Journal of
PERIODONTAL RESEARCH

J Periodont Res 2012; 47: 635–644

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2012.01476.x

Effects of herpes simplex virus type 1 infection on immune functions of human neutrophils

S-L. Hung[1,2,3,4], H-H. Chiang[1], C-Y. Wu[1], M-J. Hsu[1], Y-T. Chen[2]

[1]Institute of Oral Biology, National Yang-Ming University, Taipei, Taiwan, [2]Department of Dentistry, National Yang-Ming University, Taipei, Taiwan, [3]Department of Stomatology, Taipei Veterans General Hospital, Taipei, Taiwan and [4]Department of Education and Research, Taipei City Hospital, Taipei, Taiwan

Hung S-L, Chiang H-H, Wu C-Y, Hsu M-J, Chen Y-T. Effects of herpes simplex virus type 1 infection on immune functions of human neutrophils. J Periodont Res 2012; 47: 635–644. © 2012 John Wiley & Sons A/S

Background and Objective: Herpesviruses may play roles in the development of periodontal diseases. This study analyzed the effects of herpes simplex virus type 1 (HSV-1) infection on neutrophil function. The effects of lipopolysaccharide (LPS) from the periodontal pathogen, *Porphyromonas gingivalis*, during HSV-1 infection were also determined.

Material and Methods: Purified HSV-1 was pretreated with buffer containing no serum, with HSV-1 immunoglobulin G (IgG)-positive serum (HSV-1 antiserum) or with control serum. Neutrophils were mock-infected or infected with the pretreated HSV-1. Viral binding and phagosome formation were detected using immunostaining. Intracellular reactive oxygen species (ROS) were determined using 2′,7′-dichlorofluorescin diacetate and fluorometry. Leukotriene B_4 (LTB_4) and interleukin-8 (IL-8) were detected using enzyme immunoassays. Release of matrix metalloproteinase-9 (MMP-9) was examined using gelatin zymography. Phosphorylation of Akt/glycogen synthase kinase-3 (GSK-3) was determined using western blotting.

Results: HSV-1 bound directly to neutrophils and enhanced the release of MMP-9. HSV-1 immune complexes, formed in the HSV-1 antiserum, bound neutrophils and induced the formation of early phagosome more effectively than did HSV-1 alone. The relative levels of ROS and phosphorylation of Akt/GSK-3 were increased significantly in neutrophils after infection with HSV-1 immune complexes. Infection with HSV-1 and HSV-1 immune complexes also stimulated the production of inflammatory mediators, LTB_4 and IL-8. Moreover, LPS enhanced the HSV-1-stimulatory production of IL-8.

Conclusion: This study demonstrated differences in neutrophils infected with HSV-1 alone or with HSV-1 immune complexes, suggesting that opsonization of HSV-1 might enhance its effects on neutrophils. The *in vitro* findings suggest that HSV-1 infection may induce the inflammatory response and affect periodontal health.

Dr Yen-Ting Chen, Department of Dentistry, National Yang-Ming University, No. 155, Sec. 2, Li-Nong St., Pei-Tou, Taipei 11221, Taiwan
Tel: +886 2 2729 1157
Fax: +886 2 2826 4053
e-mail: yentingchen2@gmail.com

Key words: herpes simplex virus; neutrophil; reactive oxygen species; leukotriene B_4; interleukin-8; matrix metalloproteinase-9

Accepted for publication February 17, 2012

Primary infection with herpes simplex virus type 1 (HSV-1), an important oral pathogen, most commonly involves the mouth and/or throat, resulting in gingivostomatitis and pharyngitis (1). HSV-1 commonly attacks mucosa, skin, eyes and the nervous system. Following recovery from the primary oropharyngeal infection, patients may retain HSV DNA in the trigeminal ganglion for life and suffer recurrent attacks of herpes labialis. Studies have also revealed a possible association between HSV and periodontal diseases (2–5). HSV is related to the severity of periodontal

diseases in terms of clinical attachment loss (6). Additionally, co-infection with HSV and periodontal pathogens, such as *Porphyromonas gingivalis*, is observed in periodontal destructive sites (3,5). HSV-1 is able to infect a wide variety of cells. Cells of the oral mucosa, such as gingival keratinocytes and gingival fibroblasts, are important targets of HSV infection at the portal of entry. Organ cultures of human gingival mucosa can be infected with HSV-1 (7). In addition, human gingival keratinocytes and gingival fibroblasts support HSV replication (8–10). The interaction of viral glycoprotein D-1 (gD-1) and the cellular component, herpesvirus entry mediator (HVEM) (also known as herpesvirus entry mediator A, HveA), may mediate entry of HSV-1 into gingival keratinocytes and gingival fibroblasts (9). HSV has also been found in T lymphocytes and in monocyte/macrophage fractions from chronic periodontitis specimens (11). Local disruption of certain periodontal T-lymphocyte functions by HSV may increase the risk of destructive periodontal diseases.

Neutrophils, the most abundant circulating blood leukocytes, represent the first line of the host defense mechanism by their ingestion (phagocytosis) and killing of microorganisms. The functions of neutrophils include adherence, chemotaxis, phagocytosis and bactericidal activity (12,13). Neutrophils within the gingival crevice are able to protect the gingiva against microbial invasion through mechanisms such as phagocytosis, secretion of hydrolytic enzymes and production of oxygen radicals that are directed toward microbial killing (12,13). The production of reactive oxygen species (ROS) through the respiratory burst in neutrophils is an important pathway involved in oxidative killing (14,15). Activation of neutrophils may also lead to the release of enzymes present within cytoplasmic granules of neutrophils. A wide range of inflammatory mediators produced by neutrophils participates in the inflammatory response. Prominent among these inflammatory mediators include the arachidonic acid-derived lipid mediator, leukotriene B_4 (LTB_4) (16) and interleukin-8 (IL-8) (17). LTB_4 can enhance the recruitment of neutrophils and activate the production of lysosomal enzymes and superoxide (18). IL-8 may activate neutrophils and induce chemotaxis, the respiratory burst and exocytosis, such as release of matrix metalloproteinase-9 (MMP-9) (19). In addition to their functions against microbial infection, neutrophils are likely to be involved in the pathogenesis of periodontal destruction. Individuals with altered numbers or functions of circulating neutrophils tend to suffer severe periodontal diseases (15,20,21). Amplified activities of neutrophils may also be responsible for the tissue destruction (15).

Using a murine model of herpetic stromal keratitis, neutrophils are shown to play an important role in limiting the intraocular spread of virus in HSV-1-injected eyes (22). Neutrophils also control viral spread from the brain into the optic nerve and retina of infected eyes (22). *In vitro*, purified rabbit neutrophils bind and internalize HSV–antibody complexes (23). The results suggest an active role for neutrophils in controlling HSV infection through their ability to bind and ingest virus–antibody complexes (23). Neutrophils are capable of phagocytosis and degradation of HSV (24,25). In neutrophils, HSV-1 replication appears to be inhibited (22). However, infectious HSV-1 was still detected in neutrophils up to 36 h after exposure to HSV-1 (22). Neutrophils, which are terminally differentiated leukocytes, survive in the circulation for approximately 24–36 h before undergoing apoptosis (26). Thus, break-up of neutrophils into multiple apoptotic bodies that contain live viruses may facilitate the spread of viruses, as the apoptotic bodies are engulfed by macrophages and bypass the immune system (27). In addition, the phagocytosis of apoptotic neutrophils by macrophages prevents the release of neutrophil contents and tissue damage (28). Infectious HSV-1 remains detectable in neutrophils; however, studies on how HSV-1 may affect the functions of neutrophils are still limited. We hypothesized that HSV-1 might exaggerate the responses of neutrophils and affect periodontal health. The main purpose of this study was to determine the effects of HSV-1 infection in the absence or presence of the anti-HSV-1 serum on the immune functions of human neutrophils, including early phagosome formation, production of intracellular ROS, release of LTB_4, IL-8 and MMP-9 and phosphorylation of the Akt/glycogen synthase kinase-3 (GSK-3) signaling pathway. The possible costimulatory effects of lipopolysaccharide (LPS) from the periodontal pathogen, *Porphyromonas gingivalis*, during HSV-1 infection were also determined.

Material and methods

Preparation of neutrophils and human sera

Neutrophils were freshly purified from the venous peripheral blood of healthy volunteers (six men and seven women; mean age 23.5 ± 0.1 years; age range 23–24 years) by dextran sedimentation, followed by Ficoll density-gradient centrifugation, as previously described (29,30). The periodontal conditions of the volunteers were not examined. Written, informed consent was obtained from each donor. The research proposal was approved by the Institutional Review Board of National Yang-Ming University, Taipei, Taiwan. Purified neutrophils were washed and resuspended in Hanks' balanced salt solution (HBSS) supplemented with 1.6 mM $CaCl_2$ and 10 mM HEPES. The levels of purity were > 99%. Human serum was obtained from clotted blood that had been separated by centrifugation at 2750 *g* for 10 min. Detection of HSV-1 immunoglobulin G (IgG) in the serum was performed using a commercial HSV-1 IgG ELISA kit (Calbiotech, Spring Valley, CA, USA). The serum containing HSV-1 IgG was designated as the HSV-1 antiserum, whereas the serum with no antibody against HSV-1 was used as the control serum.

Virus preparation and infection

African green monkey kidney (Vero) cells were propagated in Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with 5% fetal calf serum and

antibiotic-antimycotic solution containing penicillin, streptomycin and amphotericin B. HSV-1 (KOS strain) from the extracellular fluid of infected Vero cells was purified using a sucrose gradient, as previously described (31). The viral plaque-forming units of purified viruses were determined on Vero cells using plaque assays. The HSV immune complexes were prepared by incubating purified HSV-1 with 10% HSV-1 antiserum. Purified HSV-1 was freshly pretreated with HBSS containing no serum, 10% HSV-1 antiserum or 10% control serum for 30 min at 37°C for each experiment. HSV-1 that was pretreated with the HSV-1 antiserum was not infectious when assayed on Vero cell monolayers. A multiplicity of infection of 100 was used to infect neutrophils in this study.

Immunostaining and confocal laser scanning microscopy

For viral binding experiments, freshly purified neutrophils were mock-infected or infected with the pretreated HSV-1 for 1 h at 4°C. For detection of early phagosomes, fresh neutrophils were mock-infected or infected with HSV-1 for 1 h at 4°C and for 5 min at 37°C. Infected neutrophils were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min. Neutrophils were then permeabilized with 0.1% Triton X-100 in PBS for 10 min and washed with PBS. To reduce nonspecific binding, neutrophils were blocked with PBS containing 20 mg/mL of goat IgG for 1–1.5 h at room temperature. For viral-binding experiments, neutrophils were incubated with mouse monoclonal anti-ICP5 Ig(1 : 100) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) overnight at 4°C, followed by incubation with goat anti-mouse IgG conjugated to fluorescein isothiocyanate isomer (GAM–FITC) (1 : 200) (Millipore, Billerica, MA, USA) for 1 h at room temperature. For detection of early phagosomes, infected neutrophils were dual-labeled with mouse monoclonal anti-ICP5 IgG1 (1 : 100) and rabbit polyclonal anti-early endosome autoantigen 1 (EEA1) IgG (1 : 100) (Abcam Inc., Cambridge, MA, USA) overnight at 4°C, followed by incubation with GAM–FITC and goat anti-rabbit IgG conjugated to Texas Red (1 : 200) (Abcam Inc.) for 1 h. Neutrophils were then incubated with 1 mg/mL of 4,6-diamidino-2-phenylindole (DAPI) for nuclei staining. Stained cells were mounted with a fluorescent mounting medium (DAKO, Glostrup, Denmark) and coverslips were attached onto slides. Samples were analyzed using a confocal laser scanning microscope (Fluoview FV1000; Olympus Corporation, Tokyo, Japan) and the analytic software (fv10-asw 1.6 viewer; Olympus Corporation). The percentage of fluorescence-positive cells was counted from the captured image.

Detection of intracellular ROS

The intracellular ROS were determined as previously described (32). The principle of the test is based on the diffusion of nonpolar 2′,7′-dichlorofluorescin diacetate (H_2DCFDA) into the cells through the cell membrane. The H_2DCFDA is then hydrolyzed to nonfluorescent dichlorofluorescin (H_2DCF). The ROS within the cell oxidize H_2DCF to a measurable fluorescent product, dichlorofluorescin. Freshly isolated neutrophils were mock-infected or infected with the pretreated HSV-1 for 1 h at 4°C and 20 min at 37°C. Treated neutrophils were centrifuged and further incubated with HBSS containing 10 μM H_2DCFDA (Molecular Probes, Inc., Eugene, OR, USA) for 30 min at 37°C and then washed with ice-cold PBS. The treated neutrophils were finally fixed with 4% paraformaldehyde in PBS and analyzed using the Fluorescence Measurement System (Multilabel counter Victor2; Perkin-Elmer, Norwalk, CT, USA) (excitation 485 nm, emission 535 nm). The relative level of intracellular ROS was calculated as follows: fluorescence intensity of the experimental sample ÷ fluorescence intensity of the control sample.

Enzyme immunoassay and ELISA

Freshly isolated neutrophils were mock-infected or infected with pretreated HSV-1 for 1 h at 4°C and 3 h at 37°C. The culture supernatants were assayed for LTB_4 using a commercially available solid-phase competition enzyme immunoassay (EIA) kit with acetylcholinesterase as the label (Cayman Chemical Company, Ann Arbor, MI, USA), according to the manufacturer's instruction. The culture supernatants were also assayed for IL-8 using a solid-phase sandwich enzyme-linked immunosorbent assay (ELISA) kit (EH2-IL-8; Pierce Biotechnology, Inc., Rockford, IL, USA). The concentration (pg/mL) of each sample was calculated using the standard curve generated.

Gelatin zymography

Freshly isolated neutrophils were mock-infected or infected with the pretreated HSV-1 for 1 h at 4°C and then for 3 h at 37°C. The gelatinolytic activity of MMP-9 released from neutrophils was investigated using gelatin zymography. Supernatant harvested from infected neutrophils was electrophoresed on a 7.5% sodium dodecyl sulfate–polyacrylamide gel containing 0.1% gelatin. The buffer controls, including HBSS containing no serum, HSV-1 antiserum or control serum, were also analyzed. After electrophoresis, the gels were washed twice in 2.5% Triton X-100 under constant mechanical shaking and then incubated for 24 h at 37°C in incubation buffer [50 mM Tris (pH 8.8), 5 mM $CaCl_2$, 0.02% NaN_3, 0.1% Triton X-100]. The gels were then stained with Coomassie Blue solution (0.1% Coomassie Brilliant Blue R-250, 50% methanol and 10% acetic acid) and destained (in a solution of 45% methanol and 10% acetic acid). Band intensities of MMP-9 monomers were quantified using the analytic software (IMAGE J, http://rsb.info.nih.gov/ij/index.html). The band intensity from the mock-infected neutrophils in the no-serum group was designated as 1. The relative expression was then calculated.

Western blotting analysis

Freshly isolated neutrophils were mock-infected or infected with pretreated HSV-1 for 1 h at 4°C and 3 h at 37°C.

Treated neutrophils were lysed with lysis buffer [1% Nonidet P-40, 0.5% sodium deoxycholate, 50 mM Tris–HCl (pH 7.5), 100 mM NaCl, 100 mM NaF, 100 mM Na_3VO_4 and 1 × protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany)]. Cell lysates were analyzed by electrophoresis on a 10% sodium dodecyl sulfate–polyacrylamide gel. Proteins were transferred onto a polyvinylidene difluoride membrane (Pall Gelman Laboratory, Ann Arbor, MI, USA) and the membrane was immunoblotted at room temperature for 1 h with the primary antibody, including the mouse monoclonal antibody against phosphorylated Akt (Ser473) (1 : 1000) (Cell Signaling Technology, Inc., Danvers, MA, USA), total Akt (1 : 1000) (sc-5298) (Santa Cruz Biotechnology), total GSK-3α/β (1 : 1000) (sc-7291) (Santa Cruz Biotechnology) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1 : 10,000) (Millipore) or the rabbit polyclonal antibody against phosphorylated GSK-3α/β (Ser21/9) (1 : 1000) (Cell Signaling Technology, Inc.). Each membrane was then incubated with the appropriate secondary antibody, GAM conjugated to horseradish peroxidase (GAM–HRP) (1 : 1000) (Millipore) or goat anti-rabbit IgG conjugated to HRP (1 : 1000) (Cell Signaling Technology, Inc.). Between each step, the membranes were washed with TBST buffer [20 mM Tris–HCl (pH 7.6), 137 mM NaCl, 0.1% Tween-20]. Proteins were revealed with the enhanced chemiluminescence detection system (SuperSignal®, West Pico Chemiluminescent substrate; Pierce Biotechnology Inc.). Band intensities were captured and quantified using the Luminescence/Fluorescence Imaging System (Fusion-SL4 X-Press; Vilber Lourmat, Marne-la-Valle' e, France) and the analytic software. The signals were normalized to those of GAPDH. The expression from the mock-infected neutrophils in the no-serum group was designated as 1. The relative expression was then calculated.

Costimulation experiments of HSV-1 and LPS from *P. gingivalis*

Freshly isolated neutrophils were mock-infected or infected with HSV-1 for 1 h at 4°C. Mock- or HSV-1-infected neutrophils were further incubated at 37°C with various concentrations of LPS purified from the periodontal pathogen, *P. gingivalis* (ATCC 33277) (InvivoGen, San Diego, CA, USA). The culture supernatants were then harvested and assayed for IL-8, LTB_4 and MMP-9, as described above.

Statistical analysis

All values were expressed as mean ± standard error of the mean. Differences between the mock-infected and HSV-1-infected neutrophils in each experimental group were analyzed using the paired *t*-test. Each experiment was repeated independently, at least three times, using neutrophils from different blood donors to ensure reproducibility. A *p*-value of < 0.05 was considered significantly different.

Results

Binding of HSV-1 to neutrophils

Green fluorescence for the viral structural protein, ICP5, was not observed for the mock-infected neutrophils in the no-serum, HSV-1-antiserum and control-serum groups (Fig. 1A–C). ICP5 was detectable when neutrophils were incubated with HSV-1 (Fig. 1D). The detection of ICP5 was enhanced significantly in neutrophils if the HSV-1 used was pretreated with the HSV-1 antiserum (Fig. 1E) but not if it was pretreated with the control serum (Fig. 1F). After HSV-1 infection, the percentages of ICP5-positive neutrophils for the no-serum, HSV-1-antiserum and control-serum groups increased from 0% to 8.5 ± 2.9% ($p < 0.05$), 43.1 ± 4.4% ($p < 0.05$) and 2.6 ± 1.8%, respectively. The results suggest that HSV-1 is able to bind to neutrophils. In addition, HSV-1 immune complexes, formed in the HSV-1 antiserum, bound neutrophils more effectively than HSV-1 alone.

Early phagosome formation in neutrophils after HSV-1 infection

EEA1 is an early phagosome marker (33). Neither ICP5 nor EEA1 were detectable in mock-infected neutrophils for the no-serum, HSV-1 antiserum or control-serum groups (data not shown). ICP5 was detectable in neutrophils after incubation with HSV-1 in all three groups (Fig. 2A, a–c). However, EEA1 was not detectable when neutrophils were infected with HSV-1 only (Fig. 2A, d). Detection of EEA1 was significantly increased in the HSV-1 antiserum group (Fig. 2A, e). ICP5 appeared to be significantly colocalized with EEA1 in the HSV-1-antiserum group (Fig. 2A, h, yellow fluorescence) but not in the other two groups (Fig. 2A, g, i). The percentages of colocalized neutrophils were 0%, 33.9 ± 9.4% and 0% for the no-serum, HSV-1-antiserum and control-serum groups, respectively. The results suggest that only HSV-1 immune complexes stimulate early phagosome formation and may enter neutrophils through phagocytosis.

Effects of HSV-1 infection on the production of intracellular ROS

After HSV-1 infection, the intracellular level of ROS was similar to that of mock-infected neutrophils when the HSV-1 used was pretreated with HBSS containing no serum or control serum (Fig. 3). However, the relative intracellular level of ROS increased significantly by 1.9 ± 0.2 fold ($p < 0.05$) in neutrophils after infection with HSV-1 that was pretreated with HSV-1 antiserum. The results suggest that HSV-1 immune complexes induce the production of intracellular ROS in neutrophils.

Effects of HSV-1 infection on the production of LTB_4 and IL-8 in neutrophils

After HSV-1 infection, the production of LTB_4 was increased in infected neutrophils compared with the mock-infected neutrophils in all three groups ($p < 0.05$), namely no serum, HSV-1 antiserum and control serum (Fig. 4A). Moreover, the production of IL-8 was significantly induced in neutrophils after infection with HSV-1 that had been pretreated with HSV-1 antiserum or control serum (Fig. 4B).

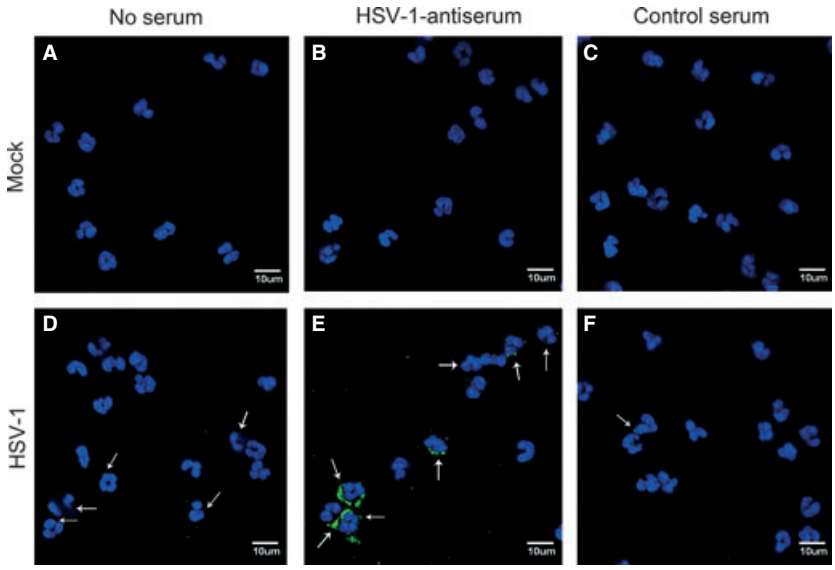

Fig. 1. Binding of herpes simplex virus type 1 (HSV-1) to neutrophils. Neutrophils were (A–C) mock infected or (D–F) infected with HSV-1 pretreated with Hanks' balanced salt solution (HBSS) containing (A, D) no serum, (B, E) HSV-1 antiserum or (C, F) control serum. After incubation for 1 h at 4°C, the binding of HSV-1 was assessed based on the detection of the viral structural protein, ICP5, using immunostaining followed by confocal laser scanning microscopy. Samples were also stained with 4,6-diamidino-2-phenylindole (DAPI) (blue fluorescence). The composite images of green and blue fluorescence shown are the representative results of three independent experiments. The arrows indicate the green fluorescent signal for ICP5-positive neutrophils.

The results suggest that HSV-1 and HSV-1 immune complexes stimulate the production of inflammatory mediators.

Effects of HSV-1 infection on the release of MMP-9 from neutrophils

The gelatinolytic activity was indicated as clear (unstained) regions (Fig. 5). Buffer controls, including HBSS containing HSV-1 antiserum or control serum, showed a very low level of MMP-9 activity (Fig. 5A). In the no-serum group, the MMP-9 activity in the supernatants from HSV-1-infected neutrophils was increased compared with the mock-infected neutrophils (Fig. 5). In the presence of HSV-1 antiserum or control serum, the release of MMP-9 from the mock-infected neutrophils was enhanced when compared with the mock-infected neutrophils in the no-serum group. However, no evident difference was observed between mock-infected and HSV-1-infected neutrophils in the HSV-1-antiserum or control-serum groups. Thus, HSV-1 alone (in the absence of serum) was able to enhance the release of MMP-9 from neutrophils.

Effects of HSV-1 on phosphorylation of Akt and GSK-3 in neutrophils

Little difference on phosphorylation of Akt and GSK-3 was observed between mock-infected and HSV-1-infected neutrophils in the no-serum and control-serum groups (Fig. 6). In the HSV-1-antiserum group, significant increases in the phosphorylation of Akt, as well as in the total amounts and phosphorylation of GSK-3α, were observed in the HSV-1-infected neutrophils when compared with the mock-infected neutrophils ($p < 0.05$) (Fig. 6). Therefore, the results indicate that infection with HSV-1 alone does not affect Akt and GSK-3. However, HSV-1 immune complexes potently triggered the phosphorylation of Akt and GSK-3 in neutrophils during HSV-1 infection.

Costimulatory effects of HSV-1 and LPS from *P. gingivalis* on the production of IL-8 in neutrophils

The possible effects of HSV-1 infection and LPS purified from a periodontal pathogen, *P. gingivalis*, were determined using the costimulation experiments. In the absence of LPS, the production of IL-8 was increased by 2.19 ± 0.12 fold in HSV-1-infected neutrophils compared with the mock-infected neutrophils ($p < 0.05$) (Fig. 7). In the presence of 1 μg/mL of LPS from *P. gingivalis*, the secretion of IL-8 was significantly increased by 2.16 ± 0.27 fold and by 3.17 ± 0.22 fold in the mock-infected and HSV-1-infected neutrophils, respectively. However, LPS from *P. gingivalis* did not alter the HSV-1-induced production of LTB_4 and MMP-9 in the costimulation experiments (data not shown). The results suggest that the cotreatment of neutrophils with HSV-1 and LPS from *P. gingivalis* (1 μg/mL) significantly enhances the production of IL-8.

Discussion

The antimicrobial capacity of neutrophils is associated with several events, including phagocytosis, the generation of ROS through the respiratory burst in neutrophils and the release of enzymatic or antimicrobial protein contents of the granules. The results of this *in vitro* study indicated that HSV-1 alone in the absence of serum was able to bind human neutrophils and enhance the release of

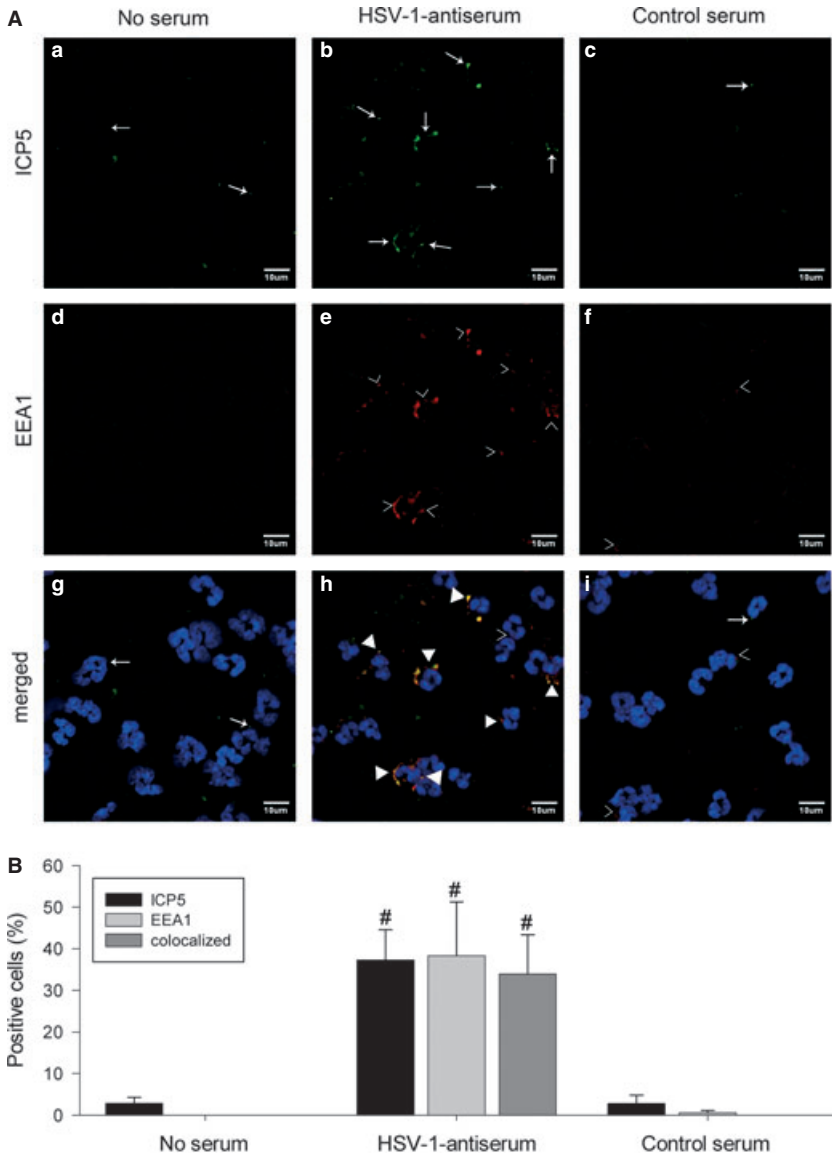

Fig. 2. Early phagosome formation in neutrophils after infection with herpes simplex virus type 1 (HSV-1). (A) Neutrophils were infected with HSV-1 pretreated with Hanks' balanced salt solution (HBSS) containing (a, d, g) no serum, (b, e, h) HSV-1 antiserum or (c, f, i) control serum. After infection for 1 h at 4°C and for 5 min at 37°C, neutrophils were dual-labeled for viral ICP5 (green fluorescence) and an early phagosome marker, early endosome autoantigen 1 (EEA1) (red fluorescence). Samples were also stained with 4,6-diamidino-2-phenylindole (DAPI) and then analyzed using confocal laser scanning microscopy. The arrows and the V marks indicate ICP5- and EEA1-positive signals, respectively. The triangles in the merged images indicate the colocalization (yellow) of green and red. (B) The percentages of positive cells shown represent the mean ± standard error of the mean from three independent experiments performed with neutrophils obtained from different donors. A significant difference compared with neutrophils in the no-serum group is indicated as # ($p < 0.05$).

MMP-9. The results also suggested that HSV-1 immune complexes, formed in the HSV-1 antiserum, may bind and enter neutrophils more effectively than HSV-1 alone. The intracellular levels of ROS and the phosphorylation of Akt and GSK-3 were increased significantly in neutrophils after infection with HSV-1 that had been pretreated with the HSV-1 antiserum. In addition, after HSV-1 infection, the production of LTB_4 and IL-8 was increased in HSV-1-infected neutrophils when compared with the mock-infected neutrophils. Moreover, LPS from *P. gingivalis* further enhanced the production of IL-8 during HSV-1 infection.

This study demonstrated that binding of HSV-1 occurred in the absence and presence of the HSV-1 immune complexes, which, however, enhanced the efficiency of viral binding. Furthermore, early phagosome formation was only apparently observed when cells were infected with HSV-1 in the format of the immune complex. Previous studies demonstrated that neutrophils are capable of phagocytosis and degradation of HSV (24,25).

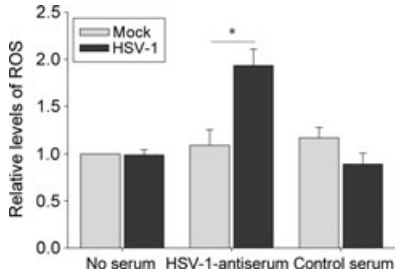

Fig. 3. Effects of herpes simplex virus type 1 (HSV-1) infection on the production of intracellular reactive oxygen species (ROS) in neutrophils. Neutrophils were mock-infected or infected with HSV-1 pretreated with Hanks' balanced salt solution (HBSS) containing no serum, HSV-1 antiserum or control serum. After infection, neutrophils were incubated with 2′,7′-dichlorofluorescin diacetate (H_2DCFDA) to detect the intracellular ROS. The fluorescence intensity of mock-infected neutrophils in the no-serum group was considered as 1. Each sample was expressed as the relative level of intracellular ROS compared with the control sample. The results shown represent the mean ± standard error of the mean from three independent experiments. A significant difference between mock-infected and HSV-1-infected neutrophils with the same treatment is indicated as * ($p < 0.05$).

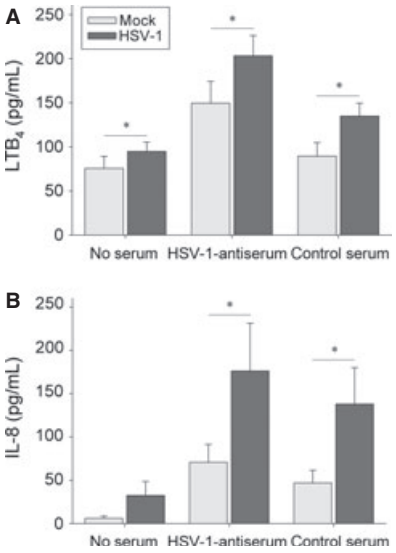

Fig. 4. Effects of herpes simplex virus type 1 (HSV-1) infection on the production of leukotriene B_4 (LTB_4) and interleukin-8 (IL-8) in neutrophils. Neutrophils were mock-infected or infected with HSV-1 pretreated with Hanks' balanced salt solution (HBSS) containing no serum, HSV-1 antiserum or control serum. After infection for 3 h, the production of (A) LTB_4 and (B) IL-8 in the supernatants was determined using an enzyme immunoassay or an enzyme-linked immunosorbent assay, respectively. The results from at least three independent experiments are expressed as mean ± standard error of the mean. A significant difference between mock-infected and HSV-1-infected neutrophils with the same treatment is indicated as * ($p < 0.05$).

Purified rabbit neutrophils have been shown to efficiently bind and internalize HSV–antibody complexes *in vitro* (23). Phagocytosis of opsonized HSV-1 is primarily enhanced through interaction with complement receptor or Fcγ receptor. HSV-1 has also been shown to interact with several cellular receptors (34), including HVEM, which is also highly expressed in neutrophils (35). LIGHT, a member of the tumor necrosis factor superfamily, is a cellular ligand for HVEM (36). The interaction of HVEM with LIGHT may enhance the bactericidal activities, including phagocytosis and the production of ROS, IL-8 and tumor necrosis factor-α, of human neutrophils (35). Viral gD-1, an envelope protein of HSV-1, inhibits the interaction of HVEM with LIGHT (36). Purified gD-1 alone also possesses the ability to enhance IL-6 secretion in gingival fibroblasts (37). Whether the interaction of gD-1 and HVEM may alter the function of neutrophils, including the changes observed in this study, remains to be examined.

The production of ROS was enhanced in neutrophils after exposure to HSV-1 that was pretreated with HSV-1 antiserum in this study. The antimicrobial efficiency of human neutrophils depends on certain events, including the generation of ROS in the form of superoxide, hydrogen peroxide and hydroxyl radical. The generation of ROS depends on the assembly and the activation of the NADPH-dependent oxidase (38). This active enzymatic complex of NADPH oxidase is able to generate superoxide anion (O_2^-), which can dismutate into H_2O_2 (39). Superoxide may function intracellularly by activating proteases within the phagolysosome (40). Production of ROS has been shown to influence the cell-cycle progression, apoptosis and chemical toxicity (41,42). Increases in the intracellular concentration of Ca^{2+} may activate protein kinase C, which regulates degranulation and production of ROS (43,44). The possible mechanisms involved in the effects of HSV-1 infection on ROS production require further investigation.

MMPs are a family of zinc-binding, calcium-dependent, proteolytic enzymes that typically degrade extracellular matrix proteins. MMP-9 degrades a variety of extracellular matrix proteins, such as type IV collagen

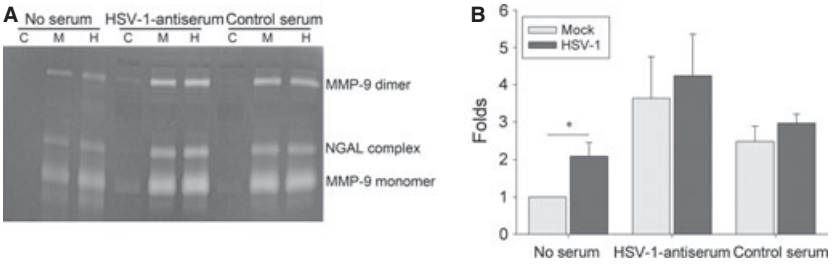

Fig. 5. Zymography of neutrophil supernatants. Neutrophils were mock-infected or infected with herpes simplex virus type 1 (HSV-1) pretreated with Hanks' balanced salt solution (HBSS) containing no serum, HSV-1 antiserum or control serum. (A) After infection at 37°C for 3 h, supernatants from mock-infected (M) or HSV-1-infected (H) neutrophils were analyzed for MMP-9 using gelatin zymography. Buffer controls (C), including HBSS containing no serum, HSV-1 antiserum and control serum, were also analyzed. (B) The relative expression of MMP-9 monomer compared with the mock-infected neutrophils in the no-serum group, shown as mean ± standard error of the mean, was calculated from four independent experiments. A significant difference between mock-infected and HSV-1-infected neutrophils with the same treatment is indicated as * ($p < 0.05$). NGAL complex, complex of MMP-9 and neutrophil gelatinase-associated lipocalin.

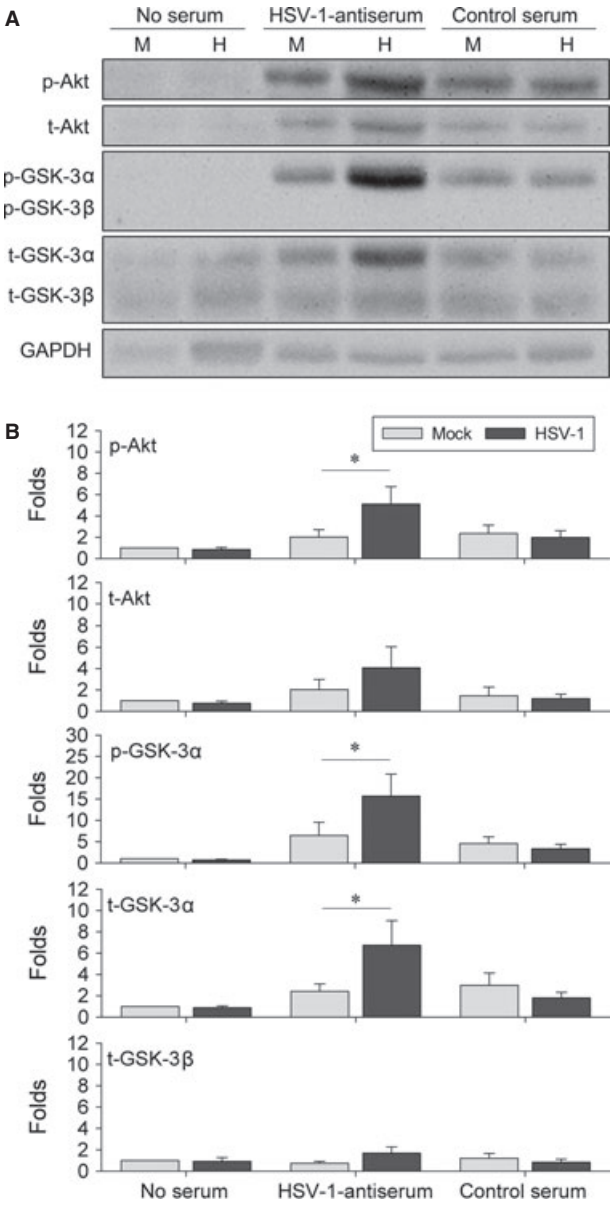

Fig. 6. Effects of herpes simplex virus type 1 (HSV-1) on phosphorylation of Akt and glycogen synthase kinase-3α/β (GSK-3α/β) in neutrophils. Neutrophils were mock-infected or infected with HSV-1 pretreated with Hanks' balanced salt solution (HBSS) containing no serum, HSV-1 antiserum or control serum. Phosphorylated (p)/total (t) Akt, phosphorylated/total GSK-3α, phosphorylated/total GSK-3β and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the mock-infected (M) or HSV-1-infected (H) neutrophils were detected using western blotting. The signals were normalized to those of GAPDH, and the protein level of the mock-infected neutrophils in the no-serum group was designated as 1. Data shown are mean ± standard error of the mean from four independent experiments. A significant difference between mock-infected and HSV-1-infected neutrophils with the same treatment is indicated as * ($p < 0.05$).

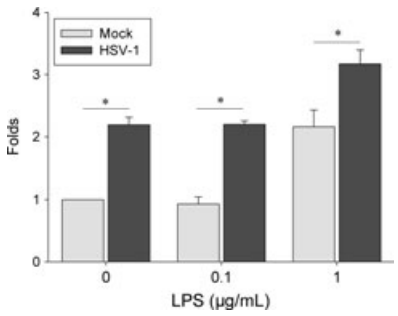

Fig. 7. Costimulatory effects of herpes simplex virus type 1 (HSV-1) and *Porphyromonas gingivalis* lipopolysaccharide (LPS) on the production of interleukin-8 (IL-8) in neutrophils. Neutrophils were mock-infected or infected with HSV-1 for 1 h at 4°C. Infected neutrophils were further incubated at 37°C for 3 h with various concentrations of LPS. The production of IL-8 relative to that found in the control neutrophils, from four independent experiments, is shown (as mean ± standard error of the mean). A significant difference between mock-infected and HSV-1-infected neutrophils at the same concentration of LPS is indicated as * ($p < 0.05$).

and gelatin (45). Neutrophils, the major source of MMP-9, produce MMP-9 constitutively and store it in their gelatinase granules. Upon stimulation, neutrophils release MMP-9 rapidly from these granules. The release of MMP-9 by neutrophils may be involved in the pathogenesis of herpetic stromal keratitis (46). IL-8 may induce the release of MMP-9 from neutrophils through two distinct pathways – one involving protein kinase C and extracellular signal-regulated kinase (ERK)1/2, and the other involving Src-family kinases (19). Activation of bovine neutrophils through complement receptor type 3 and Fc receptor may mediate the release of MMP-9 through activation of the phosphoinositide 3-kinase signaling pathway and tyrosine kinase (47). Akt is a serine/threonine protein kinase that can be activated by phosphoinositide 3-kinase (48). The increased phosphorylation of Akt and the Akt substrate, GSK-3, was only evident when neutrophils were infected with HSV-1 that had been pretreated with the HSV-1 antiserum in this study. HSV-1 infection also modulates Akt and GSK-3 in oral epithelial cells (49). The detailed molecular mechanisms involved in the effects of HSV-1 infection in this study remain to be defined.

The current results demonstrated differences when neutrophils were infected with HSV-1 alone or with HSV-1 immune complexes. These viral immune complexes not only enhanced the efficiency of the binding and internalization of HSV-1 in neutrophils, but also the production of ROS, LTB_4 and IL-8. However, HSV-1 infection only in

the absence of serum significantly enhanced the secretion of MMP-9. To the best of our knowledge, this is the first report demonstrating that HSV-1 alone can directly induce production of MMP-9 from human neutrophils. The different results were demonstrated in neutrophils infected with HSV-1 alone or with HSV-1 immune complexes, suggesting that opsonization of HSV-1 might enhance its effects on several functions of neutrophils. Moreover, costimulation with HSV-1 and LPS from *P. gingivalis* resulted in elevated levels of IL-8 in neutrophils. The herpesvirus family, including HSV, is shown to be associated with the infection of *P. gingivalis* and active periodontal breakdown (3,5). Data suggest that herpesviridae may serve as pathogenic agents in periodontal diseases by releasing destructive cytokines and inflammatory mediators. Whether neutrophils from patients respond similarly or differently to neutrophils derived from periodontally healthy controls is still not clear. The biological significance of this study during oral infection *in vivo* requires further investigation.

Acknowledgements

This investigation was supported by a grant from the Ministry of Education, Aim for the Top University Plan and by the Research Grant NSC99-2314-B-010-019-MY2 from the National Science Council, Taiwan.

References

1. Arduino PG, Porter SR. Herpes simplex virus type 1 infection: overview on relevant clinico-pathological features. *J Oral Pathol Med* 2008;**37:**107–121.
2. Contreras A, Slots J. Herpesviruses in human periodontal disease. *J Periodont Res* 2000;**35:**3–16.
3. Slots J, Kamma JJ, Sugar C. The herpesvirus-*Porphyromonas gingivalis*-periodontitis axis. *J Periodontal Res* 2003;**38:** 318–323.
4. Nishiyama SAB, Nakano V, Velasquez-Melendez G, Avila-Campos MJ. Occurrence of herpes simplex virus 1 and three periodontal bacteria in patients with chronic periodontitis and necrotic pulp. *Can J Microbiol* 2008;**54:**326–330.
5. Imbronito AV, Okuda OS, Maria de Freitas N, Moreira Lotufo RF, Nunes FD. Detection of herpesviruses and periodontal pathogens in subgingival plaque of patients with chronic periodontitis, generalized aggressive periodontitis, or gingivitis. *J Periodontol* 2008;**79:**2313–2321.
6. Ling L-J, Ho C-C, Wu C-Y, Chen Y-T, Hung S-L. Association between human herpesviruses and the severity of periodontitis. *J Periodontol* 2004;**75:**1479–1485.
7. Yura Y, Iga H, Kondo Y *et al*. Herpes simplex virus type 1 and type 2 infection in human oral mucosa in culture. *J Oral Pathol Med* 1991;**20:**68–73.
8. Rones Y, Hochman N, Ehrlich J, Zakay-Rones Z. Sensitivity of oral tissues to herpes simplex virus – in vitro. *J Periodontol* 1983;**54:**91–95.
9. Hung S-L, Cheng Y-Y, Wang Y-H, Chang K-W, Chen Y-T. Expression and roles of herpesvirus entry mediators A and C in cells of oral origin [erratum appears in *Oral Microbiol Immunol* 2002; **17**: 330]. *Oral Microbiol Immunol* 2002;**17:**215–223.
10. Hung S-L, Wang Y-H, Chen H-W, Lee P-L, Chen Y-T. Analysis of herpes simplex virus entering into cells of oral origin. *Virus Res* 2002;**86:**59–69.
11. Contreras A, Zadeh HH, Nowzari H, Slots J. Herpesvirus infection of inflammatory cells in human periodontitis. *Oral Microbiol Immunol* 1999;**14:**206–212.
12. Van Dyke TE, Hoop GA. Neutrophil function and oral disease. *Crit Rev Oral Biol Med* 1990;**1:**117–133.
13. Miyasaki KT. The neutrophil: mechanisms of controlling periodontal bacteria. *J Periodontol* 1991;**62:**761–774.
14. Smith JA. Neutrophils, host defense, and inflammation: a double-edged sword. *J Leukoc Biol* 1994;**56:**672–686.
15. Kantarci A, Oyaizu K, Van Dyke TE. Neutrophil-mediated tissue injury in periodontal disease pathogenesis: findings from localized aggressive periodontitis. *J Periodontol* 2003;**74:**66–75.
16. Offenbacher S, Odle BM, Van Dyke TE. The use of crevicular fluid prostaglandin E_2 levels as a predictor of periodontal attachment loss. *J Periodont Res* 1986;**21:** 101–112.
17. Bazzoni F, Cassatella MA, Rossi F, Ceska M, Dewald B, Baggiolini M. Phagocytosing neutrophils produce and release high amounts of the neutrophil-activating peptide 1/interleukin 8. *J Exp Med* 1991; **173:**771–774.
18. Ford-Hutchinson AW. Leukotriene B_4 in inflammation. *Crit Rev Immunol* 1990;**10:**1–12.
19. Chakrabarti S, Patel KD. Regulation of matrix metalloproteinase-9 release from IL-8-stimulated human neutrophils. *J Leukoc Biol* 2005;**78:**279–288.
20. De Beule F, Bercy P, Ferrant A. The effectiveness of a preventive regimen on the periodontal health of patients undergoing chemotherapy for leukemia and lymphoma. *J Clin Periodontol* 1991;**18:** 346–347.
21. Hart TC, Shapira L, Van Dyke TE. Neutrophil defects as risk factors for periodontal diseases. *J Periodontol* 1994;**65:** 521–529.
22. Zheng M, Fields MA, Liu Y, Cathcart H, Richter E, Atherton SS. Neutrophils protect the retina of the injected eye from infection after anterior chamber inoculation of HSV-1 in BALB/c mice. *Invest Ophthalmol Vis Sci* 2008;**49:**4018–4025.
23. Smith JW, Jachimowicz JR, Bingham EL. Binding and internalization of herpes simplex virus-antibody complexes by polymorphonuclear leukocytes. *J Med Virol* 1986;**20:**151–163.
24. Van Strijp JA, Van Kessel KP, van der Tol ME, Fluit AC, Snippe H, Verhoef J. Phagocytosis of herpes simplex virus by human granulocytes and monocytes. *Arch Virol* 1989;**104:**287–298.
25. Van Strijp JA, Miltenburg LA, van der Tol ME, Van Kessel KP, Fluit AC, Verhoef J. Degradation of herpes simplex virions by human polymorphonuclear leukocytes and monocytes. *J Gen Virol* 1990;**71:**1205–1209.
26. Savill JS, Wyllie AH, Henson JE, Walport MJ, Henson PM, Haslett C. Macrophage phagocytosis of aging neutrophils in inflammation. Programmed cell death in the neutrophil leads to its recognition by macrophages. *J Clin Invest* 1989;**83:**865–875.
27. Barber GN. Host defense, viruses and apoptosis. *Cell Death Differ* 2001;**8:**113–126.
28. Kennedy AD, DeLeo FR. Neutrophil apoptosis and the resolution of infection. *Immunol Res* 2009;**43:**25–61.
29. Hung S-L, Lee Y-Y, Liu T-Y, Peng J-L, Cheng Y-Y, Chen Y-T. Modulation of phagocytosis, chemotaxis, and adhesion of neutrophils by areca nut extracts. *J Periodontol* 2006;**77:**579–585.
30. Lai Y-L, Wu C-Y, Lee Y-Y, Chang H-W, Liu T-Y, Hung S-L. Stimulatory effects of areca nut extracts on prostaglandin E_2 production by human polymorphonuclear leukocytes. *J Periodontol* 2010;**81:**758–766.
31. Handler CG, Eisenberg RJ, Cohen GH. Oligomeric structure of glycoproteins in herpes simplex virus type 1. *J Virol* 1996;**70:**6067–6070.
32. Lai Y-L, Lin J-C, Yang S-F, Liu T-Y, Hung S-L. Areca nut extracts reduce the intracellular reactive oxygen species and release of myeloperoxidase by human polymorphonuclear leukocytes. *J Periodontal Res* 2007;**42:**69–76.
33. Scott CC, Botelho RJ, Grinstein S. Phagosome maturation: a few bugs in the system. *J Membr Biol* 2003;**193:**137–152.

34. Spear PG. Herpes simplex virus: receptors and ligands for cell entry. *Cell Microbiol* 2004;**6:**401–410.
35. Heo SK, Ju SA, Lee SC *et al*. LIGHT enhances the bactericidal activity of human monocytes and neutrophils via HVEM. *J Leukoc Biol* 2006;**79:**330–338.
36. Mauri DN, Ebner R, Montgomery RI *et al*. LIGHT, a new member of the TNF superfamily, and lymphotoxin alpha are ligands for herpesvirus entry mediator. *Immunity* 1998;**8:**21–30.
37. Teng Y-C, Cheng Y-Y, Chen Y-T, Hung S-L. Requirements for the induction of interleukin-6 by herpes simplex virus-infected gingival fibroblasts. *Viral Immunol* 2005;**18:**170–178.
38. Babior BM. NADPH oxidase: an update. *Blood* 1999;**93:**1464–1476.
39. Babior BM. The respiratory burst of phagocytes. *J Clin Invest* 1984;**73:**599–601.
40. Reeves EP, Lu H, Jacobs HL *et al*. Killing activity of neutrophils is mediated through activation of proteases by K^+ flux. *Nature* 2002;**416:**291–297.
41. Clutton S. The importance of oxidative stress in apoptosis. *Br Med Bull* 1997;**53:**662–668.
42. Shackelford RE, Kaufmann WK, Paules RS. Cell cycle control, checkpoint mechanisms, and genotoxic stress. *Environ Health Perspect* 1999;**107:**5–24.
43. Bokoch GM. Chemoattractant signaling and leukocyte activation. *Blood* 1995;**86:**1649–1660.
44. Nishizuka Y. Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. *Science* 1992;**258:**607–614.
45. Opdenakker G, Van den Steen PE, Dubois B *et al*. Gelatinase B functions as regulator and effector in leukocyte biology. *J Leukoc Biol* 2001;**69:**851–859.
46. Lee S, Zheng M, Kim B, Rouse BT. Role of matrix metalloproteinase-9 in angiogenesis caused by ocular infection with herpes simplex virus. *J Clin Invest* 2002;**110:**1105–1111.
47. Higuchi H, Ishizaka M, Nagahata H. Complement receptor type 3 (CR3)- and Fc receptor (FcR)-mediated matrix metalloproteinase 9 (MMP-9) secretion and their intracellular signalling of bovine neutrophils. *Vet Res Commun* 2007;**31:**985–991.
48. Alessi DR, Andjelkovic M, Caudwell B *et al*. Mechanism of activation of protein kinase B by insulin and IGF-1. *EMBO J* 1996;**15:**6541–6551.
49. Hsu M-J, Wu C-Y, Chiang H-H, Lai Y-L, Hung S-L. PI3K/Akt signaling mediated apoptosis blockage and viral gene expression in oral epithelial cells during herpes simplex virus infection. *Virus Res* 2010;**153:**36–43.