation (Pourtois et al., 1991), but our results suggest that this enhancement of the salivary peroxidase system may provide inhibition against other viruses as well.

Acknowledgements

This work was supported by the Academy of Finland, the Sigrid Juselius Foundation, and the Finnish Dental Society. Dr. Hannu Larjava (The University of British Columbia, Vancouver, Canada) is gratefully acknowledged for providing the human gingival fibroblast cell lines for viral cultivations.

References

Archibald, D.W. and Cole, G.A. (1990) In vitro inhibition of HIV-1 infectivity by human salivas. AIDS Res. Hum. Retroviruses 6, 1425–1432.

Archibald, D.W., Hebert, C.A., Gregory, K.L. and Lewis, G.K. (1993) Effects of human salivas on recombinant HIV-1 proteins. Crit. Rev. Oral Biol. Med. 4, 475–478.

Aune, T.M. and Thomas, E.L. (1977) Accumulation of hypothiocyanite ion during peroxidase-catalyzed oxidation of thiocyanate ion. Eur. J. Biochem. 80, 209–214.

Auvinen, P. and Hyypiä, T. (1990) Echoviruses include genetically distinct serotypes. J. Gen. Virol. 71, 2133–2139.

Belding, M.E., Klebanoff, S.J. and Ray, C.G. (1970) Peroxidase-mediated virucidal systems. Science 167, 195–196.

Bergey, E.J., Gu, M., Collins, A.R., Bradway, S.D. and Levine, M.J. (1993) Modulation of herpes simplex virus type 1 replication by human salivary secretions. Oral Microbiol. Immunol. 8, 89–93.

Brandtzaeg, P. (1989) Salivary immunoglobulins. In: J. Tenovuo (Ed.), Human Saliva: Clinical Chemistry and Microbiology, Vol. II. CRC Press, Boca Raton, FL, pp. 1–54.

Courtois, P., Van Beers, D., De Foor, M., Mandelbaum, I.M. and Pourtois, M. (1990) Abolition of herpes simplex cytopathic effect after treatment with peroxidase generated hypothiocyanite. J. Biol. Buccale 18, 71–74.

Fox, P.C. (1992) Salivary gland involvement in HIV-1 infection. Oral Surg. Oral Med. Oral Pathol. 73, 168–170.

Fox, P.C., Wolff, A., Yeh, C.-K., Atkinson, J.C. and Baum, B.J. (1988) Saliva inhibits HIV-1 infectivity. J. Am. Dent. Assoc. 116, 635–637.

Gyselink, R., Coles, D., Ash, R.J. and Fritz, M.E. (1978) Salivary neutralizing activity against herpes simplex virus type 1. J. Infect. Dis. 137, 583–586.

Hedner, E. (1993) Herpes simplex virus type 1 and intraoral wound healing (dissertation). Univ. of Goethenborg, Sweden.

Hoogendoorn, H. (1985) Activation of the salivary peroxidase antimicrobial system: clinical studies. In: K.M. Pruitt and J. Tenovuo (Eds.), The Lactoperoxidase System: Chemistry and Biological Significance. Marcel Dekker, New York, pp. 217–227.

Kähäri, V.M., Larjava, H. and Uitto, J. (1991) Differential regulation of extracellular matrix proteoglycan (PG) gene expression. Transforming growth factor-β1 up-regulates biglycan (PGI), and versican (large fibroblast PG) but down-regulates decorin (PGII) mRNA levels in human fibroblasts in culture. J. Biol. Chem. 266, 10608–10615.

Kameyama, T., Sujaku, C., Yamamoto, S., Hwang, C.B.C. and Shillitoe, E.J. (1988) Shedding of herpes simplex virus type 1 into saliva. J. Oral Pathol. 17, 478–481.

Lenander-Lumikari, M. (1992) Inhibition of *Candida albicans* by the peroxidase/SCN^-/H_2O_2 system. Oral Microbiol. Immunol. 7, 315–320.

Lenander-Lumikari, M., Tenovuo, J. and Mikola, H. (1993) Effects of a lactoperoxidase system-containing toothpaste on levels of hypothiocyanite and bacteria in saliva. Caries Res. 27, 285–291.

Mandel, I.D. (1987) The functions of saliva. J. Dent. Res. 66, 623–627.

Månsson-Rahemtulla, B., Rahemtulla, F., Baldone, D.C., Pruitt, K.M. and Hjerpe, A. (1988) Purification and characterization of human salivary peroxidase. Biochemistry 27, 233–239.

Månsson-Rahemtulla, B., Techanitiswad, T., Rahemtulla, F., McMillan, T.O., Bradley, E.L. and Wahlin, Y.B. (1992) Analyses of salivary components in leukemia patients receiving chemotherapy. Oral Surg. Oral Med. Oral Pathol. 73, 35–46.

Möller, Å. (1966) Microbiological examination of root canals and periapical tissues of human teeth. Scand. J. Dent. Res. 74, 1–380.

Nahmias, A.J. and Roizman, B. (1973) Infection with herpes simplex viruses 1 and 2. New Engl. J. Med. 289, 667–674.

Norrild, B. (1985) Humoral response to herpes simplex virus infections. In: B. Riozman and C. Lopez (Eds.), The Herpesviruses, Plenum Press, New York, pp. 69–86.

Pedersen, A., Madsen, H.O., Vestergaard, B.F. and Ryder, L.P. (1993) Varicella-zoster virus DNA in recurrent aphthous ulcers. Scand. J. Dent. Res. 101, 311–313.

Pourtois, M., Binet, C., Van Tieghem, N., Courtois, P., Vandenabbeele, A. and Thiry, L. (1990) Inhibition of HIV infectivity by lactoperoxidase-produced hypothiocyanite. J. Biol. Buccale 18, 251–253.

Pourtois, M., Binet, C., Van Tieghem, N., Courtois, P., Vandenabbeele, A. and Thiry, L. (1991) Saliva can contribute in quick inhibition of HIV infectivity. AIDS 5, 589–600.

Pruitt, K.M. and Reiter, B. (1985) Biochemistry of peroxidase system: antimicrobial effects. In: K.M. Pruitt and J. Tenovuo (Eds.), The Lactoperoxidase System: Chemistry and Biological Significance, Marcel Dekker, New York, pp. 143–178.

Pruitt, K.M., Månsson-Rahemtulla, B. and Tenovuo, J. (1983) Detection of the hypothiocyanite ($OSCN^-$) ion in human parotid saliva and the effect of pH on $OSCN^-$ generation in the salivary peroxidase antimicrobial system. Arch. Oral Biol. 28, 517–525.

Pruitt, K.M., Tenovuo, J., Månsson-Rahemtulla, B., Harrington, P. and Baldone, D.C. (1986) Is thiocyanate peroxidation at equilibrium in vivo? Biochim. Biophys. Acta 870, 385–391.

Robinovitch, M.R., Iversen, J.M. and Resnick, L. (1993) Anti-infectivity activity of human salivary secretions toward human immunodeficiency virus. Crit. Rev. Oral Biol. Med. 4, 455–459.

Scully, C. and Bagg, J. (1992) Viral infections in dentistry. Curr. Opin. Dent. 2, 102–115.

Tenovuo, J. (1989) Nonimmunoglobulin defense factors in human saliva. In: J. Tenovuo (Ed.), Human Saliva: Clinical Chemistry and Microbiology, Vol II. CRC Press, Boca Raton, FL, pp. 55–92.

Tenovuo, J. (1991) Salivary peroxidase. In: J. Everse, K.E. Everse and M.B. Grisham (Eds.), Peroxidases in Chemistry and Biology, Vol I. CRC Press, Boca Raton, FL, pp. 181–197.

Tenovuo, J. and Larjava, H. (1984) The protective effect of peroxidase and thiocyanate against hydrogen

peroxide toxicity assessed by the uptake of [^{3}H]-thymidine by human gingival fibroblasts cultured in vitro. Arch. Oral Biol. 29, 445–451.

Tenovuo, J., Pruitt, K.M. and Thomas, E.L. (1982) Peroxidase antimicrobial system of human saliva: hypothiocyanite levels in resting and stimulated saliva. J. Dent. Res. 61, 982–985.

Tenovuo, J., Anttila, O., Lumikari, M. and Sievers, G. (1988) Antibacterial effect of myeloperoxidase against *Streptococcus mutans*. Oral Microbiol. Immunol. 3, 68–71.

Thomas, E.L., Pera, K.A., Smith, K.W. and Chwang, A.K. (1983) Inhibition of *Streptococcus mutans* by the lactoperoxidase antimicrobial system. Infect. Immun. 39, 767–778.

Waris, M., Ziegler, T., Kivivirta, M. and Ruuskanen, O. (1990) Rapid detection of respiratory syncytial virus and influenza A virus in cell cultures by immunoperoxidase staining with monoclonal antibodies. J. Clin. Microbiol. 28, 1159–1162.

Wilson, M.B. and Nakane, P.K. (1978) Recent developments in the method of conjugating horseradish peroxidase (HRPO) to antibodies. In: W. Knapp, K. Holubar and G. Wick (Eds.), Immunofluoresence and Related Staining Techniques, Elsevier, Amsterdam, pp. 215–224.

Yamaguchi, Y., Semmel, M., Stanislawski, L., Strosberg, A.D. and Stanislawski, M. (1993) Virucidal effects of glucose oxidase and peroxidase or their protein conjugates on human immunodeficiency virus type 1. Antimicrob. Agents Chemother. 37, 26–31.

Ziegler, T., Hukkanen, V., Arstila, P., Auvinen, P., Jalava, A. and Hyypiä, T. (1985) Typing of herpes simplex virus isolates with monoclonal antibodies and by nucleic acid spot hybridization. J. Virol. Methods 12, 169–177.